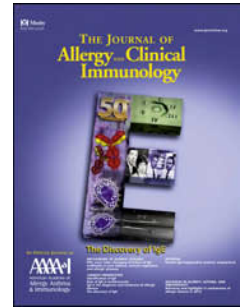


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TNFAIP3 levels in lung dendritic cells instruct Th2 or Th17 cell differentiation in eosinophilic or neutrophilic asthma

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Abstract (max 250 words: 245):

Background: It is currently unknown, why allergen exposure or environmental triggers in mild to moderate asthma patients results in Th2-mediated eosinophilic inflammation, whereas severe asthma patients often present with Th17-mediated neutrophilic inflammation. The activation state of dendritic cells (DCs) is crucial for both Th2 and Th17-cell differentiation, and is mediated through NF- κ B activation. Ablation of TNFAIP3, one of the crucial negative regulators of NF- κ B activation in myeloid cells and DCs was shown to control DC activation.

Objective: In this study we investigated the precise role of TNFAIP3 in myeloid cells for the development of Th2 and Th17-cell mediated asthma.

Methods: We exposed mice with conditional deletion of the *Tnfaip3* gene in either myeloid cells (using the *LysM* promotor) or specifically in DCs (using the *Cd11c* promotor) to acute and chronic house dust mite (HDM)-driven asthma models.

Results: We demonstrated that reduced *Tnfaip3* gene expression in DCs in either *Tnfaip3*^{CD11c} or *Tnfaip3*^{LysM} mice dose-dependently controlled development of Th17-mediated neutrophilic severe asthma in both acute and chronic HDM-driven models, whereas wildtype mice developed a purely Th2-mediated eosinophilic inflammation. TNFAIP3-deficient DCs induced HDM-specific Th17-cell differentiation, through increased expression of Th17-instructing cytokines, IL-1 β , IL-6 and IL-23, whereas HDM-specific Th2-cell differentiation was hampered by the increased IL-12 and IL-6 production.

Conclusions: These data show that the extent of TNFAIP3 expression in DCs controls Th2/Th17-cell differentiation. This implies that reducing DC activation could be a new

pharmacological intervention to treat severe asthma patients that present with a Th17-mediated neutrophilic inflammation.

Abbreviations used:

AHR (Airway hyperresponsiveness), APC (Antigen presenting cell), BAL (broncho-alveolar lavage), BM (bone marrow), cDC (conventional dendritic cells), DC (Dendritic cell), GM-CSF (Granulocyte-macrophage colony-stimulating factor), HDM (House dust mite), IL (Interleukin), i.t. (intra-tracheal), MLN (mediastinal lymph nodes), moDCs (monocyte-derived dendritic cells), PPR (Pattern recognition receptor), Tg (transgenic), Th (T helper), TLR (Toll like receptor), TNFa (tumor necrosis factor α), TNFAIP3 (TNFa-induced protein 3), TNIP, (TNFAIP3 interacting protein), PCA (Principal Component Analysis Principal Component Analysis).

Keywords: Asthma, T helper 2 cells, T helper 17 cells, Dendritic cells, Neutrophils

Key Messages:

- TNFAIP3 levels in DCs critically regulate development of either Th2-mediated eosinophil asthma or Th17-mediated neutrophilic asthma.
- DCs deficient in TNFAIP3 secrete high levels of Th17-promoting cytokines, IL-1 β , IL-6 and IL-23 upon HDM stimulation.

Introduction:

Asthma is a heterogeneous chronic inflammatory disorder of the airways, that can present with T helper 2 (Th2) cell driven eosinophilic, Th17-driven neutrophilic, or even agranulocytic inflammation(1). In general, Th2-mediated asthma can be well treated by inhalation of corticosteroids, whereas patients that display with Th17-mediated neutrophilic asthma are often unresponsive to corticosteroid treatment, resulting in poor disease control and frequent exacerbations(2).

Myeloid cells, such as macrophages, monocytes and dendritic cells (DCs) have antigen presenting functions and control naive CD4⁺ T cell differentiation into effector Th2 and Th17-cells in asthma, but also mediate tolerance to inhaled antigens(3, 4). Th-cell differentiation depends on antigen dosing, expression of co-stimulatory molecules and the cytokine milieu. Th2-cell differentiation is favoured with a low antigen dose(5, 6) and expression of OX40L and Jagged on antigen presenting cells (APCs)(7-10), in a cytokine environment high in interleukin (IL)-4 and low in IL-12(11). Conversely, Th17-cell differentiation is induced by a high antigen dose(12), expression of CD40 and CD86(13), and an IL-1 β , IL-6, and TGF- β -rich environment(14). IL-23 supports the maintenance and function of differentiated Th17-cells(15).

Polarization of antigen-specific naïve T-cells into Th1, Th2 or Th17-cells is a specialized function of migratory conventional DCs (cDCs) that derive from circulating pre-DCs(3, 16). DCs also regulate effector T-cell responses, as monocyte-derived DCs (moDCs) control effector T-cell responses to allergens in the lungs, due to the production of chemokines that attract effector cells to the lungs(3, 17).

DC activation is essential for proper Th-cell differentiation into either Th2 or Th17-cells(18). Activation of DCs in the context of asthma can occur directly by allergen exposure triggering pattern recognition receptors (PRRs) on DCs(19, 20). In addition to direct activation, DCs are also activated by cytokines secreted by the airway epithelium upon Toll-like receptor 4 (TLR4) ligation(21). PRR triggering on DCs activates the transcription factor NF- κ B, and thereby initiates transcription of pro-inflammatory cytokines, such as IL-1 and IL-12(22, 23). The activation threshold of DCs and NF- κ B levels is carefully regulated, as overt activation can lead to immune responses to self-antigens in addition to allergens. NF- κ B activation is negatively regulated by TNFAIP3 (TNF α -induced protein 3, also known as A20), a ubiquitin modifying enzyme that deubiquitinates several key intermediate NF- κ B signalling molecules, and thus acts as a brake on DC activation(24, 25). In particular, it has been shown that conditional deletion of TNFAIP3/A20 in macrophages or dendritic cells (using Cd11c-cre or LysM-cre mediated deletion in mice respectively) results in increased phosphorylation and degradation of I κ B (25, 26). Genetic polymorphisms in *TNFAIP3* and TNFAIP3 interacting protein (*TNIP*) have been associated with risk of developing asthma and allergies(27, 28). How alterations in TNFAIP3 levels might control asthma development remains enigmatic, as TNFAIP3 has very diverse effects in many different cell types. TNFAIP3 controls the production of Th-cell skewing cytokines in lung epithelial cells and a high expression (induced by environmental exposure to farm dust) is beneficial to protect from allergic asthma development(27). Lack of TNFAIP3 in mast cells controls the activation state of the cell, causing increased plasma extravasation and features of eosinophilic asthma upon allergen challenge(29). The precise role of TNFAIP3 in myeloid cells in asthma is currently unknown, however absence of TNFAIP3 in myeloid cells or

CD11c^{hi} DCs provokes Th17 induction(26). These studies indicate that TNFAIP3 levels in different cell types can control Th-cell differentiation.

As the role of TNFAIP3 in myeloid cells in the development on Th2 immune responses is unknown at present, we addressed the precise role of TNFAIP3 in myeloid cells in asthma. To determine this, we used mice that lack *Tnfaip3* in either myeloid cells or in DCs, and exposed these mice to either acute and chronic house dust mite (HDM)-driven asthma models. Our data shows that TNFAIP3 levels in DCs control whether HDM induces a predominantly Th2-mediated eosinophilic or Th17-mediated neutrophilic form of the disease.

Materials and Methods:

Mice

Tnfaip3^{LysM} and *Tnfaip3*^{CD11c} mice were bred and housed at Erasmus MC. *Tnfaip3*^{LysM} mice were crossed to ROSA26^{fl}EYFP mice, and *Tnfaip3*^{CD11c} mice were crossed to IL-23^{YFP} mice. 1-Der mice were provided by B.N. Lambrecht (VIB, Ghent, Belgium). Female C57BL/6 mice were from Harlan. Experiments were performed on mice backcrossed into C57BL/6 genetic background for at least ten generations and housed under SPF conditions. Mice were analyzed at 6-12 weeks of age. All experiments were performed under approval by the animal ethics committee of the Erasmus MC.

HDM-induced allergic airway inflammation

During HDM exposures, mice were anesthetized using isoflurane. For the acute allergic HDM model, mice were sensitized intranasally with 1 µg/40 µl HDM (Greer) or 40 µl PBS (GIBCO Life Technologies) as control on day 1, and challenged on day 7-11 with 10 µg/40 µl HDM. Mice were sacrificed on day 15. In the chronic HDM-driven asthma model, mice were treated intranasally with 25 µg/40 µl HDM or 40 µl PBS as control three times per week for five consecutive weeks(30). Mice were sacrificed for analysis at day 37. Lung function was measured using a whole body plethysmograph (EMKA) under urethane sedation. BAL was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA (Sigma-Aldrich). Lung were inflated with either PBS/OCT (1:1) solution and placed in 4% PFA and embedded in paraffin.

HDM-induced innate immune responses and Th-cell polarization

To determine allergen uptake and migration by pulmonary DCs, HDM was labelled with AF647 (Invitrogen, Molecular Probes). Mice were treated with 50 µg HDM-AF647 extract intratracheally. 24 and 72 hours later allergen uptake was evaluated in pulmonary DC subsets and migration in DC subsets in the MLN. Single cell suspensions were obtained from lungs by digesting the lungs using DNase and Liberase for 30 minutes at 37°C(3). Upon digestion, the lungs were homogenized through a 100 µm cell strainer (BD Biosciences). Red blood cells were lysed using osmotic lysis buffer (8.3% NH₄CL, 1% KHCO₃ and 0.04% NA₂EDTA in Milli-Q).

To determine the innate effect of HDM exposure on cytokine production by pulmonary DCs, mice were treated with 100 µg of HDM extract intratracheally. Cytokine production was evaluated 24 hours later in pulmonary DC subsets.

To examine Th-cell proliferation and differentiation in naïve HDM-specific T-cells, we adoptively transferred 2x10⁶ CFSE-labelled 1-DER T-cells, specific to the Der p 1WAFSGVAAT peptide (30) prior to 50 µg of HDM extract. Th-cell proliferation and differentiation were determined 3 days later by flow cytometry.

Generation of BM-Derived DCs

To obtain monocyte-derived DCs, bone marrow (BM) cells were cultured for 9 days in DC culture medium (DC-CM, RPMI 1640 containing GlutaMAX (Invitrogen) supplemented with 5% FCS (Hyclone), 50 µM 2-mercaptoethanol (Sigma) and 50 µg/ml gentamycin (Invitrogen) containing 20 ng/ml of GM-CSF, as previously described. 5 µg/mL HDM was added 18 hr prior to harvesting the cells.

RNA sequencing

Total RNA was extracted from monocyte-derived DCs using the miRNAeasy mini kit (Qiagen Sciences). Approximately 1 ug of RNA from each sample was used to generate RNA-seq cDNA libraries for sequencing using the TruSeq RNA sample Prep Kit v2 (Illumina). Sample preparation followed the manufacturer's protocol. Sequencing of SR42 bp paired reads was performed with an Illumina HiSeq HT instrument at the Bio Informatics department of the Erasmus MC. Single-end reads were aligned to the mouse genome (UCSC Genome Browser mm9) using TopHat (Tophat version 2.0.8). Gene expression levels as fragments per kilobase of a transcript per million mapped reads (FPKMs) were calculated using Cufflinks.

RNA-Seq Data Analysis

Differential gene expression assessment was done in the R environment (version 3.1.1) with edgeR (version 3.6.8)(31), as earlier described by Nota *et al.* (32) on TIGR multiexperiment viewer (MEV) software(33). MeV software was used to generate heatmaps and principal component analysis. Canonical pathway analysis was performed using the MSigDB database.

Flow cytometry procedures

Single cell suspensions were prepared from BAL and MLN using standard procedures. MLNs were homogenized through a 100 µm cell strainer (BD Biosciences). Flow cytometry surface and intracellular staining procedures have been described previously(34). Monoclonal antibodies used for flow cytometric analyses are listed in Table S1. For all experiments, dead cells were excluded using Live-dead Aqua (Invitrogen). Cytokine production was measured by flow cytometry. To measure cytokine production by T-cells, cells were stimulated at 37°C using 10 ng/ml PMA (Sigma-Aldrich), 250 ng/ml ionomycin (Sigma-Aldrich) and GolgiStop (BD Bioscience), for 4 hr. To measure cytokine production by DCs, cells were stimulated at 37°C with

GolgiPlug (BD Biosciences) for 4 hours. Data were acquired using a LSR II flow cytometer (Beckton Dickinson) and FACSDiva™ software (Beckton Dickinson) and analyzed by FlowJo version 9 (Tree Star Inc software).

Lung histology

5 µm-thick paraffin embedded lung sections were stained with hematoxylin/eosin.

ELISA

Antigen-specific IgE was measured in serum. Plates were coated overnight with anti-IgE capture antibody (Opteia, BD Biosciences). Serum was incubated for 2 hours at 37°C. Detection was performed using biotinylated-HDM, and streptavidin-HRP was used to develop the ELISA, according to the manufacturer's protocol (Opteia, BD Biosciences). IL-5 (Ready Set Go, eBioscience), IL-13, IL-17 (R&D systems), and IFN γ (Opteia, BD Biosciences) levels were measured in supernatant of HDM restimulated MLN cells. IL-1 β (Opteia, BD Biosciences), IL-23 and IL-12p70 (Ready Set Go, eBioscience) were measured in supernatant of DC cultures, 18 hours after stimulation with HDM.

Statistical analysis

Mann-Whitney U tests were used for comparison between two groups and a p-value of <0.05 was considered statistically significant. RNA sequencing analyses were performed using MEV software, and all other analyses were performed using Prism (GraphPad Software, USA).

Results:

LysM expression targets pulmonary DC subsets

As the lysozyme M promotor is active in several myeloid cell types(35), we crossed *Tnfaip3*^{LysM} mice to Rosa26-stop^{fl}EYFP mice, to visualize TNFAIP3 deletion in various myeloid cell types. As described before, YFP expression was found in pulmonary macrophages, neutrophils, monocytes, and DCs(35)(Fig 1A). LysM-driven cre-recombinase activity in DCs has been reported previously(35), but is exceptional as cDCs seem to derive from a dedicated pre-cDC progenitor(36, 37). Pulmonary DCs can be subdivided into cDCs (CD103⁺ cDC1s and CD11b⁺ cDC2) and CD64⁺ moDCs(37). Both moDCs, cDC2s and cDC1s showed similar YFP expression as pulmonary monocytes (Fig. 1B-C). LysM-dependent (YFP⁺) DCs functioned similar to LysM-independent (YFP⁻) DCs in WT mice, e.g. antigen uptake and migration (Fig. S1). To determine whether TNFAIP3-deletion would alter the proportions of YFP⁺ cells, YFP expression was determined in *Tnfaip3*^{LysM-WT}, *Tnfaip3*^{LysM-HZ} and *Tnfaip3*^{LysM-KO} mice. The proportions of YFP-expressing cDC2s, moDCs, and monocytes were enhanced in *Tnfaip3*^{LysM-KO} xROSA26-stop^{fl}EYFP mice compared with *Tnfaip3*^{LysM-WT} xROSA26-stop^{fl}EYFP mice (Fig. 1A/1C). Not only the proportions of YFP⁺ cells increased, also total DCs, cDC1s, and moDC numbers were elevated in naïve *Tnfaip3*^{LysM-KO} mice compared with both *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-HZ} mice (Fig. 1D). Ablation of TNFAIP3 in myeloid cells enhances NF-κB signalling indicated by increased p-IκB, and increased expression of various co-stimulatory molecules as MHCII, CD40, CD80 and CD86(25). CD86 expression was evaluated in pulmonary DC subsets as a surrogate marker to examine NF-κB activation *in vivo*. Pulmonary DCs exhibited increased CD86 expression in *Tnfaip3*^{LysM-HZ} and *Tnfaip3*^{LysM-KO} mice compared to WT mice (Fig. 1E), suggesting that both intermediate and absent TNFAIP3 expression influence DC activation.

Reduced TNFAIP3 expression in DCs did not significantly influence antigen uptake and DC migration (Fig. S1B).

These data indicate that cre-recombinase under the Lysozyme M promotor not only deletes *Tnfaip3* in macrophages, monocytes and neutrophils, but also targets pulmonary cDC subsets to a similar extent as monocytes. Absence of TNFAIP3 selectively increases the numbers of cDC1s and moDCs, indicating that TNFAIP3 deletion in myeloid cells increases their recruitment or survival. Intermediate TNFAIP3 expression already influences DC numbers and activation status.

Myeloid specific deletion of *Tnfaip3* induces Th17-mediated neutrophilic airway inflammation in a HDM-driven model

To address whether TNFAIP3 expression in myeloid cells controls asthma development, we used *Tnfaip3*^{LysM} mice(26) and exposed them to an acute HDM-driven asthma model (Fig. 2A). As expected, HDM-sensitized *Tnfaip3*^{LysM-WT} mice had increased eosinophils in the broncho-alveolar lavage (BAL) compared to PBS-treated controls (Fig. 2C). In contrast, HDM-sensitized *Tnfaip3*^{LysM-KO} mice developed only a very mild eosinophilic inflammation, but neutrophilic inflammation was increased compared to WT controls. Moreover, using *Tnfaip3*^{LysM-HZ} mice, a gene dose effect was observed, as these mice developed a mixed eosinophilic/neutrophilic inflammation (Fig. 2C). T-cells, B-cells, DC, and macrophages influx did not differ between *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-KO} mice, yet B cells were increased in HDM-sensitized *Tnfaip3*^{LysM-HZ} mice compared to HDM-sensitized *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-KO} mice (Fig. 2D-E).

Eosinophilia is generally associated with a type 2 immune response, whereas neutrophilia is a sign of IL-17-producing cells. Whereas in BAL fluid of HDM-sensitized *Tnfaip3*^{LysM-WT} mice the numbers of IL-5 and IL-13-expressing Th2-cells were increased, *Tnfaip3*^{LysM-KO} mice mainly showed an increase in IL17-expressing Th17 and interferon γ (IFN γ)-expressing Th1-cells (Fig. 2F and Fig S2A). IL-17/IFN γ -double-expressing CD4 T-cells were not induced (data not shown). *Tnfaip3*^{LysM-HZ} mice had elevated numbers of Th2-cells compared to *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-KO} mice, and similar numbers of Th17-cells compared to *Tnfaip3*^{LysM-KO} mice (Fig. 2F). Effector T cells are typically found in the lungs, whereas central memory T cells are found in lung draining mediastinal lymph nodes (MLN). IL-17 and IFN γ secretion were also increased in *in vitro* HDM-restimulated MLN cells of HDM-sensitized *Tnfaip3*^{LysM-KO} mice compared with both *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-HZ} mice. IL-13 levels were elevated in *in vitro* HDM-restimulated MLN cells of HDM-sensitized *Tnfaip3*^{LysM-HZ} mice compared with HDM-sensitized *Tnfaip3*^{LysM-WT} mice (Fig. 2G).

In summary, ablation of TNFAIP3 levels in myeloid cells induces a neutrophilic, Th17-associated airway inflammation rather than a Th2-associated eosinophilic airway inflammation in an HDM-driven airway inflammation model. Strikingly, intermediate TNFAIP3 expression in myeloid cells increased both the Th2 and the Th17 response after HDM exposures.

***Tnfaip3*^{LysM-KO} mice develop severe asthma upon chronic HDM exposure**

Uncontrolled severe asthma patients display increased neutrophil numbers, IL-17 levels in serum and BAL fluid, and increased airway remodelling(38). To more closely mimic severe asthma and to induce structural airway remodelling in mice, we exposed *Tnfaip3*^{LysM} mice to HDM over

period of 5 weeks (Fig. 3A)(39). Upon chronic HDM exposure, eosinophilic and B-cell inflammation was increased in both *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-KO} mice compared to PBS controls. Neutrophils and total T-cell numbers were higher in HDM-treated *Tnfaip3*^{LysM-KO} mice compared to HDM-treated *Tnfaip3*^{LysM-WT} mice (Fig. 3C-D). No differences were found Th2-cell numbers in BAL fluid of *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-KO} mice, whereas Th17 and Th1-cell numbers were strongly increased in *Tnfaip3*^{LysM-KO} mice compared with HDM-treated *Tnfaip3*^{LysM-WT} (Fig. 3E). Serum levels of HDM-specific IgE were similar between HMD-treated *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-KO} mice (Fig. 3F). KC levels in BAL fluid were only induced in HDM-treated *Tnfaip3*^{LysM-KO} mice (Fig. 3G). HDM-treated *Tnfaip3*^{LysM-KO} mice showed denser pulmonary inflammatory sites compared to HDM-treated *Tnfaip3*^{LysM-WT} mice (Fig. 3H). Lung resistance was even higher in HDM-treated *Tnfaip3*^{LysM-KO} mice compared to HDM-exposed *Tnfaip3*^{LysM-WT} mice (Fig. 3I). Surprisingly, no differences in IL-13-expressing CD4⁺ T cells were observed between HDM-exposed *Tnfaip3*^{LysM-WT} and *Tnfaip3*^{LysM-KO} mice, a key cytokine for increased airway hyper responsiveness (AHR).

In conclusion, upon chronic HDM exposure, absence of TNFAIP3 in myeloid cells induces development of a severe Th2/Th17-cell-associated eosinophilic and neutrophilic asthma phenotype with increased lung resistance.

DC-specific deletion of *Tnfaip3* also induces Th17-mediated neutrophilic asthma

As shown before, deletion of *Tnfaip3* using the LysM promotor affected many cell types, including neutrophils that accumulated in the airways of *Tnfaip3*^{LysM-KO} mice after HDM challenge. To specify whether reduced TNFAIP3 levels only in CD11c^{hi} DCs were responsible

for the development of Th17-mediated neutrophilic asthma phenotype, we exposed *Tnfaip3*^{CD11c} mice to the acute HDM-driven asthma protocol (Fig. 2A). In these mice, *Tnfaip3* is specifically deleted in all DC subsets and alveolar macrophages as previously shown by Kool *et al*(25). Pulmonary cDC subsets are decreased in *Tnfaip3*^{CD11c-HZ} and *Tnfaip3*^{CD11c-KO} mice compared to *Tnfaip3*^{CD11c-WT} mice (Fig. S3). In contrast, moDCs are increased in naïve *Tnfaip3*^{CD11c-KO} mice compared to *Tnfaip3*^{CD11c-WT} mice (Fig. S3). HDM-sensitized *Tnfaip3*^{CD11c-KO} mice were resistant to development of eosinophilic inflammation, yet a strong neutrophilic inflammation was induced compared with HDM-sensitized WT controls. Like the *Tnfaip3*^{LysM-HZ} mice, HDM-treated *Tnfaip3*^{CD11c-HZ} mice developed a mixed eosinophilic/neutrophilic inflammation (Fig. 4A-B). Numbers of T-cells and macrophages in BAL were not significantly different between HDM-sensitized *Tnfaip3*^{CD11c-WT}, *Tnfaip3*^{CD11c-HZ} and *Tnfaip3*^{CD11c-KO} mice, but B cells and DCs were reduced in HDM-sensitized *Tnfaip3*^{CD11c-KO} mice compared with both HDM-sensitized *Tnfaip3*^{CD11c-WT} and *Tnfaip3*^{CD11c-HZ} mice (Fig. 4C-D). Th2-cell numbers in BAL fluid of HDM-sensitized *Tnfaip3*^{CD11c-WT} and *Tnfaip3*^{CD11c-HZ} mice were increased compared to HDM-sensitized *Tnfaip3*^{CD11c-KO} mice (Fig. 4E, Fig. S2B). HDM-sensitized *Tnfaip3*^{CD11c-KO} mice showed increased Th17-cells in BAL fluid compared to HDM-sensitized *Tnfaip3*^{CD11c-WT} mice (Fig. 4E). MLN cells of both HDM-sensitized *Tnfaip3*^{CD11c-WT} and *Tnfaip3*^{CD11c-HZ} mice produced IL-13 in response to HDM-restimulation *in vitro*, whereas no IL-13 production was found in HDM-restimulated MLN cells of HDM-sensitized *Tnfaip3*^{CD11c-KO} mice. IL-17A levels were higher in HDM-restimulated MLN cells of HDM-sensitized *Tnfaip3*^{CD11c-KO} mice than of HDM-sensitized *Tnfaip3*^{CD11c-WT} mice (Fig. 4F).

Therefore, we conclude that specific ablation of TNFAIP3 in CD11c^{hi} DCs (and/or alveolar macrophages), but not in neutrophils induced development of Th17-associated neutrophilic airway inflammation.

Instruction of Th17 differentiation of HDM-specific T-cells by *Tnfaip3*-deficient DCs

To investigate more directly whether *Tnfaip3*^{CD11c-KO} mice induced HDM-specific Th17 differentiation, *Tnfaip3*^{CD11c} mice received Der p1-specific TCR transgenic (Tg) CD4⁺ T cells obtained from 1-DER mouse intravenously. 1-DER CD4⁺ T cells express a transgenic TCR encoding a Vβ4 and Vα8 chain, leading to a precursor frequency of 60-70% of Der p1-reactive CD4⁺ T cells(30).

On day 0, CFSE-labelled 1-DER CD4⁺ T-cells were transferred into *Tnfaip3*^{CD11c} mice, to determine their proliferative capacity. *Tnfaip3*^{CD11c} mice were subsequently treated with 50 µg HDM intra-tracheally at day 1 (Fig 5A). At day 4, 1-DER T-cells that were transferred into *Tnfaip3*^{CD11c-KO} mice showed reduced proliferation in the MLN, compared with those transferred into *Tnfaip3*^{CD11c-WT} and *Tnfaip3*^{CD11c-HZ} mice (Fig. 5B-5C). However, the replication index in the dividing 1-DER T-cells in *Tnfaip3*^{CD11c-HZ} and *Tnfaip3*^{CD11c-KO} mice was increased compared to dividing 1-DER T-cells in *Tnfaip3*^{CD11c-WT} mice (Fig. 5D). Indicating that once 1-DER T-cells start proliferation, the amount of proliferation cycles is higher in *Tnfaip3*^{CD11c-KO} mice compared to *Tnfaip3*^{CD11c-WT} mice.

The expression of lineage defining transcription factors like RORγT, GATA3 and T-bet were used to study differentiation of Th17, Th2 and Th1-cells, respectively. Strikingly, the percentage of RORγT⁺ 1-DER T-cells was increased in both undivided and divided 1-DER T-cells in *Tnfaip3*^{CD11c-KO} mice compared to both *Tnfaip3*^{CD11c-WT} and *Tnfaip3*^{CD11c-HZ} mice (Fig.

5E-F). The MFI of GATA3 and T-bet was elevated in divided 1-DER T-cells compared to undivided 1-DER T-cells in all mice, however both undivided as divided 1-DER T-cells in *Tnfaip3*^{CD11c-KO} mice had decreased levels of GATA3 compared with *Tnfaip3*^{CD11c-WT} and *Tnfaip3*^{CD11c-HZ} mice (Fig. 5F). No differences were found in the expression levels of T-bet in 1-DER T-cells in *Tnfaip3*^{CD11c-WT}, *Tnfaip3*^{CD11c-HZ}, and *Tnfaip3*^{CD11c-KO} mice (Fig. 5F).

This indicates that HDM induces Th17-cell differentiation of Der p1-specific T-cells in *Tnfaip3*^{CD11c-KO} mice that lack TNFAIP3 expression in DCs and alveolar macrophages, whereas in wild type mice it predominantly induces a Th2 response.

Adoptive transfer of *Tnfaip3*-sufficient or deficient DCs drives eosinophilic or neutrophilic asthma

The *Cd11c* promotor used to excise *Tnfaip3* can also lead to excision of this gene in other cells like alveolar macrophages, CD8 T-cells, plasmacytoid DCs and plasmablasts, caused by *Cd11c* gene expression in these cell types(40). We therefore resorted to a model of intra-tracheal adoptive transfer of bone marrow (BM)-derived DCs to induce asthma(41). This would exclude any effects due to expression outside the DC lineage. It became clear that GM-CSF-stimulated BM cultures contains a mix of cDCs and macrophage-like cells, but we did not observe large shifts in the relative distribution of these cell subsets in cultures of mice of different genotypes (Fig. S4)(42). GM-CSF BM-cultured *Tnfaip3*^{KO} DCs lacked expression of exon 4 and 5 of the *Tnfaip3* gene, preventing formation of functional TNFAIP3 protein, and confirming effective Cre-mediated recombination *in vitro* (Fig. S5). We intra-tracheally sensitized C57BL/6 mice by adoptive transfer of HDM or PBS-stimulated BM-derived DCs from *Tnfaip3*^{CD11c-WT} and *Tnfaip3*^{CD11c-KO} mice, followed by 5 HDM challenges (Fig. 6A). C57BL/6 mice sensitized with

HDM-stimulated *Tnfaip3*^{WT} DCs or *Tnfaip3*^{HZ} DCs developed an increased eosinophilic airway inflammation both in absolute numbers as in percentages compared to mice sensitized with PBS-stimulated *Tnfaip3*^{WT} DCs (Fig. 6B). Eosinophilic inflammation was not induced in mice sensitized with HDM-stimulated *Tnfaip3*^{KO} DCs. Neutrophilic inflammation was increased in absolute count in mice sensitized with either HDM-stimulated *Tnfaip3*^{WT}, *Tnfaip3*^{HZ}, and *Tnfaip3*^{KO} DCs compared to mice sensitized with unstimulated DC, but was not different between mice sensitized with HDM-stimulated *Tnfaip3*^{WT} or *Tnfaip3*^{KO} DCs. Furthermore, neutrophil influx was enhanced in mice sensitized with HDM-stimulated *Tnfaip3*^{HZ} DCs compared to mice sensitized with HDM-stimulated *Tnfaip3*^{KO} DCs (Fig. 6C). Mice that received HDM-stimulated *Tnfaip3*^{KO} DCs had a decreased percentage of Th2-cells and an increased percentage of Th17-cells in BAL compared with HDM-stimulated *Tnfaip3*^{WT} or *Tnfaip3*^{HZ} DC-sensitized mice. The percentage of IFN γ -expressing CD4⁺ T-cells was not different between all mice (Fig. 6D). Furthermore, *in vitro* co-culture of antigen-pulsed *Tnfaip3*^{KO} DCs with antigen-TCR-specific CD4⁺ T cells showed that *Tnfaip3*^{KO} DCs induced IL-17 production by Th cells in contrast to Th2 differentiation by *Tnfaip3*^{WT} DCs (Fig. S6A). Interleukin-17 production was specifically induced by GM-CSF-cultured BM-DCs, and not by FLt3L-cultured cDC1s, cDC2s, or pDCs (Fig. S6B).

In conclusion, TNFAIP3 deletion dose dependently induced Th17 differentiation while hampering Th2 differentiation, resulting in an absence of eosinophilic inflammation in mice sensitized with *Tnfaip3*-deficient DCs.

Tnfaip3*^{KO} DCs display a pro-Th17-cell signature upon HDM stimulation *in vitro* and *in vivo

387 To gain insight into the mechanism by which *Tnfaip3*^{KO} DCs induced Th17-cell differentiation,
 388 we performed gene expression profiling of PBS or HDM-stimulated GM-CSF BM-cultured
 389 *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs. Both unstimulated as HDM-stimulated GM-CSF BM-cultured
 390 *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs differed in their expression profile as observed in the Principal
 391 Component Analysis (PCA) (Fig. 7A). In the unstimulated situation, 934 genes were
 392 differentially expressed between *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs (Fig S7). Canonical pathway
 393 analysis was consistent with our *in vivo* findings and showed no alterations in genes implicated
 394 in survival, migration and allergen uptake between *Tnfaip3*^{WT} or *Tnfaip3*^{KO} DCs (Table S2-3).
 395 HDM stimulation induced 1290 genes differentially expressed genes in either *Tnfaip3*^{WT} or
 396 *Tnfaip3*^{KO} DCs (Fig. S8). Of these 1290 genes, 1054 were differentially expressed in HDM
 397 versus unstimulated *Tnfaip3*^{WT} DCs, 174 genes in both *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs, and 62
 398 genes only in *Tnfaip3*^{KO} DCs (Fig. 7B). No compensatory mechanisms to regulate NF-κB
 399 activation (e.g. other negative regulators) were induced *Tnfaip3*^{HZ} and *Tnfaip3*^{KO} DCs (Fig. S9).
 400 Clustering of these 1290 genes, revealed that multiple genes involved in Th17 differentiation,
 401 such as IL-6 and IL-1β were upregulated upon HDM stimulation specifically in *Tnfaip3*^{KO} DCs.
 402 Also CXCL3, a known chemoattractant for neutrophils was increased in HDM-stimulated
 403 *Tnfaip3*^{KO} DCs(43). Furthermore, IL-12b, a cytokine known to inhibit Th2 differentiation when
 404 expressed in DCs(44) was specifically induced in HDM-stimulated *Tnfaip3*^{KO} DCs (Fig. 7C-D).
 405 IL-1β, IL-23 and IL-12 protein expression in culture supernatants of BM-derived DC cultures
 406 was also increased upon HDM stimulation, whereas IL-6 protein expression was already
 407 increased in unstimulated GM-CSF BM-cultured *Tnfaip3*^{KO} DCs compared to HDM-stimulated
 408 GM-CSF BM-cultured *Tnfaip3*^{WT} DCs (Fig. 7E). HDM-exposed *Tnfaip3*^{HZ} DCs showed an

intermediate production of most cytokines compared to *Tnfaip3*^{WT} DCs and *Tnfaip3*^{KO} DCs (Fig. 7E).

Since GM-CSF-cultured DCs displayed a Th17-cell-promoting phenotype upon HDM stimulation *in vitro*, we wondered whether Th17-cell differentiation instructive cytokines would also be increased in DCs of *Tnfaip3*^{CD11c} mice that received HDM *in vivo*. To determine this, IL-23^{YFP} mice were crossed to *Tnfaip3*^{CD11c} mice, generating IL-23^{YFP}*xTnfaip3*^{CD11c} mice. IL-23^{YFP}*xTnfaip3*^{CD11c} mice received an intranasal administration of 100 µg of HDM and cytokine expression by different pulmonary DC subsets was determined 24 hours later. The numbers of IL-6 and IL-23 producing moDCs in IL-23^{YFP}*xTnfaip3*^{CD11c-KO} mice was increased compared to IL-23^{YFP}*xTnfaip3*^{CD11c-WT} mice (Fig. 8A-B). cDC2s in IL-23^{YFP}*xTnfaip3*^{CD11c-KO} mice only had significantly increased IL-23 production, whereas IL-6 and IL-23 production was not different between cDC1s in IL-23^{YFP}*xTnfaip3*^{CD11c-WT} and IL-23^{YFP}*xTnfaip3*^{CD11c-KO} mice (Fig. 8B).

In summary, these data show that *Tnfaip3*-deficient moDCs and cDC2s express increased levels of cytokines that promote Th17 differentiation in response to HDM.

Discussion:

Asthma is a heterogeneous disorder. Mild to moderate asthma patients often display Th2-cell-mediated eosinophilic inflammation, whereas severe asthma patients often present Th17-cell-mediated neutrophilic inflammation(1). DCs are essential for both the initiation and maintenance of Th2-cell-mediated asthma, and can also induce pulmonary Th17-cell responses(45, 46). However, the underlying mechanisms driving Th17-cell-driven neutrophilic asthma are still undetermined. We and others have previously shown that DCs activation is crucial for Th-cell differentiation and activation, and is mediated through NF- κ B activation driving expression of key cytokine pathways. Ablating the negative feedback regulator of NF- κ B signalling, TNFAIP3, in myeloid cells and DCs was shown to be essential for Th17 differentiation(25, 26).

Our results demonstrate that TNFAIP3 levels in DCs control development of Th17-cell-mediated neutrophilic asthma in an acute HMD-driven asthma model, and a mixed Th2/Th17-mediated severe eosinophilic/neutrophilic asthma upon a chronic HDM-driven asthma model. TNFAIP3-deficient DCs induced HDM-specific Th17-cell differentiation and hampered HDM-specific Th2-cell differentiation. TNFAIP3 deficiency in DCs does not affect their capability take up allergens and migrate towards LN, but it affects the number of DC subsets. TNFAIP3-deficient cDC2s and moDCs produce cytokines implicated in Th17-cell differentiation, such as IL-1 β , IL-6 and IL-23(47). Furthermore, both increased IL-12 and IL-6 by TNFAIP3-deficient DCs could hamper Th2 immunity (43, 44). IL-6 inhibits Th2 immunity either directly by inhibiting GATA3 expression, or indirectly by restricting IL-4 expression by follicular Th-cells(48). IL-12 strongly promotes Th1 differentiation and thereby hampers Th2 differentiation(43).

Ablating TNFAIP3 expression in DCs increased AHR upon chronic exposure to HDM, whereas the AHR-inducing cytokine IL-13 was similar between KO and WT mice, however IL-17 levels are specifically increased in mice lacking TNFAIP3 in DCs. Transfer of both Th2 as Th17-cells has been shown to elicit AHR(49), suggesting that combined expression of Th2 and Th17-cell cytokines further enhances AHR. Furthermore, suppression of IL-13 and IL-17 simultaneously decreases AHR more efficiently(50). Blockade of IL-17A during the chronic HDM protocol restored neutrophil inflammation in *Tnfaip3*^{LysM-KO} mice to similar neutrophil numbers as observed in *Tnfaip3*^{LysM-WT} mice (data not shown), which could suggest that AHR is also depending on IL17A in our model.

Tnfaip3^{LysM-KO} mice develop a mild eosinophilic inflammation upon HDM sensitization, which is completely absent in *Tnfaip3*^{CD11c-KO} mice. Differences in eosinophilic inflammation between these two transgenic mouse strains is probably mediated by different induction of IL-5-producing CD4⁺ T cells, which were present in *Tnfaip3*^{LysM-KO} mice and entirely lacking in *Tnfaip3*^{CD11c-KO} mice (Fig. S2). The different Th2-cell induction could be caused by the different percentage of DCs targeted for TNFAIP3 deletion in *Tnfaip3*^{LysM} and *Tnfaip3*^{CD11c} mice. The LysM-CRE targets 40-60% of the DC subsets for Tnfaip3-deletion (Fig. 1), whereas the CD11c-CRE excises TNFAIP3 in 90-100% of all DC subsets(51).

In addition to controlling NF-κB activation, TNFAIP3 also regulates apoptosis susceptibility(52). Our deep-sequencing data showed that TNFAIP3 deficiency does not affect survival pathways in cultured moDCs, however cDC1s and cDC2s could be differently susceptible. In support, 90-100% deletion of TNFAIP3 using CD11c-CRE impairs the number of pulmonary cDC1s and cDC2s, whereas no alterations in cDC1 and cDC2 numbers were observed in LysM-CRE mice.

Sensitization of C57BL/6 mice with TNFAIP3-deficient DCs also induced Th17-cell differentiation, suggesting that our observed phenotype was indeed due to genetic alterations selectively in DCs, and not in neutrophils.

Our data indicate that TNFAIP3 levels directly control secretion of Th17-polarizing cytokines in moDCs and cDC2s both *in vivo* and *in vitro*, whereas these cytokines are not increased in cDC1s. This together with the fact cDC2s and moDCs are essential for Th2 differentiation and maintenance in asthma(45), shows that these DC subsets are crucial for the balance of Th2/Th17-cell differentiation in asthmatic lungs.

Ablation of TNFAIP3 in DCs results in constitutive NF- κ B activation(25, 53). NF- κ B directly promotes transcription of pro-inflammatory Th17 differentiation target genes IL-1 β and IL-6(54), which indicates that TNFAIP3 deletion in DCs directly enables the transcription of Th17-promoting cytokines through increased NF- κ B signalling.

It is conceivable that environmental triggers might also alter *Tnfaip3* expression in DCs, as combined exposure with allergens and environmental factors (such as diesel exhaust particles, cigarette smoke and viral infections) are essential for DC-induced Th17-mediated severe asthma, by induction of Th17-cell differentiating cytokines(18, 55). It will thus be very important to study if any of these environmental co-regulators might suppress TNFAIP3 gene and/or protein expression in DCs. There is already evidence for such environmental regulation of TNFAIP3 in lung epithelial cells *in vivo*(27). TNFAIP3 seems to be strongly regulated by microRNAs (mir125 family and mir221)(56), and microRNA levels can vary greatly in response to chronic environmental stimulation.

In conclusion, our data demonstrate that loss of TNFAIP3 specifically in pulmonary cDC2s and moDCs, amplifies NF- κ B-controlled gene expression of IL-6 and IL-23 in response to allergen exposure. Increase in these Th17-cell-polarizing cytokines promotes Th17-cell differentiation and Th17-cell-mediated severe neutrophilic asthma phenotype, accompanied by increased lung resistance. As a result, our data imply that increasing TNFAIP3 levels and thereby decreasing DC activation could be a new pharmacological intervention to treat severe Th17-cell-mediated neutrophilic asthma.

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Author contributions

HV, RWH, MK designed the experiments. HV, IB, JvH, MvN, DvU, SYP, MS performed experiments and analyzed data. HV, HH, BNL, RWH and MK wrote the manuscript. All authors read and approved the final manuscripts.

Conflict of interest statement

The authors declare no conflict of interest.

Figure legends

Figure 1: LysM expression targets all pulmonary DC subsets. (A) Percentage YFP positive cells within the gated population. (B) Gating strategy for different pulmonary DC subsets using flow cytometry. (C) Percentage YFP positive cells within the gated population in *Tnfaip3*^{LysM-WT}, *Tnfaip3*^{LysM-HZ} and *Tnfaip3*^{LysM-KO} mice. (D) Quantification of total DCs, cDC1s, cDC2s, and moDCs by flow cytometry. (E) Quantification of the mean fluorescent intensity (MFI) of CD86 of lung DC subsets by flow cytometry. Results are presented as mean $\pm$ SEM of n=3-4 per group and representative of two or more independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 2: Myeloid specific deletion of *Tnfaip3* induces Th17 neutrophilic asthma upon an acute HDM-driven asthma protocol. (A) Mice were sensitized i.n. with PBS or 1 μ g HDM on day 0 and challenged i.n. with 10 μ g HDM daily between days 7 and 11 to induce allergic asthma. Analysis was performed at day 15. (B-E) Quantification of total BAL inflammation, eosinophils, neutrophils, DCs, macrophages, B-cells and T-cells by flow cytometry. (F) Number of IL-13⁺, IL-17⁺ and IFN γ ⁺ CD4⁺ T-cells in the BAL were analyzed by flow cytometry. (G) Quantification of IL-13, IL-17 and IFN γ in supernatant of HDM-restimulated MLN cells by ELISA. Results are presented as mean $\pm$ SEM of n=4-7 per group and representative of two or more independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 3: *Tnfaip3*^{LysM-KO} mice develop severe corticosteroid resistant asthma upon chronic HDM exposure. (A) Mice were exposed to a chronic HDM model, and treated with PBS or HDM at indicated time points. Analysis was performed at day 37. (B-D) Quantification of total BAL inflammation, eosinophils, neutrophils, B-cells and T-cells by flow cytometry. (E) Number

of IL-13⁺, IL-17⁺ and IFN γ ⁺ CD4⁺ T-cells in the BAL were analyzed by flow cytometry. (F) Serum HDM-specific IgE levels. (G) BAL fluid levels of KC measured for ELISA. (H) Histologic analysis of lung inflammation by H&E staining. (I) Bronchial hyper-responsiveness was measured upon increasing doses of inhaled methacholine. Results are presented as mean $\pm$ SEM of n=3-5 per group and representative of two or more independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 4: DC specific deletion of *Tnfaip3* also induces Th17 mediated neutrophilic asthma.

(A-D) Quantification of total BAL inflammation, eosinophils, neutrophils, DCs, macrophages, B cells and T-cells by flow cytometry. (E) Number of IL-13⁺, IL-17⁺ and IFN γ ⁺ CD4⁺ T-cells in the BAL were analyzed by flow cytometry. (F) Quantification of IL-13, IL-17 and IFN γ in supernatant of HDM-restimulated MLN cells by ELISA. Results are presented as mean $\pm$ SEM of n=3-7 per group and representative of two or more independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 5: HDM specific differentiation of Th17-cells by *Tnfaip3* deficient DCs. (A) Scheme for intravenous transfer of CFSE labelled 1-DER T-cells into *Tnfaip3*^{CD11c} mice and subsequent exposure to HDM intranasal at indicated time points. Analysis was performed at day 5. (B) Flow cytometric gating of proliferating 1-DER T-cells in the MLN. (C) Quantification of the % proliferating 1-DER T-cells in the MLN by flow cytometric analysis software FlowJo. (D) Quantification of the replication index of 1-DER T cells in the MLN by flow cytometric analysis software FlowJo. (E) Flow cytometric gating of ROR γ T⁺ dividing 1-DER T-cells in the MLN. (F) Quantification of the percentage of ROR γ T⁺ Th17-cells and MFI of GATA3 and T-bet in

undivided and divided 1-DER T-cells in the MLN by flow cytometry. Results are presented as mean $\pm$ SEM of n=4 per group. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 6: Adoptive transfer of *Tnfaip3*-sufficient or deficient DCs drives eosinophilic or neutrophilic asthma. (A) C57bl/6 mice were sensitized on day 1 i.n. with 30.000 PBS/HDM stimulated GM-CSF cultured DCs from bone marrow of *Tnfaip3*^{CD11c} mice. All mice were challenged i.n. with 10 μ g HDM daily between days 7 and 11 to induce allergic asthma. Analysis was performed at day 15. (B-C) Quantification of total BAL inflammation, eosinophils, neutrophils by flow cytometry. (D) Number of IL-13⁺, IL-17⁺ and IFN γ ⁺ CD4⁺ T-cells in the BAL were analyzed by flow cytometry. Results are presented as mean $\pm$ SEM of n=4-6 per group and are representative of two independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 7: GM-CSF cultured *Tnfaip3*^{KO} DCs display a Th17-instructive signature after HDM stimulation. (A) Principal Component Analysis of PBS/HDM stimulated GM-CSF BM-cultured *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs. (B) RNA sequencing analysis of total mRNA from PBS/HDM stimulated GM-CSF BM-cultured DCs. EdgeR analysis identified 1290 significantly different genes. (C) MeV hierarchical clustering of a selection of the 1290 differentially expressed genes are represented in the heatmap. (D) FPKM values of PBS/HDM stimulated GM-CSF BM-cultured DCs. (E) Analysis of IL1 β , IL23, IL-6 and IL12 in supernatant of PBS/HDM stimulated GM-CSF bone marrow cultured DCs using ELISA. Results are presented as mean $\pm$ SEM of n=4-6 per group. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 8: MoDCs and cDC2s in IL-23^{YFP} $\times$ *TNFAIP3*^{CD11c-KO} mice display a pro-Th17-signature upon HDM stimulation *in vivo*. (A) Flow cytometric gating of IL-6 and IL-23 producing pulmonary DCs in IL-23^{YFP} $\times$ *Tnfaip3*^{CD11c} mice 24 hours after HDM exposure. (B)

Quantification of total IL-6-producing and IL-23-producing pulmonary DCs. Results are presented as mean $\pm$ SEM of n=2-4 mice per group. Similar results were found in *Tnfaip3*^{LysM} mice. * = p<0.05, ** = p<0.01, *** = p<0.001.

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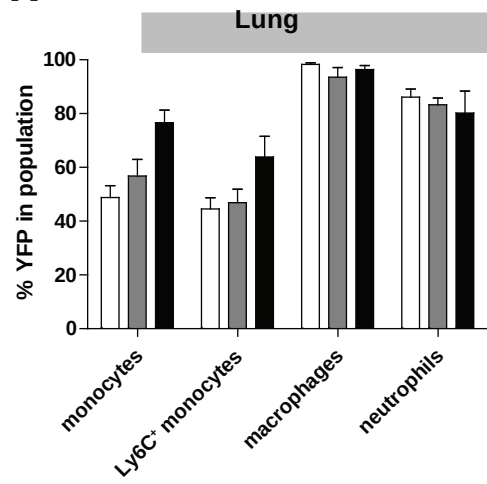
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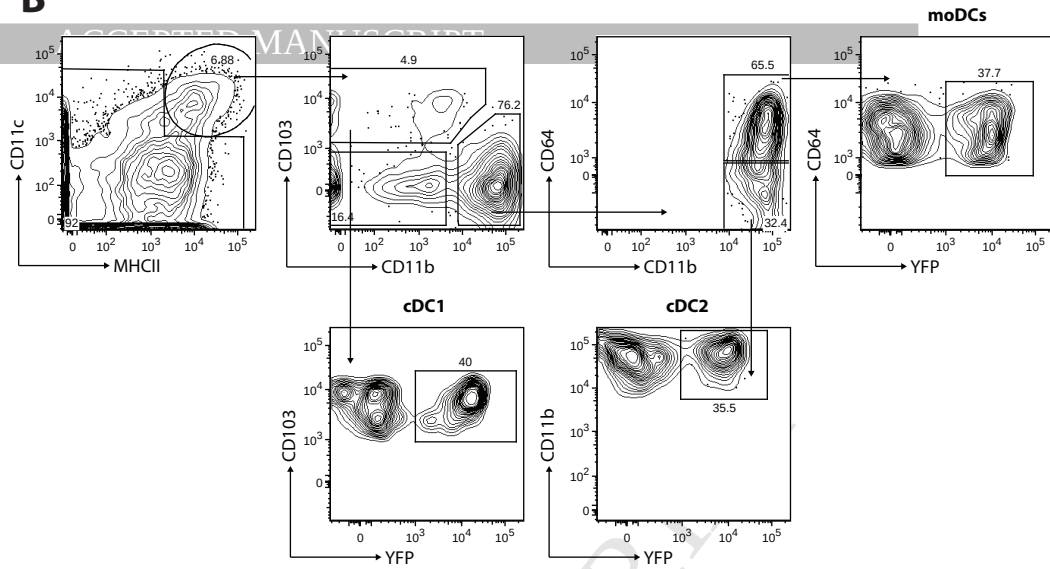
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Figure 1 LysM expression Lung

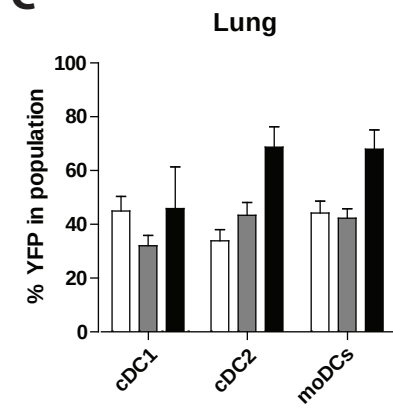
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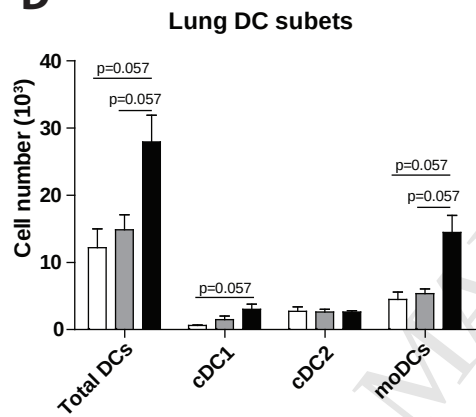
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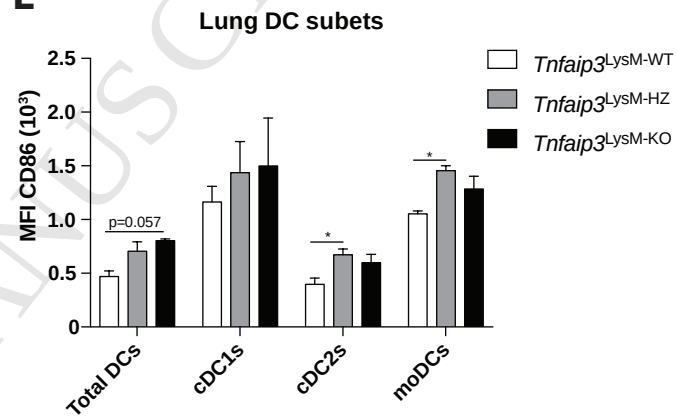
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Legend:
 □ *Tnfaip3*^{LysM-WT}
 ■ *Tnfaip3*^{LysM-HZ}
 ■ *Tnfaip3*^{LysM-KO}

Figure 2: LysM data Acute

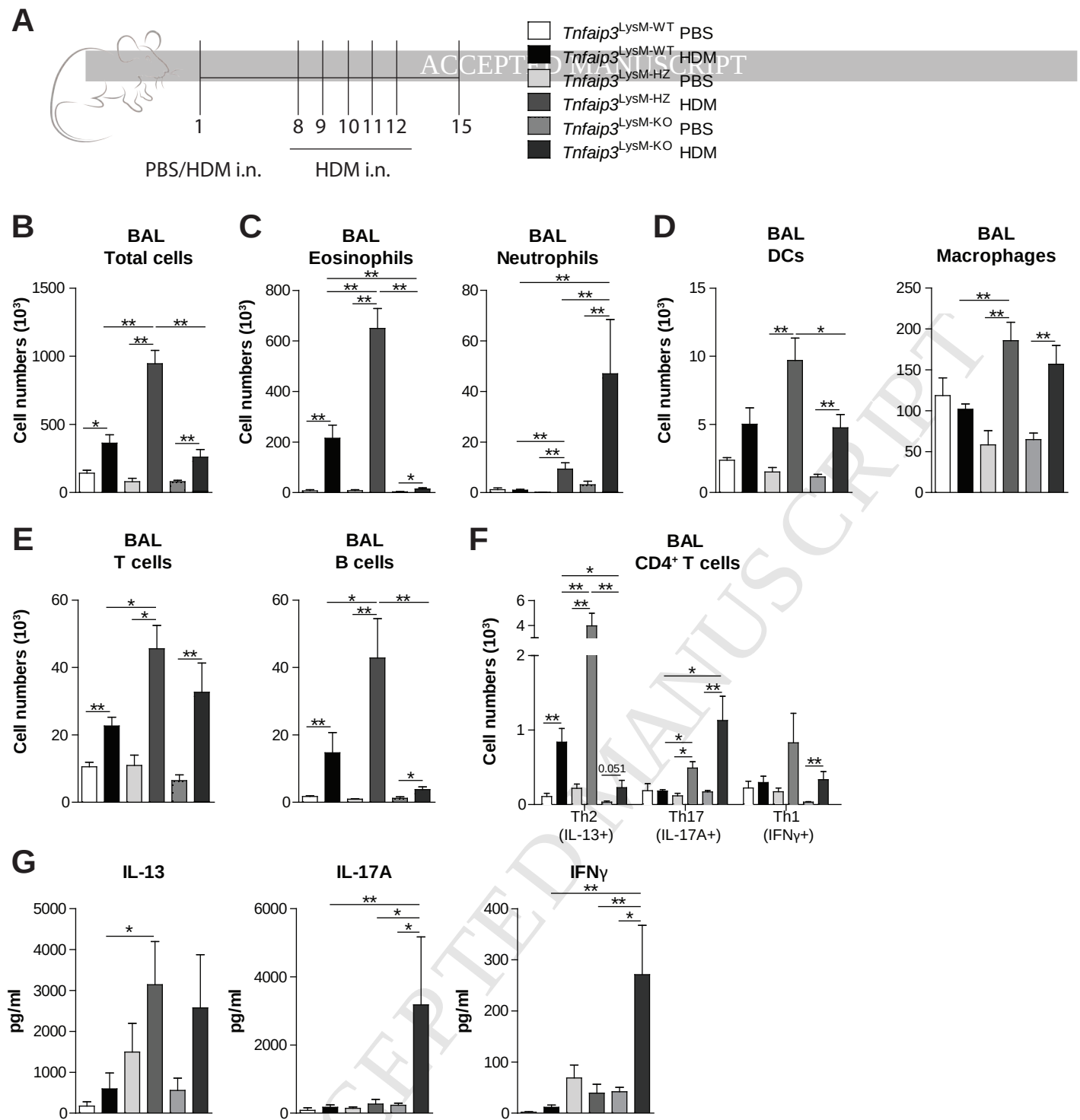
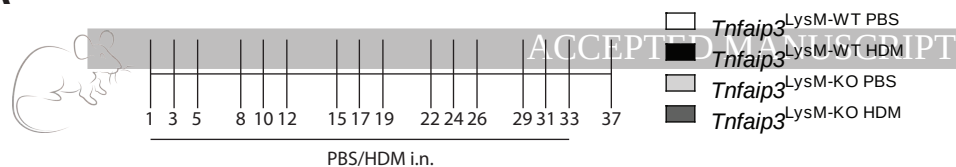
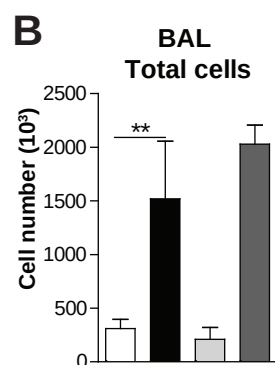


Figure 3: Th17-mediated neutrophilic inflammation in chronically HDM treated *Tnfaip3*^{LysM-KO} mice

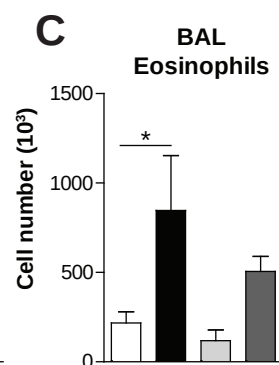
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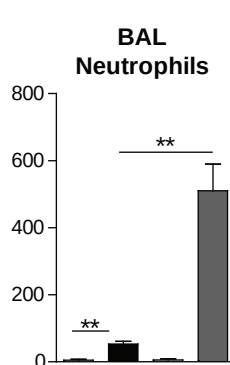
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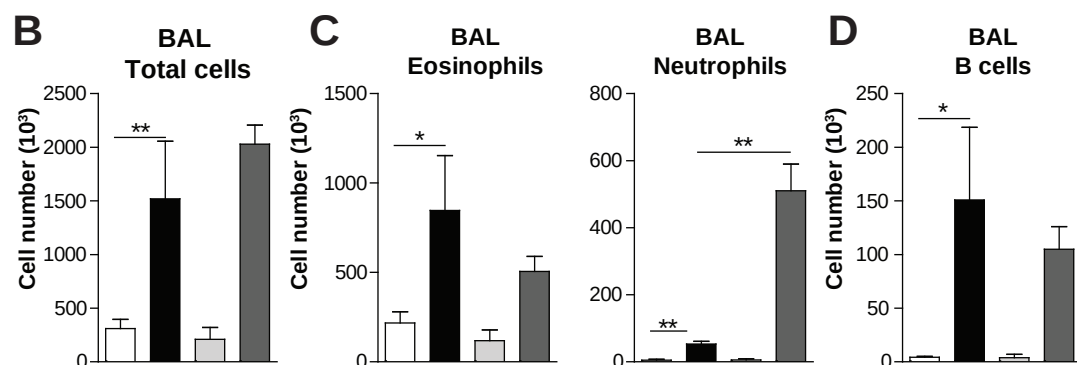
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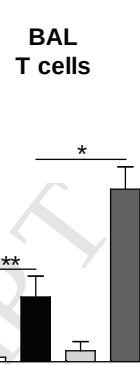
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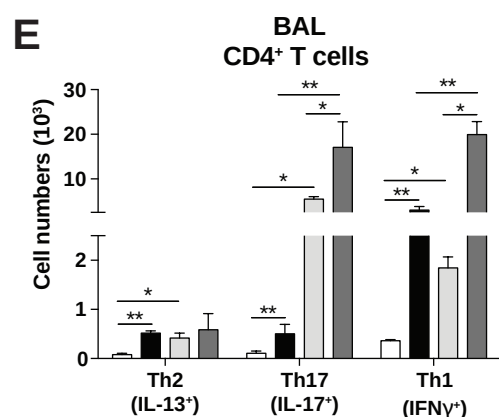
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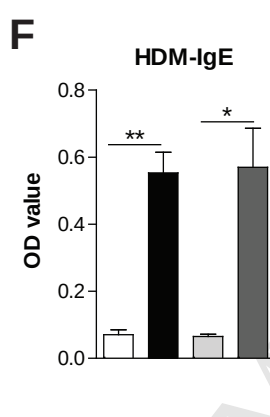
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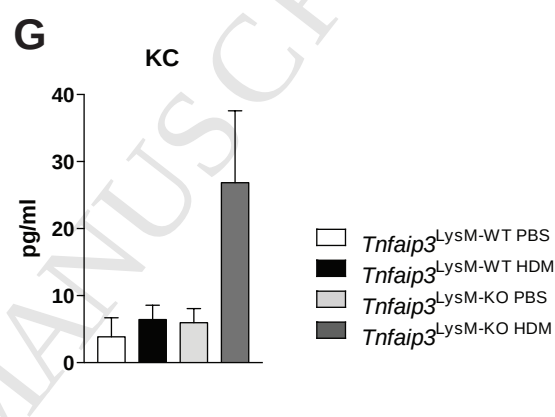
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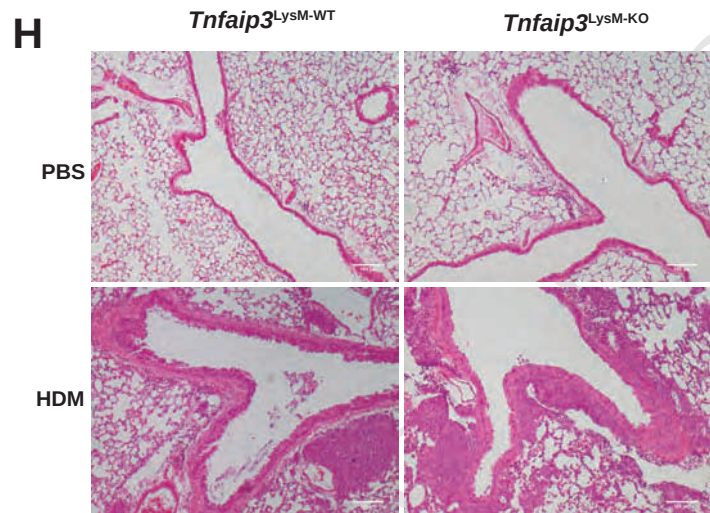
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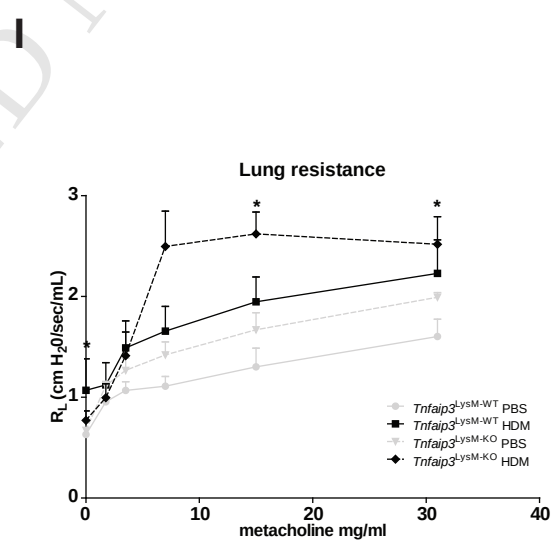


Figure 4: CD11c data Acute

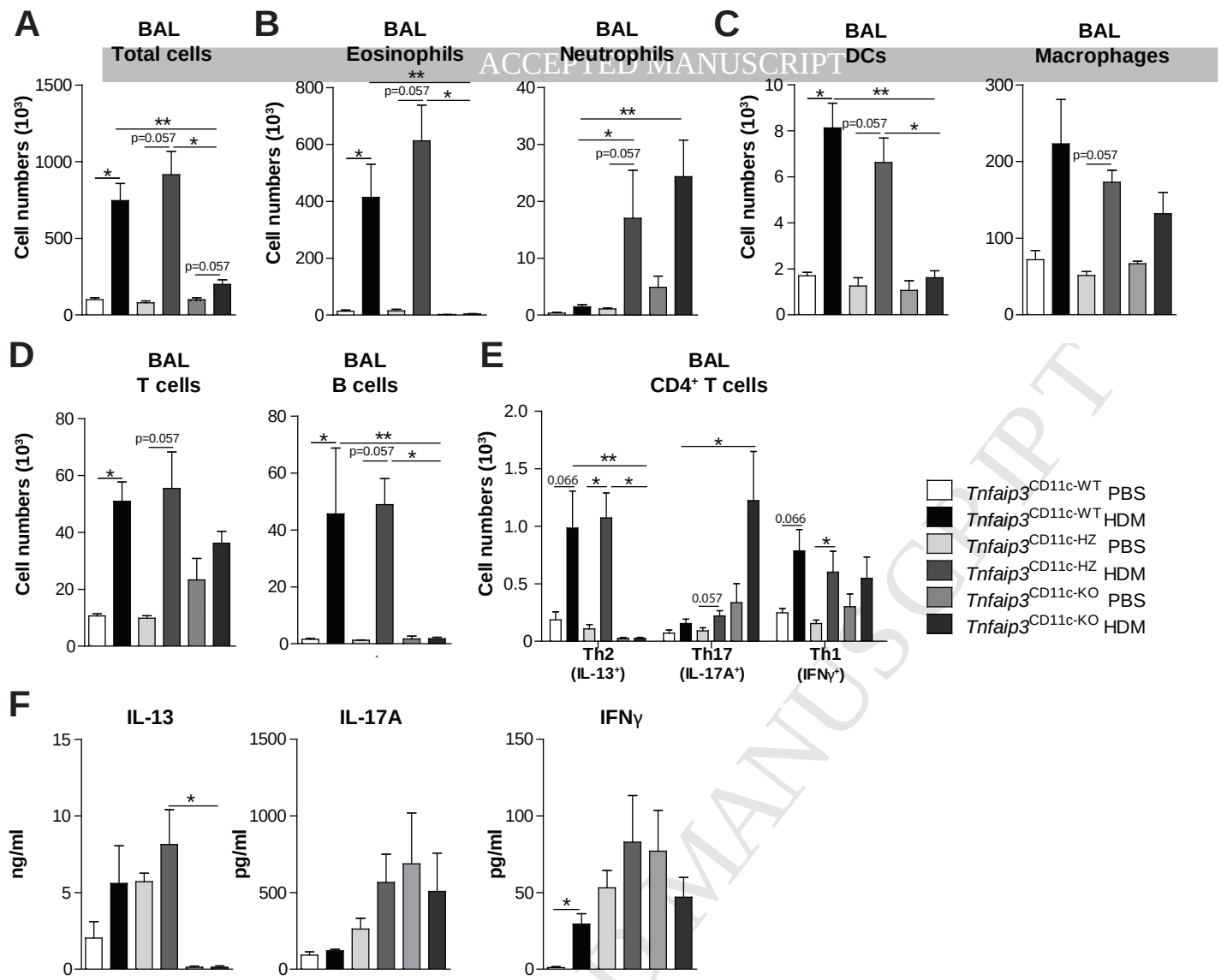


Figure 5: HDM specific Th17 cell differentiation in *Tnfaip3*^{CD11c-KO} mice

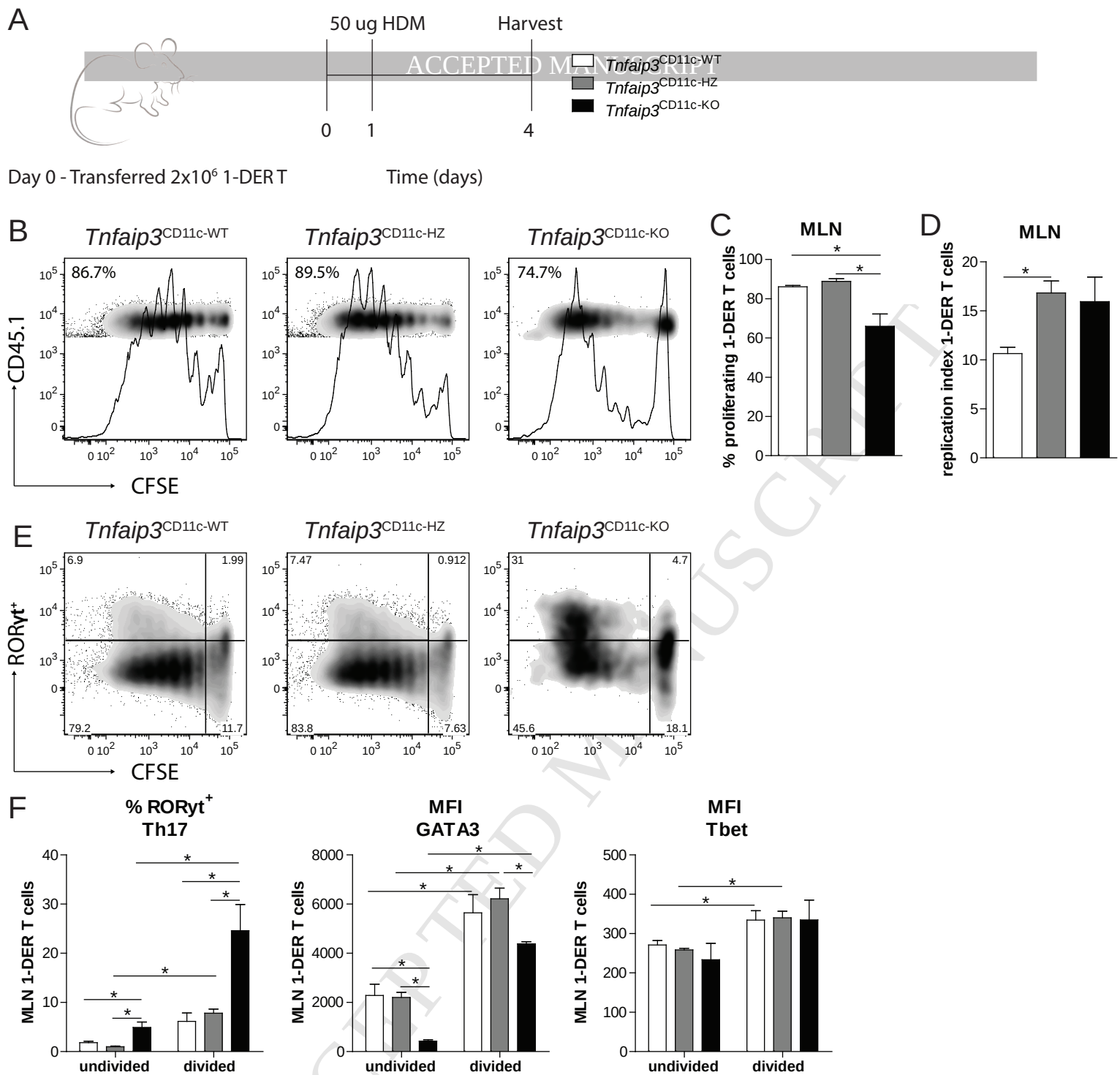


Figure 6: Adoptive transfer of *Tnfaip3*-sufficient or deficient DCs drives eosinophilic or neutrophilic asthma

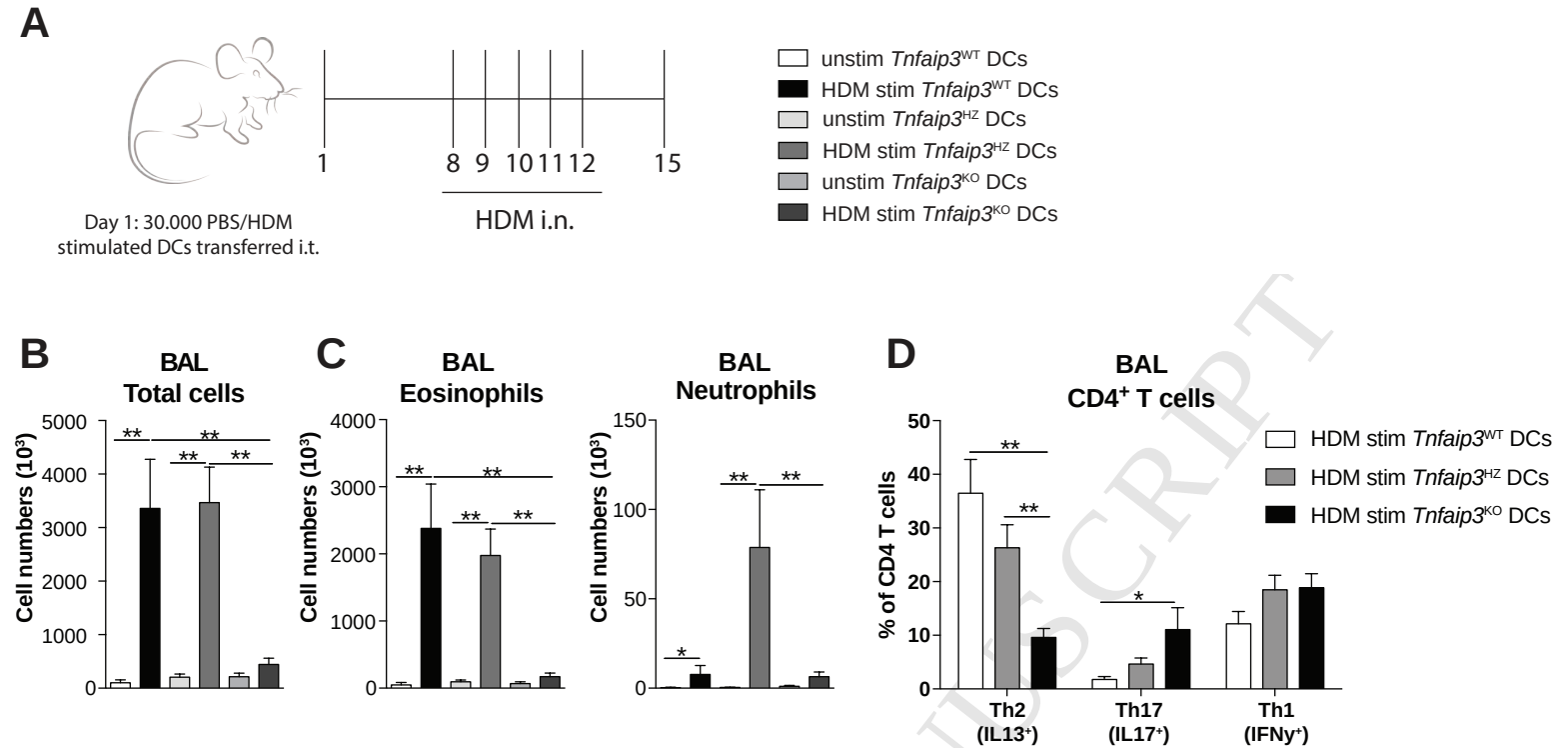


Figure 7: *Tnfaip3*KO DCs display a Th17 signature upon HDM stimulation

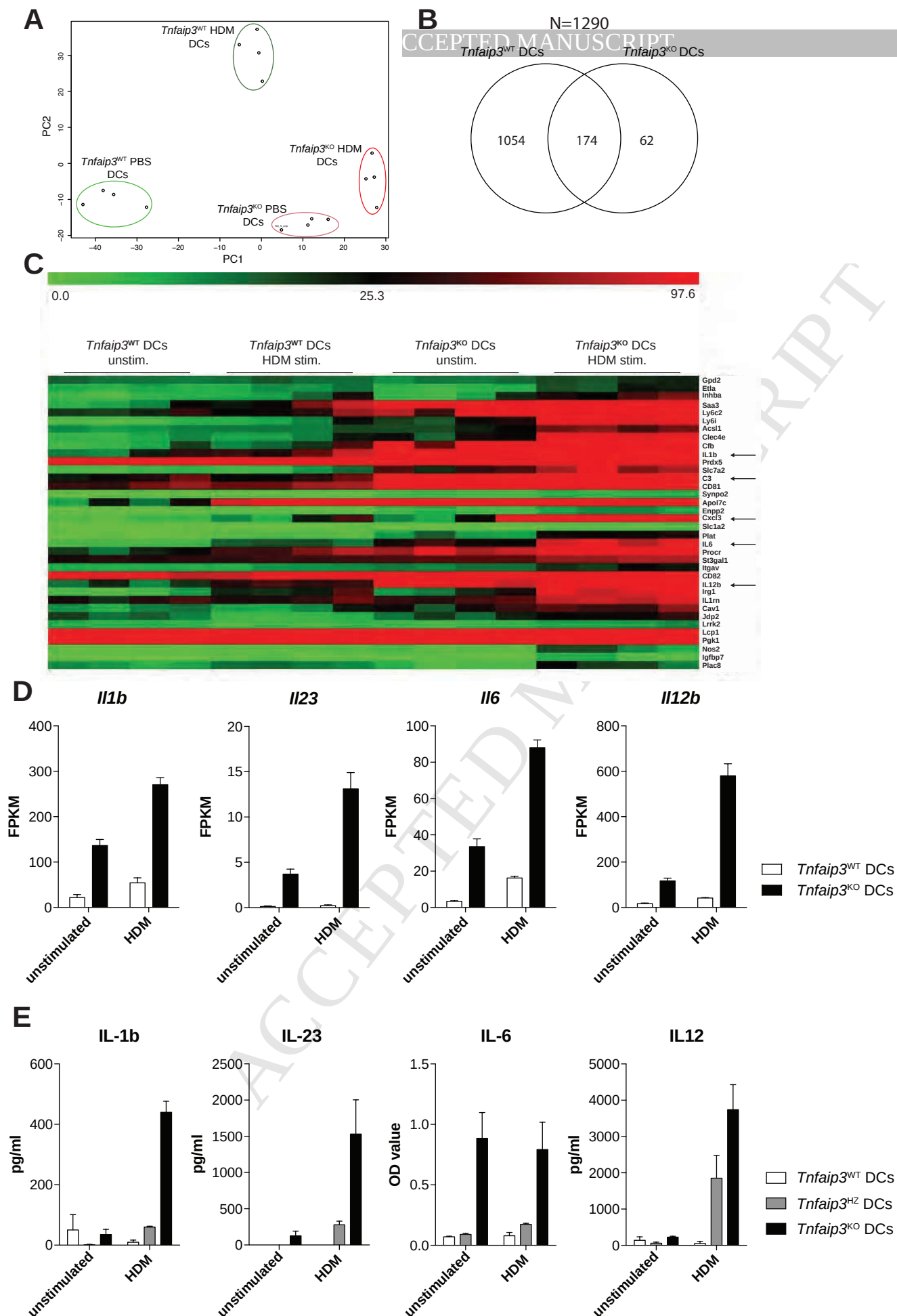


Figure 8: increased expression of Th17 polarizing cytokines in IL-23^{YFP} x *Tnfaip3*^{CD11c-KO} mice

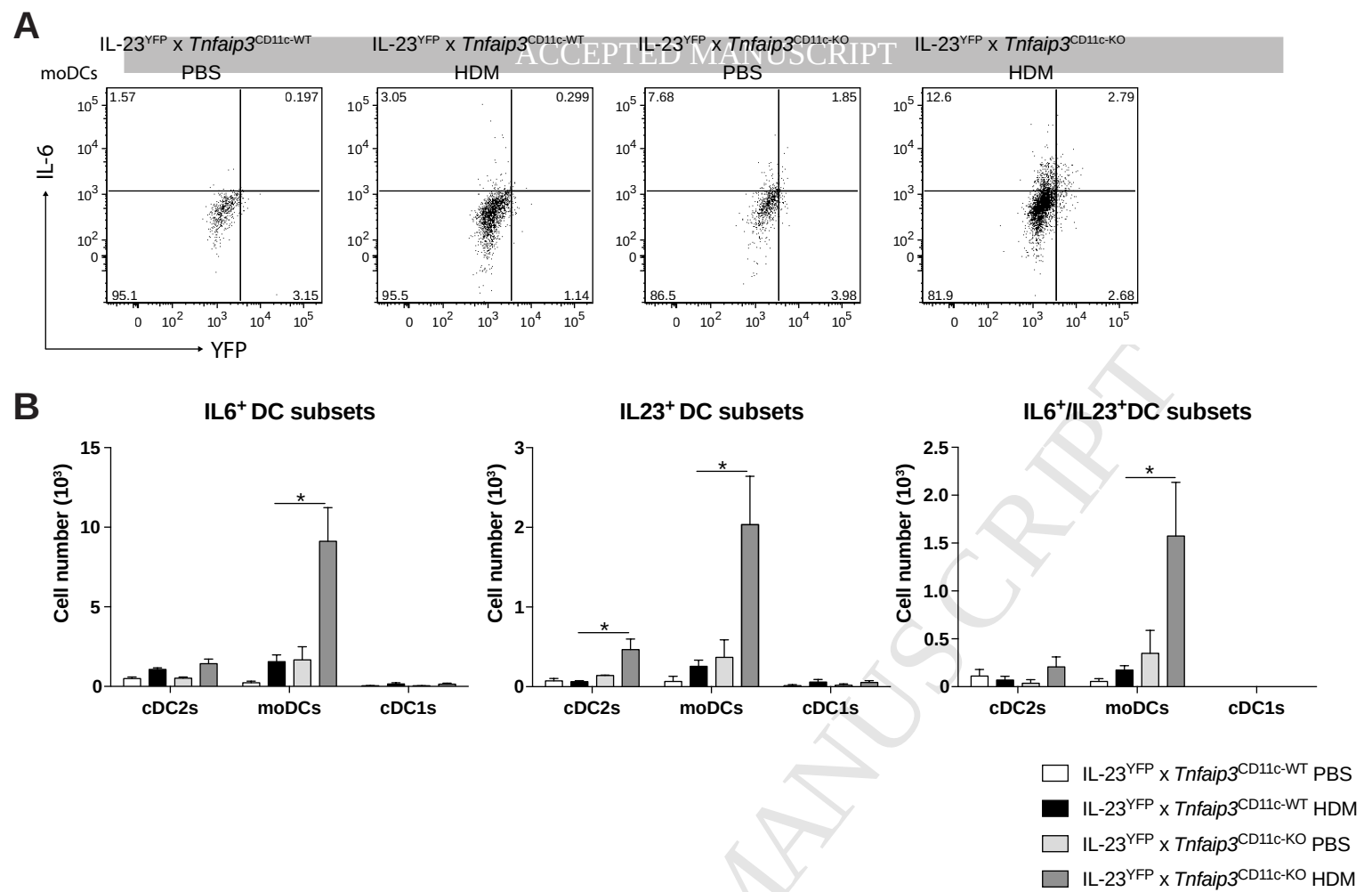


Table S1 Antibodies used for flow cytometry

Antibody	Conjugate	Clone	Company
CD103	eF450	2E7	eBioscience
CD11b	PerCP-Cy5.5	M1/70	BD Biosciences
CD11c	PE-TxR	N418	eBiosciences
CD19	APC-eFluor 780	ID3	eBiosciences
CD3	PE-CF594	145-2C11	BD Biosciences
CD3	APC-eF780	17A2	eBiosciences
CD4	BV711	RM4-5	BD Biosciences
CD4	BV605	L3T4	BD Biosciences
CD45.1	APC-Cy7	A20	BD Biosciences
CD45.2	BV711	104	BD Biosciences
CD64	BV711	X54-5/7.1	Biolegend
CD8	PE-Cy7	53-6.7	eBioscience
FoxP3	PE-Cy7	FJK-16s	eBioscience
GATA3	APC	TWAI-14	eBioscience
GR-1	Pe-Cy7	RB6-8C5	eBiosciences
IFN γ	EF450	XMG1.2	eBioscience
IL-13	EF660	eBio13A	eBioscience
IL-17A	AF700	TC11-18H10.1	BD Biosciences
IL-5	PE	TRFK-5	BD Biosciences
IL-6	PE	MP5-20F3	BD Biosciences
MHCII	Alexa Fluor 700	M5/114.15.2	eBiosciences
RoRgt	PE	Q31-378	BD Biosciences
Siglec F	PE	E50-2440	BD Biosciences
T-bet	eF450	O4-46	BD Biosciences

Gene Set Name	# Genes in Gene Set (K)	# Genes in Overlap (k)	k/K	p-value	FDR q-value
IMMUNE_SYSTEM	933	43	0.0461	1.34E-20	1.78E-17
MATRISOME	1028	44	0.0428	7.8E-20	5.19E-17
LYSOSOME	121	19	0.1570	1.91E-19	8.46E-17
MATRISOME_ASSOCIATED	753	36	0.0478	6.77E-18	2.25E-15
INNATE_IMMUNE_SYSTEM	279	22	0.0789	9.00E-16	2.39E-13
HIF1_TFPATHWAY	66	12	0.1818	1.45E-13	3.21E-11
METABOLISM_OF_LIPIDS_AND_LIPOPR OTEINS	478	24	0.0502	9.07E-13	1.72E-10
CYTOKINE_CYTOKINE_RECEPTOR_INTE RACTION	267	18	0.0674	5.45E-12	8.1E-10
TRAFFICKING_AND_PROCESSING_OF_E NDOSOMAL_TLR	14	7	0.5000	5.48E-12	8.1E-10
ECM_REGULATORS	238	17	0.0714	8.52E-12	1.13E-9
ADAPTIVE_IMMUNE_SYSTEM	539	24	0.0445	1.13E-11	1.36E-9
TNF_PATHWAY	46	9	0.1957	8.37E-11	8.21E-9
LIPID_DIGESTION_MOBILIZATION_AND _TRANSPORT	46	9	0.1957	8.37E-11	8.21E-9
CD40_PATHWAY	31	8	0.2581	8.65E-11	8.21E-9
TOLL_RECEPTOR_CASCADES	118	12	0.1017	1.75E-10	1.55E-8
TOLL_LIKE_RECEPTOR_SIGNALING_PAT HWAY	102	11	0.1078	5.36E-10	4.45E-8
HEMOSTASIS	466	20	0.0429	1.14E-9	8.93E-8
PATHWAYS_IN_CANCER	328	17	0.0518	1.24E-9	9.17E-8
LIPOPROTEIN_METABOLISM	28	7	0.2500	1.72E-9	1.2E-7
INTEGRIN2_PATHWAY	29	7	0.2414	2.25E-9	1.5E-7

*Top 20 upregulated canonical pathways in *Tnfaip3*-KO DCs (from MSigDB database)

Gene Set Name	# Genes in Gene Set (K)	# Genes in Overlap (k)	k/K	p-value	FDR q-value
IMMUNE_SYSTEM	933	54	0.0579	3.23E-23	4.29E-20
HEMOSTASIS	466	33	0.0708	3.9E-17	2.59E-14
ADAPTIVE_IMMUNE_SYSTEM	539	35	0.0649	6.22E-17	2.74E-14
METABOLISM_OF_LIPIDS_AND_LIPOPR OTEINS	478	33	0.0690	8.24E-17	2.74E-14
REGULATION_OF_ACTIN_CYTOSKELET ON	216	20	0.0926	4.88E-13	1.3E-10
CDC42_PATHWAY	70	13	0.1857	7.65E-13	1.7E-10
LEUKOCYTE_TRANSENDOTHELIAL_MIG RATION	118	15	0.1271	4.18E-12	7.94E-10
ECADHERIN_NASCENT_AJ_PATHWAY	39	10	0.2564	1.1E-11	1.66E-9
ERBB1_DOWNSTREAM_PATHWAY	105	14	0.1333	1.12E-11	1.66E-9
PDGFRB_PATHWAY	129	15	0.1163	1.55E-11	2.06E-9
PLATELET_ACTIVATION_SIGNALING_A ND_AGGREGATION	208	18	0.0865	2.23E-11	2.7E-9
GASTRIN_CREB_SIGNALLING_PATHWA Y_VIA_PKC_AND_MAPK	205	17	0.0829	1.57E-10	1.74E-8
RAC1_PATHWAY	54	10	0.1852	3.58E-10	3.58E-8
MHC_CLASS_II_ANTIGEN_PRESENTATI ON	91	12	0.1319	3.83E-10	3.58E-8
SIGNALING_BY_RHO_GTPASES	113	13	0.1150	4.04E-10	3.58E-8
AXON_GUIDANCE	251	18	0.0717	4.88E-10	4.05E-8
FATTY_ACYL_COA_BIOSYNTHESIS	18	7	0.3889	5.25E-10	4.1E-8
B_CELL_RECEPTOR_SIGNALING_PATH WAY	75	11	0.1467	6.45E-10	4.76E-8
DEVELOPMENTAL_BIOLOGY	396	22	0.0556	7.66E-10	5.36E-8
FC_GAMMA_R_MEDIATED_PHAGOCYT OSIS	97	12	0.1237	8.15E-10	5.42E-8

*Top 20 downregulated canonical pathways in *Tnfrsf3*-KO DCs
(from MSigDB database)

Supplementary Figure Legends

Supplementary figure 1: Allergen uptake and migration is not affected in *Tnfaip3*^{LysM} mice. (A) Percentage YFP-expressing cDC1s, cDC2s and moDCs in lung or MLN of PBS or 100 µg HDM-treated *Tnfaip3*^{LysM} mice by flow cytometry. (B) Percentage of HDM-expressing cDC1, cDC2 or moDCs in the lungs of *Tnfaip3*^{LysM} mice, 1 or 3 days after 100 µg HDM-treatment as measured by flow cytometry. Percentage of HDM-expressing cDC1, cDC2 or moDCs in the MLN of *Tnfaip3*^{LysM} mice, 1 or 3 days after 100 µg HDM-treatment as measured by flow cytometry. Results are presented as mean ±SEM of n=3-4 mice per group.

Supplementary figure 2: IL-5-expressing CD4⁺ T cells in the BAL of *Tnfaip3*^{LysM} and *Tnfaip3*^{CD11c} mice IL-5-expressing CD4⁺ T cells in the BAL of *Tnfaip3*^{LysM} mice (A) and *Tnfaip3*^{CD11c} mice (B) measured by intracellular flow cytometry. Results are presented as mean ±SEM of n=4-6 per group. * = p<0.05, ** = p<0.01.

Supplementary figure 3: TNFAIP3 deletion increases the number of pulmonary moDCs. Quantification of pulmonary DC subsets in naïve *Tnfaip3*^{CD11c} mice by flow cytometry. Results are presented as mean ±SEM of n=3-5 mice per group. * = p<0.05.

Supplementary figure 4: Composition of moDCs and cDCs in GM-CSF cultured BM-DCs. Flow cytometric analysis of the composition of moDCs and cDCs in GM-CSF cultured BM-DCs.

Supplementary figure 5: Deletion of exon 4 and 5 in TNFAIP3-deficient GM-CSF cultured BM DCs. Reads of RNA sequencing of total mRNA from GM-CSF BM-cultured *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs mapped to *Tnfaip3* gene, visualized with IGV.

Supplementary figure 6: Cultured TNFAIP3-deficient moDCs preferentially induce IL-17 expression by OT-II cells. (A) GM-CSF-cultured *Tnfaip3*^{WT}, *Tnfaip3*^{HZ} and *Tnfaip3*^{KO} moDCs were stimulated with HDM/OVA overnight and thereafter placed in a 4-day co-culture with OVA-TCR Tg (OT-II) Th cells. IL-5, IL-13, and IL-17A production were determined by intracellular staining. (B) GM-CSF-cultured moDCs or sorted Flt3L-cultured cDC1s, cDC2s and pDCs were stimulated with OVA/LPS and placed in coculture with OT-II cells. IL-17 expression was measured by intracellular flow cytometry. Results are presented as mean $\pm$ SEM of n=2-4 mice per group.

Supplementary figure 7: Heatmap of differentially expressed genes between unstimulated TNFAIP3-sufficient and TNFAIP3-deficient GM-CSF cultured BM DCs. RNA sequencing analysis of total mRNA from unstimulated GM-CSF BM-cultured DCs. EdgeR analysis identified 934 significantly different genes. Gene expression differences are represented in the heatmap based on MeV hierarchical clustering.

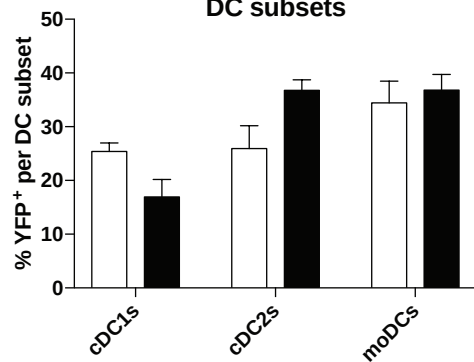
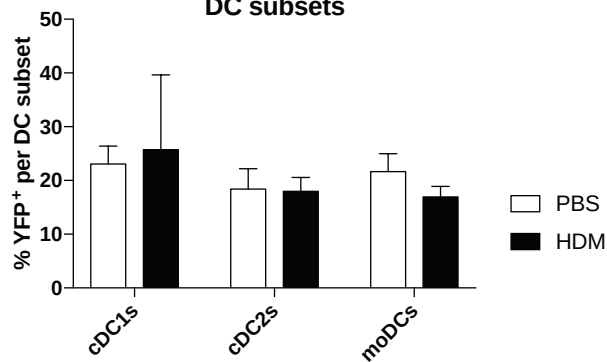
Supplementary figure 8: Heatmap of differentially expressed genes between house dust mite and unstimulated TNFAIP3-sufficient and TNFAIP3-deficient GM-CSF cultured BM-DCs. RNA sequencing analysis of total mRNA from PBS/HDM stimulated GM-CSF BM-cultured DCs. EdgeR analysis identified 1290 significantly different genes. Gene expression differences are represented in the heatmap based on MeV hierarchical clustering.

Supplementary figure 9: Expression of negative regulators of NF- κ B signalling. FPKM values of Tollip, Sigirr, Socs1 and Irak3 in GM-CSF-cultured *Tnfaip3*^{WT}, *Tnfaip3*^{HZ}, and *Tnfaip3*^{KO} moDCs.

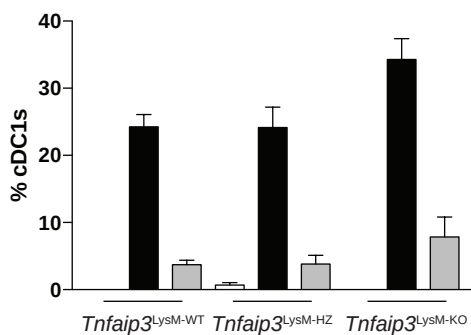
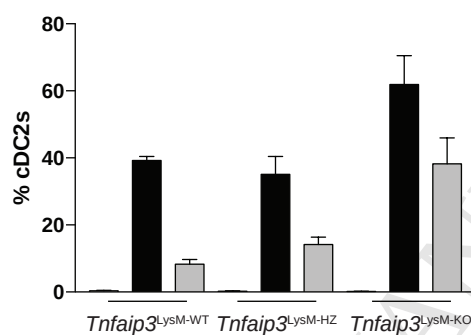
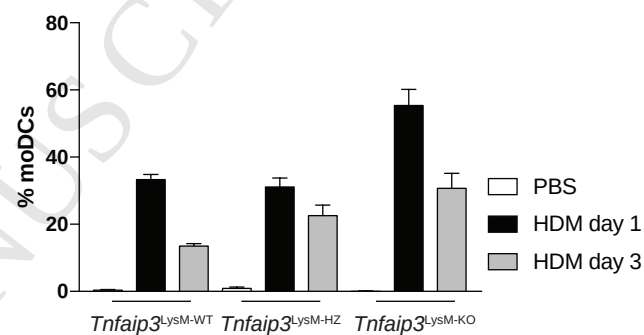
Supplementary Table Legends:**Table S1: Antibodies used for flow cytometry**

Table S2: Canonical pathways upregulated in TNFAIP3-deficient GM-CSF cultured moDCs. Top 20 upregulated canonical pathways between GM-CSF cultured *Tnfaip3*^{WT} and *Tnfaip3*^{KO} moDCs (from MSigDB database).

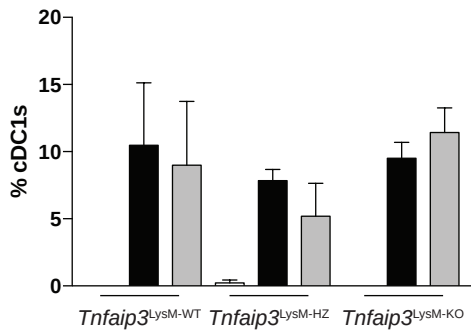
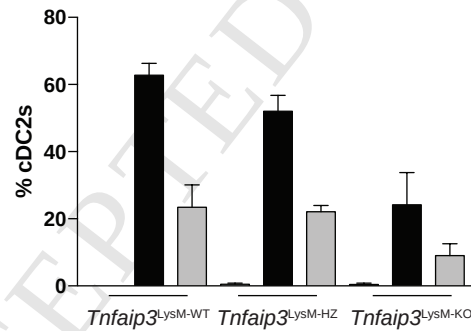
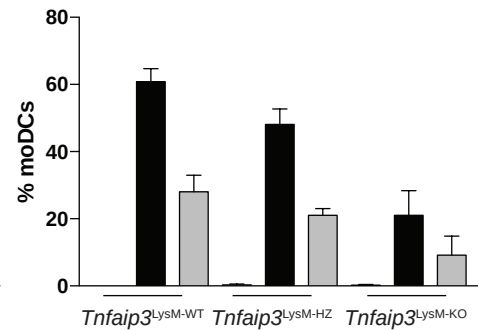
Table S3: Canonical pathways downregulated in TNFAIP3-deficient GM-CSF cultured moDCs. Top 20 downregulated canonical pathways between GM-CSF cultured *Tnfaip3*^{WT} and *Tnfaip3*^{KO} moDCs (from MSigDB database).

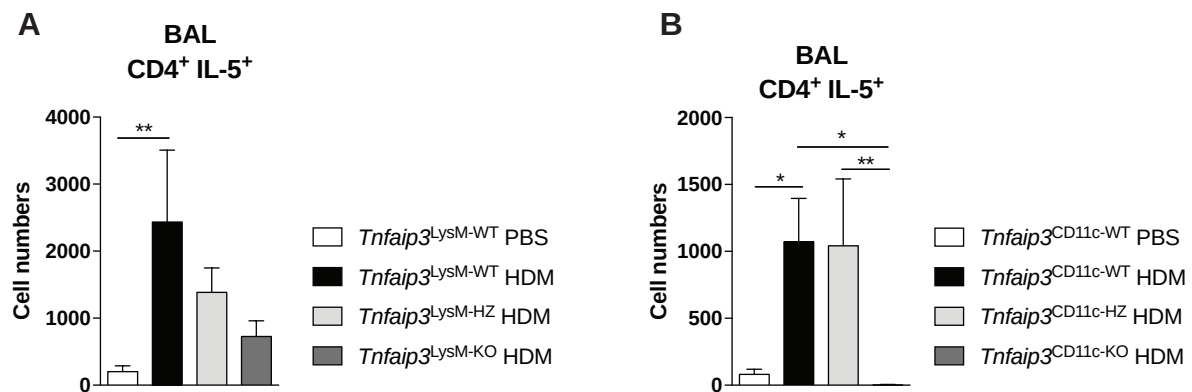
AAllergen uptake**Lung
DC subsets**Migration**MLN
DC subsets**

□ PBS
■ HDM

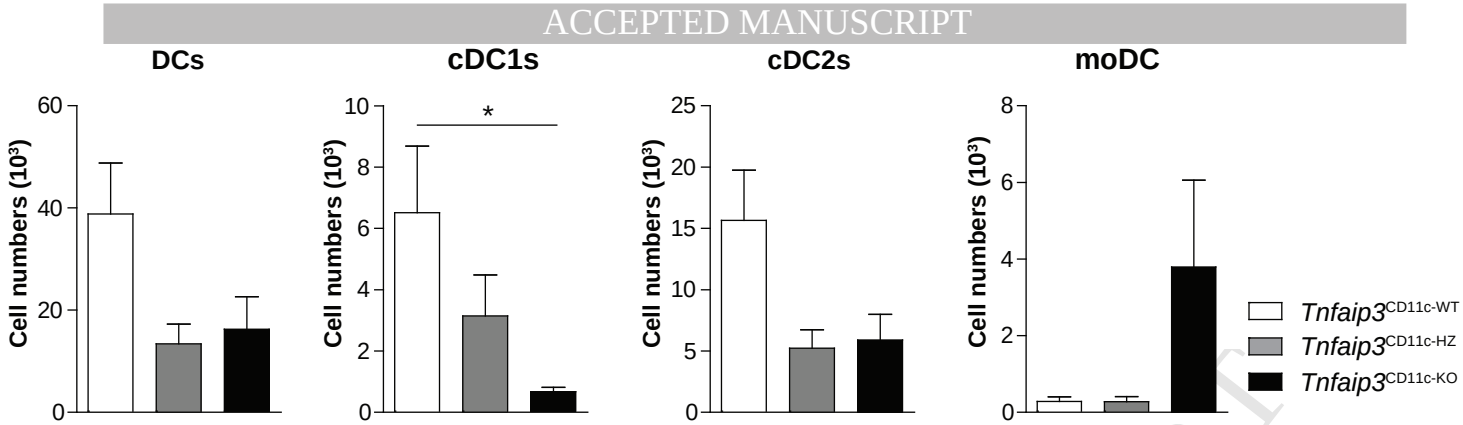
BAllergen uptake**Lung
HDM+ cDC1s****Lung
HDM+ cDC2s****Lung
HDM+ moDCs**

□ PBS
■ HDM day 1
■ HDM day 3

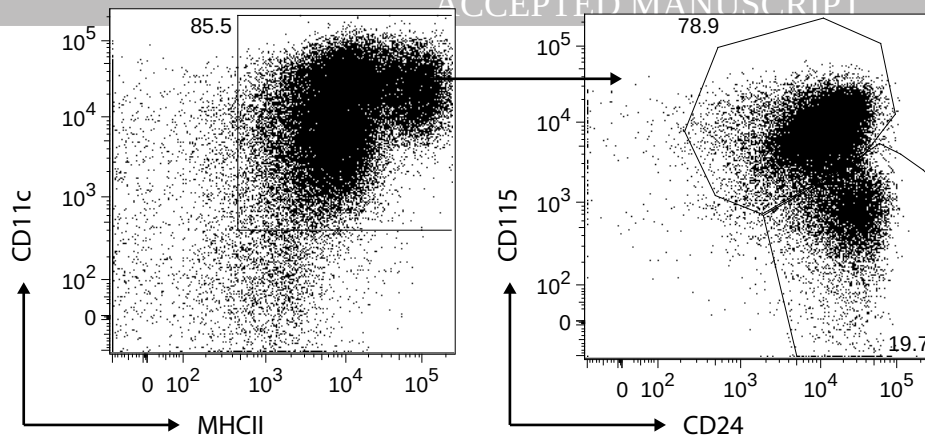
CMigration**MLN
HDM+ cDC1s****MLN
HDM+ cDC2s****MLN
HDM+ moDCs**



Supplementary Figure 3: DC subsets in naive *Tnfaip3*^{CD11c} mice



Tnfrp3^{CD11c-WT} BM-derived DCs



Tnfrp3^{CD11c-KO} BM-derived DCs

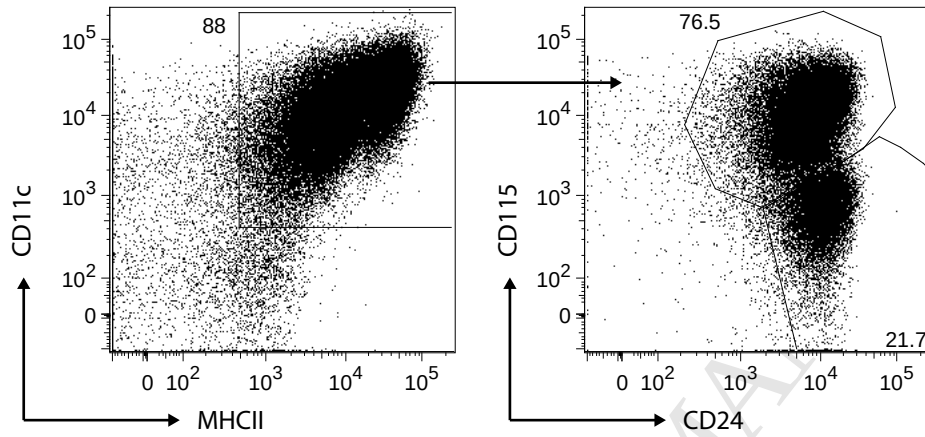
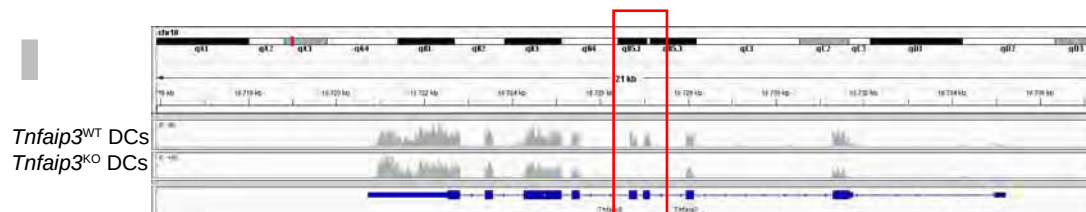


Figure S5: Exon 4 and 5 are deleted in *Tnfaip3*^{KO} DCs



ACCEPTED MANUSCRIPT

Supplementary Figure 6: Cultured A20-KO moDCs preferentially induce IL-17 secretion in OT-II cells

