Heterogeneity in Asthma

Implications for Dendritic Cell Activation?

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The work described in this thesis was conducted at the Department of Pulmonary Medicine, Erasmus MC, Rotterdam, The Netherlands.

Longfonds and Stichting Astma Bestrijding provided financial support for the printing of this thesis.

Layout: Ridderprint BV - www.ridderprint.nl
Printed by: Ridderprint BV - www.ridderprint.nl

ISBN: 978-94-6299-583-3

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Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

Vrijdag 23 juni 2017 om 13.30 uur

Heleen Vroman
geboren te Goes

Erasmus University Rotterdam

Erasmus

PROMOTIECOMMISSIE:

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Part of Introduction I was published as a review in Immunobiology 2015; Feb;220(2):254-61

MODE OF DENDRITIC CELL ACTIVATION: THE DECISIVE HAND IN TH2/TH17 CELL DIFFERENTIATION. IMPLICATION IN ASTHMA SEVERITY?

Asthma is a heterogeneous chronic inflammatory disease of the airways, which is initiated by exposure to environmental factors (1). Phenotypically, asthma can be classified as mild, moderate or severe according to National Asthma Education and Prevention Program, Global Initiative for Asthma (GINA), or American Thoracic Society (ATS) guidelines (2,3). Mild to moderate asthma is initiated by an allergic response to allergens and often referred to as allergic asthma. It is defined by airway hyper responsiveness (AHR) of the bronchioles to aspecific stimuli, increased mucus production and airway remodelling. T helper (Th) 2 cells initiate allergic immune responses in mild to moderate asthma. Th2 cytokines are produced by both Th2 cells (IL-4, IL-5, and IL-13) and type 2 innate lymphoid cells (ILCs) (IL-5 and IL-13). These cytokines facilitate the classical allergic responses, such as IgE class switching by B cells (IL-4), eosinophilic infiltration (IL-5), and goblet cell hyperplasia (IL-13) (4,5). In contrast, severe asthmatic patients display an aggravated asthmatic phenotype, with severe and increased number of exacerbations and patients are less responsive to corticosteroid treatment (6). Severe asthma patients are often non-allergic, but display asthmatic symptoms in response to other stimuli such as diesel exhaust particles (DEP), viruses, and cigarette smoke (7,8). Inflammation in severe asthma patients is generally defined by a neutrophilic or mixed eosinophilic/neutrophilic infiltration. The neutrophilic influx is thought to be mediated by Th17 cells, however Th1 cells are also associated with severe asthma. Moderate and severe asthma patients display elevated levels of IL-17A in both bronchoalveolar lavage (BAL) fluid and lung biopsies (9,10). Th17 cytokines mediate an increased infiltration of neutrophils by activating the epithelium to secrete the neutrophil chemo-attractant CXCL8 (11). Additionally, Th17 cytokines will mediate increased mucus production (IL-17) and severe airway remodelling (IL-17/IL-22) leading to recurrent exacerbations (12). Dendritic cells (DCs) are potent antigen presenting cells (APCs), which are essential for inducing and priming of proper Th responses (13,14). DCs are crucial for both the initiation as well as the maintenance of the inflammatory asthmatic response (15,16). In this introduction, we will discuss a potential role for DC activation as initiator for Th2, Th1 or Th17-driven asthma.

IMMUNOLOGY OF HOUSE DUST MITE DRIVEN ASTHMA

Sensitization to house dust mite is pivotal for the development of most acute childhood asthma, since up to 85% of patients with recurrent asthma are allergic to HDM during hospitalization (17,18). HDM contains at least 22 different allergen groups, recently reviewed by Jacquet *et al* (19). Besides HDM allergens, microbial pattern associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), chitin and β-glucans are frequently found in mite

excretes. Several allergen groups within HDM, like Derp1 and Derp3 display protease activity which can damage the airway epithelial layer and allow entrance to the lungs (19). Airway DCs are exposed to HDM, take up the allergen, become activated, and undergo maturation. During activation, CCR7 will be upregulated, necessary for DC migration towards the draining mediastinal lymph node (MLN) (20). In the MLN, fully matured DCs activate naïve T cells to proliferate and differentiate into effector T helper cells (21) (**Figure 1**).

LUNG DC SUBSETS

Lungs can be divided into the large conducting airways and the interstitium containing alveolar septa and capillaries. It has become clear that at least four $CD11c^{+}MHCII^{+}$ DC subsets can be found in the lungs of mice. During steady state, three different DC subsets can be found. These three DC subsets contain conventional DCs (cDCs), consisting of 2 subsets: $CD103^{+}$ cDC1s and $CD11b^{+}$ cDC2s, and plasmacytoid DCs (pDCs) (22). cDC1s underline the epithelium of the large conducting airways and have the potential to protrude their dendrites into the lumen of the airways in an active search for antigens. cDC2s can be found in the lamina propria underneath the epithelium (21). For optimal Th2 cell priming after HDM exposure, this cDC2 subset is fundamental (23). During an inflammatory response, a fourth DC subset arises in the lung, the inflammatory monocyte-derived DC (moDC), which will amplify the secondary local inflammatory response. Lung DC subsets are reviewed in more detail by Lambrecht *et al* (21,23).

HOUSE DUST MITE MEDIATED RECOGNITION, UPTAKE AND ACTIVATION OF DCS

HDM can be recognized by specific pattern recognition receptors (PPRs) that are expressed by both immune cells as well by epithelial cells. PPRs can be subdivided into C-type lectin receptors (CLRs), Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (19,24). PPRs recognize PAMPs as well as damage-associated molecular patterns (DAMPs). Beside containing several PAMPs, HDM can also lead to DAMP release in a TLR4 dependent manner by airway epithelial cells, such as heat shock proteins, ATP, and uric acid and enhance Th2 immunity (13,25). For instance, uric acid is released after allergen exposure in mice and asthmatic patients and is critical for Th2 sensitization and the secondary inflammatory response (13).

HDM recognition and uptake by DCs is mediated through binding with specific CLRs. The mannose receptor (MR) mediates recognition and uptake of Derp1 (26), whereas both Derp1 and Derp2 are recognized and taken up by DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (27-29). DCs of HDM-allergic patients show a higher MR expres-

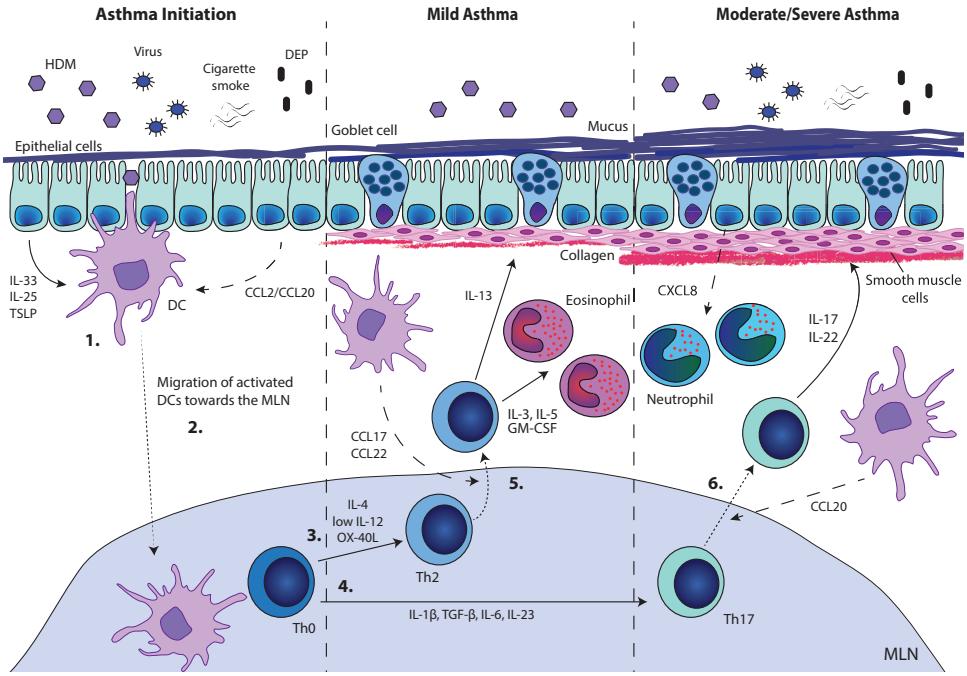

Figure 1: Asthma pathogenesis in both mild as moderate to severe asthma. Upon primary exposure with HDM, with or without viruses, cigarette smoke and DEP, the airway epithelium and underlying DCs are activated. (1) Activated airway epithelial cells will secrete CCL2 and CCL20, necessary for the attraction of immature DCs to the lungs. IL-33, IL-25, and TSLP secreted by airway epithelial cells will activate DCs. (2) Activated DCs upregulate CCR7 and migrate to the MLN. (3) In mild asthma, activated DCs will prime naïve T cells to differentiate into Th2 cells through expression of OX-40L, and a cytokine milieu consisting of high levels of IL-4 and absence of IL-12. (4) In moderate to severe asthma, Th17 differentiation will be initiated by IL-1β, TGF-β, IL-6. IL-23 production will enhance Th17 cell function. (5) Th2 cells migrate towards the lungs in response to CCL17 and CCL22 expression. Th2 cytokines induces eosinophilia (IL-3, IL-5, GM-CSF), goblet cell hyperplasia (IL-13) and airway hyperresponsiveness (IL-13). (6) Primed Th17 cells will migrate towards the lung in response to CCL20, produced by DCs. Th17 cytokine secretion activates airway epithelial cells (IL-17) to secrete CXCL8, a chemoattractant for neutrophils. Furthermore, IL-17 and IL-22 will induce airway remodelling by increasing smooth muscle cell hyperplasia and collagen deposition.

sion and are more efficient in the uptake of HDM in comparison to DCs of non-allergic patients (26). Allergen uptake by MR also promotes Th2 immunity, whereas MR-deficient DCs promote Th1 responses and in turn protect from Th2 driven allergic responses (30). This is opposite for DC-SIGN; uptake of Derp1 by DC-SIGN induces Th1 differentiation, while DC-SIGN-deficient DCs induce Th2 differentiation (27). Using its enzymatic activity, Derp1 is able to cleave DC-SIGN and thereby promote Th2 cell differentiation conditions (31,32). Low expression of DC-SIGN is also observed on DCs of HDM allergic asthma patients (31), suggesting that DCs of allergic asthma patients are intrinsically biased to induce Th2 cell differentiation.

Derp2 has been shown to signal via TLR2 (33) and TLR4 (34). Due to its homology with myeloid differentiation 2 (MD2), which participates in the recognition of LPS by TLR4, Derp2 forms a complex with TLR4 that signals similarly to the MD2/TLR4 complex and activates the innate immune system. Recent reports reveal the importance of TLR4 signalling in the development of allergic asthma. TLR4-deficient mice were not capable of mounting HDM-driven Th2 mediated allergic asthma (35,36). This was mediated by reduced expression of the Th2 promoting co-stimulatory molecule OX-40L on cDCs (36). However, Hammad *et al* (35) showed that TLR4 expression was dispensable on DCs but crucial on airway epithelium cells for the development of HDM-driven allergic asthma (35). Ligation of TLR2 and TLR4 will trigger a downstream signalling cascade leading to NF-κB activation. NF-κB activation will promote transcription of both pro-inflammatory cytokines as well as co-stimulatory molecules imperative for DC maturation.

T HELPER CELL PRIMING CONDITIONS BY DCS

Fully activated mature DCs express high levels of MHCII molecules that present allergen (Ag)-derived peptides. These MHC-II-Ag complexes will engage with T-cell receptors (TCR) on naïve Ag-specific $CD4^+$ Th cells. This activation together with expression of co-stimulatory molecules and cytokines will determine Th cell differentiation. At this moment, at least five different Th cell subsets have been described (Th1, Th2, Th17, follicular T helper cells (Tfh) and regulatory T cells (Tregs)). Each Th cell subset expresses a unique set of transcription factors and cytokines. Th cell differentiation requires the expression of a master transcription factor together with particular signal transducer and activator of transcription (STAT) proteins. Th2 cells are associated with GATA-binding protein 3 (GATA-3)/STAT5, Th1 cells with T-box transcription factor expressed in T cells (T-bet)/STAT4, Th17 cells with retinoid orphan receptor γt (RORγt)/STAT3, Tfh cells with B-cell lymphoma (Bcl-6)/STAT3 and finally Tregs with forkhead box protein 3 (Foxp3)/STAT5 (37).

Th2 cell priming conditions mediated by DCs

Th2 cells play an important role in the defence against helminth infections, but are also implicated in allergic diseases, such as atopy and asthma. Th2 cell differentiation is induced upon TCR stimulation in combination with Th2 cell favoring co-stimulatory molecules and cytokines expressed by DCs. Low dose antigen has been reported to favor specific Th2 cell differentiation (38,39). Besides low antigen dose, co-stimulatory molecules such as OX-40L and Jagged expression on DCs are required for optimal Th2 cell priming(40-43). OX-40L is induced after CD40L-CD40 interaction (41). CD40 expression itself is important for Th2 induction, as CD40-deficient DCs were incapable of inducing Th2 cell responses to helminth antigens (44). Additionally, differentiation of Th2 cells depends on a high IL-4 and low IL-12 environment.

IL-4 is not produced by DCs, but is produced by other cells, such as mast cells and basophils (45). The master transcription factor for Th2 cells GATA-3 is induced upon low antigen dose stimulation in naïve T cells. GATA-3 activation will induce IL-2 production. IL-2 expression in turn activates STAT5 in differentiating Th2 cells. STAT5 in combination with GATA-3 induces TCR dependent, IL-4 transcription. This endogenous produced IL-4 will continue STAT5 activation and determine Th2 cell fate (46). Furthermore, IL-21-producing Tfh cells can enhance Th2 cell function, as eosinophilia is reduced in mice lacking the IL-21 receptor (47). Moreover, evidence was provided supporting that Th2 cells can originate from IL-4-producing Tfh cells that upon secondary exposure to HDM further differentiate into IL-4 and IL-13-producing Th2 cells (48).

Th1 cell priming conditions by DCs

Th1 cells play an important role in fighting intracellular microbial and viral infections, but are also implicated in asthma. In contrast to Th2 cells, Th1 cells require a high antigen dose for their differentiation (38,39) and the expression of the co-stimulatory molecule Delta (43,49). DCs activated through CD40 or high dose antigens produce increased amounts of IL-12 and type I interferons which drive Th1 cell differentiation (50,51). IL-12 and type I interferon will activate STAT4 in naïve Th cells, whereas strong TCR signalling directly activates STAT1. STAT1 will induce T-bet expression, whereas STAT4 will activate IFNγ production. Induction of T-bet increases the expression of IL-12R, providing a positive feedback loop for further Th1 cell differentiation (52).

Th17 cell priming conditions by DCs

Th17 cells play an important role in protection against bacterial and fungal infections, and in the development of autoimmune diseases. Recent evidence has shown that Th17 cells are also implicated in moderate to severe asthma (11). In contrast to both Th1 and Th2 development, Th17 generation requires an even higher antigen concentration (53). High antigen concentration increases CD40L expression on naïve T cells (53). CD40 signalling also appears to be crucial in the Th17 cell induction since CD40-deficient DCs were incapable to induce Th17 differentiation (54). Also CD86 expression is crucial as blocking CD86 downregulates DC-dependent Th17 differentiation *in vitro* (54). Differentiation of Th17 cells from naïve T cells is also determined by the cytokine milieu. IL-1β, IL-6, and TGF-β in combination with TCR stimulation will program Th17 cell differentiation. These cytokines will induce IL-23R and RORγt expression in naïve Th cells (54,55). IL-23 will support maintenance and function of Th17 cells (56).

Regulatory T cell priming conditions by DCs

Regulatory T cells (Tregs) can be classified into naturally occurring Tregs (nTregs) and inducible Tregs (iTregs), which both dampen inflammatory immune responses and are essential to maintain self-tolerance (57,58). nTregs develop in the thymus as a distinct Th cell population,

whereas iTregs differentiate in the periphery from $CD4^+$ T cells (59), and can differentiate into ICOS-expressing Tregs. Thymic Tregs are induced by intermediate TCR stimulation and IL-2 and TGF-b signalling, leading to FoxP3 expression. In the periphery, multiple inducible Treg subpopulations are found, such as inducible/adapter Tregs (iTregs), IL-10-producing type 1 Tregs (Tr1 cells), IL-17-producing Tregs, and $CD8^+$ Tregs. Induced Tregs, IL-17-producing and $CD8^+$ Tregs share the expression of FoxP3, and all iTreg subsets secrete IL-10 with or without TGF-b (60). Inducible Treg differentiation from naïve $CD4^+$ T cells is initiated by weak TCR signals and absence of pro-inflammatory cytokines. TGF-b induces Smad binding to the FoxP3 locus and promotes transcription in iTregs (61-63). Furthermore, also retinoic acid and PPARg are also implicated in the differentiation of HDM-induced Treg differentiation (64,65). Interestingly, it was shown in mice that recent thymic emigrants are the preferential precursors of regulatory T cells that differentiate in the periphery (66).

TH2-INSTRUCTIVE SIGNALS FOR DC ACTIVATION

It is well described that HDM-driven activation or damage of the airway epithelium is crucial for DC activation in priming efficient Th2 cell immune responses. β-glucan structures in HDM activate dectin-1 on airway epithelial cells, initiating CCL2 and CCL20 secretion (67,68). These chemokines attract immature DCs and DC precursors from the bone marrow towards the lungs (67,68). HDM exposure will trigger cytokine production by airway epithelial cells, such as IL-1α, IL-33, thymic stromal lymphopoietin (TSLP), IL-25, and granulocyte-macrophage colony-stimulating factor (GM-CSF) that contribute to DC activation and provide Th2-instructive signals to the DCs (14,69,70). IL-33 and IL-1RL1 (which encodes ST2/IL-33R) are also identified by Genome Wide Association Studies (GWAS) as asthma susceptibility loci (71), indicating the importance of IL-33 and IL-33R for the development of Th2-driven asthma. IL-33 signalling through IL-33R/ST-2 on DCs leads to the production of Th2-skewing co-stimulatory molecules and cytokines/chemokines, such as OX-40L, CD40 and IL-6, IL-1β, TNFα, and CCL17 (72-74). Importantly, no IL-12 and/or IL-23 are induced (75), and in neonatal mice, IL-33 even suppresses IL-12p35 expression in cDC2s (74). Thus, IL-33-stimulated DCs particularly induce Th2 differentiation from naïve Th cells (73). DCs can also produce IL-33 themselves, which can act in an autocrine manner to further amplify the Th2 driving capacity (76). DCs stimulated with Th2 promoting stimuli, such as low dose HDM induce interferon regulatory factor-4 (IRF4) signalling (77). IRF4 signalling in DCs induces IL-33 and IL-10 secretion contributing to Th2 cell differentiation. Moreover, IRF4 is crucial for Th2-driven allergic asthma, since mice deficient of IRF4 in DCs fail to develop allergic asthma (77). Classically IRF4 and IRF8 are thought to be involved in the development of different DC subsets. Current evidence shows that IRF molecules are also

implication in directing Th differentiation by DCs (77,78), as IRF4 drives DCs to promote Th2 immune responses (77,79) (**Figure 2A**).

Next to IL-33, TSLP is an additional Th2 instructing cytokine for DC activation. Inhaled allergen exposure increased the expression of TSLP receptor on bone marrow derived cDCs of asthmatic subjects (80). TSLP stimulates Th2 differentiation by inducing OX-40L expression on DCs in the absence of IL-12 (81-83). TSLP-activated DCs will also produce CCL17 and CCL22, necessary for effector Th2 cell migration towards the lungs (81,84,85). TSLP signalling induces activation of STAT6, which programs DCs to secrete these chemokines. In addition, TSLP signalling limits activation of STAT4 and IRF-8, essential factors for the production of the Th1 polarizing cytokine IL-12 (78). Next to IL-33, TSLP also induces IRF4 expression through STAT5 activation in human DCs (82,86). TSLP can also directly influence T cells, since it is a potent growth and survival factor for Th2 cells (87,88).

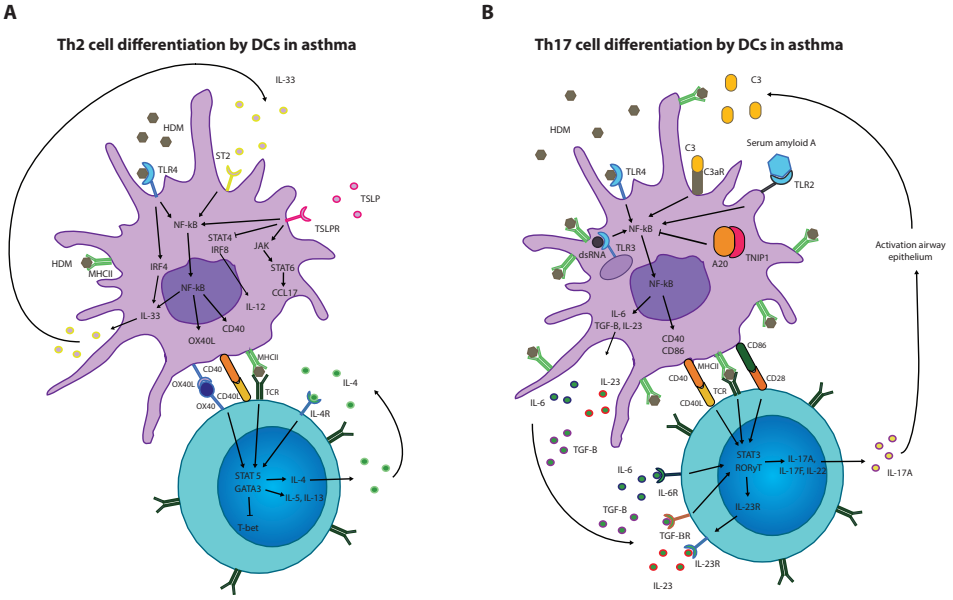

Figure 2: T helper cell differentiation mediated by DCs in asthma. DC activation is crucial for efficient Th cell differentiation. (A) Th2 cell differentiation is induced upon low antigen dose, OX-40L expression and IL-4 presence in the absence of IL-12. Activation of DCs in a Th2 promoting manner is initiated by cytokines secreted by the airway epithelium, like TSLP and IL-33. TSLP signalling specifically suppresses IL-12 secretion while inducing CCL17 expression. TLR4 signalling initiated by HDM induces increased OX-40L and IL-33 expression. IL-33 can signal in an autocrine manner, increasing the Th2 promoting activation of DCs. (B) Th17 cell differentiation is induced by high antigen dose, CD40 and CD86 expression in addition with IL-6 and TGF-β. Expression of IL-6 and TGF-β are induced upon strong NF-κB signalling. Increased NF-κB signalling can be initiated by combined signalling of e.g. TLR3/TSLP, C3aR, TLR2 and TLR4. Subsequent expression of IL-23 will maintain Th17 cell function.

The third Th2-instructive cytokine IL-25 can be produced by basophils and epithelial cells in response to allergens (89,90). Inhaled allergen exposure increased the expression of IL-17RB (IL-25 receptor) on both myeloid and pDCs of mild atopic asthma patients (91). Mice overexpressing IL-25 in lung epithelial cells show increased allergic inflammation (89). IL-25 increases both OX-40L and Jagged1 expression by DCs, which contributes to Th2 differentiation (75,90,92). IL-25 expression is also associated with increased levels of CD80 and CD86 on DCs, indicating that IL-25 activates DCs (93). However, Jagged1 and Jagged2 expression on DCs was dispensable for HDM-induced Th2-mediated eosinophilic inflammation (94). IL-25 treatment of DCs induces CCL17 expression, attracting Th9 cells towards the lung (95).

Along this line, also GM-CSF can be produced by airway epithelium cells (96,97). DCs cultured in the presence of GM-CSF are very potent inducers of Th2 cell differentiation (16). HDM-mediated Th2 sensitization in the airways is partially mediated through GM-CSF (98) as GM-CSF neutralization showed reduced Th2 cell responses (99,100).

Recent research has provided better insight in Th2 cell promoting cytokines secreted by the airway epithelium and DCs. However, much remains unknown about underlying signalling pathways initiating the expression of these cytokines and co-stimulatory molecules and their interactions. Research exploring these mechanisms will provide more insight and possible new therapeutic targets.

TH1 MEDIATED IMMUNE RESPONSES IN ASTHMA BY ACTIVATED DCS

Neutrophilic inflammation observed in moderate and severe asthmatic patients has long been associated with a Th1 cell immune response. T-bet-deficient mice develop spontaneous T cell dependent lung inflammation and AHR indicating that Th1 cells are dispensable for allergic asthma (101). However, Th1 cells are increased in severe asthma patients and during asthma exacerbations. Severe asthma patients with mixed granulocytic inflammation display increased levels of IFNγ, TNFα and IL-17 (102-104). These increased levels of IFNγ and TNFα generated the idea that Th1 cells mediate neutrophil inflammation (105). More recent evidence has shown that neutrophil inflammation is mediated by IL-17. IL-17 activates airway epithelium and initiates secretion of CXCL8, the chemo attractant for neutrophils (12). Therefore, neutrophil inflammation observed in moderate to severe asthmatics is now thought to be dependent on a Th17 immune response.

TH17 INSTRUCTIVE SIGNALS FOR DC ACTIVATION IN ASTHMA

The role of Th17 cells in severe asthma is a relative new concept. Both exposure to HDM and environmental triggers, such as DEP and cigarette smoke have been shown to activate the airway epithelium and DCs in a Th17 promoting state. Severe Th17 cell or mixed Th2/Th17 cell-mediated asthma is associated with exposure to DEP. DEP exposure decreases lung function and increases neutrophil biomarkers (106). Exposure of young and adult mice to a combination of DEP and HDM has been shown to provoke an exaggerated Th2 and mixed Th2/Th17 cell mediated inflammatory response in the airways (107,108). Strikingly, collagen content is increased in the lungs of DEP/HDM exposed adult mice, indicative of lung remodelling. Children with allergic asthma show increased IL-17A serum levels upon DEP exposure (109). The underlying mechanism how DEP/HDM exposure results in a mixed Th2/Th17 or a more Th17 immune response remains poorly understood. Induction of Th2 and Th17 cytokines upon combined exposure of OVA and ultrafine particles was Jagged 1 dependent, as Jagged 1 blockage restores this increase in IL-4, IL-13, IFNg and IL-17A (110). DEP exposure induces DC maturation(111,112) through activation of airway epithelium (111). Airway epithelial cell exposure to DEP increases secretion of CCL2 and CCL20 necessary for DC recruitment, and TSLP that is Th2 instructive (107,112,113). In addition, DEP exposure enhances DC migration towards the MLN in a CCR7 dependent manner (112), whereas moDC accumulation upon DEP exposure was dependent on CCR2 and CCR6 (114). Acute infiltration of monocytes and moDCs upon DEP exposure was dependent on G-coupled receptor chemR23 (115). Whether DEP exposure also induces secretion of Th17 promoting cytokines by DCs, such as IL-1β, IL-6 and IL-23 needs further investigation.

In addition to DEP also cigarette smoking is associated with asthma development in non-atopic asthma patients (116). Cigarette smoking asthmatic patients develop more severe asthmatic symptoms, such as corticosteroid resistance, increased neutrophil counts, decreased FEV1 and severe lung remodelling (7,117). Acrolein, one of the chemicals present in mainstream cigarette smoke has been shown to reduce neutrophil apoptosis when administered in combination with LPS. Acrolein induces epithelial barrier dysfunction, thereby promoting neutrophil migration (118). Combined exposure of acrolein and ovalbumin increases neutrophilic airway inflammation (119). Furthermore, acrolein promotes the production of reactive oxygen species (ROS), implicated in degranulation in mast cells (120). Cigarette smoke also increases TLR3 expression in lungs of mice. TLR3 stimulation with Poly I:C (dsRNA) results in exacerbated neutrophil infiltration and AHR (121). Indicating that cigarette smoke enhances susceptibility for viral infections that could attenuate severe asthma exacerbations. Furthermore, cigarette smoking COPD patients show increased levels of IL-22 in serum and IL-17 and IL-22 in sputum. This implies that cigarette smoke specifically leads to increased expression of Th17 cell cytokines (122,123).

Besides environmental factors as DEP and cigarette smoke also other factors are recently associated with severe asthma. Recent evidence suggests a role for NLRP3 inflammasome in severe asthma associated Th17 cell differentiation. NLRP3 is activated by serum amyloid A (SAA), acute phase protein. SAA is produced by airway epithelial cells upon exposure to microbes or microbial particles, such as LPS and pro-inflammatory cytokines. SAA is increased in BAL fluid of asthma patients with a Th2/Th17-predominant phenotype (124). SAA induces production of IL-1α, IL-1β, IL-6, PGE2, and IL-23 by DCs, which are instructive for Th17 cell differentiation (125). Th17-driven severe asthma is associated with corticosteroid resistance and strikingly SAA-stimulated DCs are capable of stimulating Th17 cell responses even after treatment with corticosteroids (126). Moreover, severe asthma patients display higher SAA serum concentrations (127,128), suggestive of a prominent role for SAA and NLRP3 activation in Th17 cell-driven severe asthma. Recently, innate cells producing IL-17, such as ILC3, and the NLRP3 inflammasome have been implicated in obesity-associated airway hyperreactivity (129).

In addition to NLRP3 activation, high dose LPS exposure is also associated with Th1/Th17 mediated severe asthma (130). High dose LPS exposure specifically induces VEGF secretion by the airway epithelium. VEGF induces DC maturation and secretion of Th17 promoting cytokines like IL-6 (131). Up to 50% of asthma exacerbations are associated with viral infections (132). Binding of viruses has been shown to specifically induce CXCL8 secretion by airway epithelium cells, thereby inducing neutrophil inflammation (133). Implicating that viral infection could be a potential driver of neutrophil-associated severe asthma. Administration of Poly I:C (dsRNA, TLR3 ligand) in combination with OVA also increases VEGF secretion, leading to subsequent IL-6 expression and Th17 cell-driven asthma (134). Stimulation of human DCs with TSLP/PolyI:C induces IL-23 production and increases Th17 differentiation (135).

Polymorphisms in complement (C) 3 and C3aR are positively associated with severe asthma, whereas polymorphisms in C5 are negatively associated (136,137). Asthmatic patients show increased C3a and C5a levels after allergen challenge in BAL fluid (138). Asthma patients with a Th2/Th17 high phenotype have increased C3a levels in BAL fluid (138). Thereby, recent interest is arising in role of complement in allergic asthma. Complement signalling has also been shown to promote Th cell effector responses (139,140). Several well-known triggers for asthma, such as HDM activate C3(141). Lajoie *et al* (142) have nicely shown that C3aR signalling induces IL-23 secretion through NF-κB activation, indicating that C3aR signalling promotes Th17 cell function. IL-17 expression by Th17 cells enhances C3 secretion by airway epithelial cells. This provides a positive feedback loop that will further enhance IL-23 production by DCs. In contrast, C5aR signalling induced IL-10 expression via activation of JNK signalling. IL-10 activates AP-1 expression. AP-1 blocks accessibility of the IL-23a promotor region and inhibits Th17 cell function by DCs and maintains homeostasis (142) (**Figure 2B**).

As mentioned above, NF-κB signalling in DCs is crucial for TLR and cytokine driven DC activation and subsequent cytokine expression. Recently single nucleotide polymorphisms (SNPs) in tumor necrosis factor alpha interacting protein 3 (TNFAIP3) interacting protein 1 (TNIP1) were found to be associated with severe asthma (143). TNFAIP3 is a deubiquinating enzyme and deubiquinates signalling molecules downstream of TLR and TNFαR activation, abrogating NF-κB signalling (144) (See box 1). This further indicates the essential role of cell activation in the potential pathogenesis of severe asthma.

TREG INSTRUCTIVE SIGNALS FOR DC ACTIVATION IN ASTHMA

As immunosuppressive cells, Tregs are essential for maintaining immunological tolerance to airborne allergens such as HDM. Depletion of Tregs only at the initiation phase of an HDM-induced allergic asthma model is sufficient to increase airway hyperresponsiveness. Treg depletion increased the frequency and activation status of pulmonary DCs, indicating Tregs not only directly suppress Th2 immune responses through the consumption of IL-2 and secretion of IL-10, but also indirectly by hampering the Th2 differentiating capacity of pulmonary DCs (145). Furthermore, in an ovalbumin-induced asthma model, transfer of Tregs during the peak of airway inflammation was even able to reverse already established airway inflammation and remodelling, whereas no effects were observed on airway hyperresponsiveness (146). The numbers of IL-10-expressing cells are decreased in atopic allergic patients compared to healthy controls (147). Tregs of atopic individuals were also less capable of suppressing allergen-stimulated T cells (148).

Der p 1 treatment on DCs isolated from HDM-sensitive patients decreased indoleamine 2,3-dioxygenase (IDO) expression, an immunoregulatory enzyme involved in Treg differentiation, leading to increased Th2 cell differentiation, whereas IDO expression was increased in Der p 1 treated DCs of non-allergic patients and induced development of IL-10-producing Tregs (149). Treating cultured moDCs with a combination of Der p 2 and IL-10 decreased Th2 differentiation; instead it led to the generation type 1 regulatory T cells (150). Combining Der p 1 treatment with Glucuronoxylomannan, a microbial compound that can modulate immune responses increased TGF-β secretion in DCs and promoted the generation of antigen-specific Tregs (151). Inhaled antigens have also been shown to induce retinaldehyde dehydrogenase 2 (aldh1a2), an enzyme involved in the production of retinoic acid (RA), specifically in pulmonary $CD103^+$ cDC1s. RA is a cofactor for TGF-β induced FoxP3 induction and subsequent Treg development (64). The β-subunit of CK2 in Tregs is also essential to control Th2 differentiation by cDC2s, as Treg-specific deletion of the β-subunit of CK2 induced proliferation of ILT3-expressing Treg cell subpopulation. These Tregs are unable to control IRF4 expression in cDC2s, leading to spontaneous development of pulmonary Th2 cell airway inflammation (152). Furthermore,

CD103-deficient mice develop exaggerated airway inflammation upon exposure to both HDM and ovalbumin-induced allergic asthma models (153). Unfortunately, the effect of CD103-depletion on Tregs was not evaluated. However, the data implicate that $CD103^{+}$ cDC1s and CD103-expressing T cells are essential to maintain airway tolerance.

CONCLUSION

Underlying mechanisms responsible for different immune responses observed in mild and moderate/severe asthma patients are only partially unraveled. However, it is well established that DCs prime Th cell subset differentiation. Recent evidence indicates that the mode of DC activation is of crucial importance for cytokine and co-stimulatory molecule expression. Allergens activate both airway epithelium and DCs, initiating the secretion of IL-33, TSLP, IL-25 and GM-CSF. These Th2 cell promoting cytokines inhibit IL-12 production via IRF8, while inducing OX-40L and Jagged1 expression. In contrast, allergen exposure in combination with environmental factors and/or genetic variations further increase DC activation and induce secretion of IL-1β, IL-6, and IL-23 driving Th17 cell differentiation. How differentiation of Th cells is directed by antigen strength, co-stimulatory molecules, and cytokines expressed by DCs is currently well established. However, the exact molecular signalling pathways responsible for the production of these cytokines and co-stimulatory molecules remain largely unknown. Therefore, molecular signalling pathways activated by various environmental triggers need more investigation. Better insight in the underlying mechanisms driving Th cell differentiation could improve our current understanding of different asthma pathologies.

AIMS AND OUTLINE OF THE THESIS

It is becoming more and more clear that asthma is not a purely Th2-mediated inflammatory disease, but also other Th cell subsets, such as Tfh and Th17 cells contribute to the asthmatic disease. Therefore, in this thesis, we will investigate the importance of DCs as underlying mechanism controlling asthmatic Th2-mediated eosinophilic, Th17-mediated neutrophilic inflammation, or suppress allergic airway inflammation.

Recently it has been shown that Tfh cells can enhance Th2 cell function (47) and that IL-4-expressing Tfh cells have the capacity to differentiate into IL-4 and IL-13 double-expressing Th2 cells upon secondary HDM exposure (48). However, both these studies used acute HDM-mediated allergic asthma models that consist of a sensitization and a challenge phase, which does not correspond to the continuous allergen exposure as occurs in patients. Furthermore, acute HDM-mediated asthma models do not strongly induce airway remodelling as observed in asthma patients. Therefore, we examined in **Chapter 2**, whether Tfh cells and germinal center reactions are also essential for the development of Th2-mediated eosinophilic inflammation in a chronic HDM-driven asthma model in which animals are continuously treated with allergens, resembling the natural exposure of asthma patients. In **Chapter 2**, we observed that follicular T helper (Tfh) cells and B cells are not required for the development of eosinophilic inflammation in the airways in a chronic HDM-driven asthma model. This indicates that the experimental model and antigen dose make an essential difference in how Th2-mediated eosinophilic airway inflammation develops.

As described above, not all asthmatic patients display with a Th2-mediated eosinophilic inflammation. Especially, severe asthma patients can display mixed eosinophilic/neutrophilic or neutrophilic inflammation or even without detectable granulocytic inflammation. Neutrophils observed in severe asthma patients are most likely attracted to the lung due to Th17 cell activation. Patients with severe asthma display elevated levels of IL-17A in both bronchoalveolar lavage (BAL) fluid and lung biopsies (9). Underlying mechanisms that control the development of these two asthma phenotypes are currently unknown. DCs as antigen-presenting cells are unique in their capacities to induce Th cell differentiation and are therefore likely to control the differentiation of either Th2 and/or Th17 cell differentiation in these two different asthma subtypes. The mode of DC activation is of crucial importance for cytokine and co-stimulatory molecule expression, and as described above, Th17 cell differentiation in general requires a higher state of DC activation than Th2 cell differentiation.

In **Chapter 4**, we investigated whether the activation status of DCs affects Th2 and Th17-mediated asthmatic airway inflammation. To investigate this, we used a mouse model in which TNFAIP3, one of the negative regulators of NF-kB, was removed from DCs, leading to increased DC activation (Box 1), and exposed the mice to both acute and chronic HDM-driven asthma models.

Box 1: Canonical NF-κB activation is essential for proper DC activation, which is triggered by TLR activation by for instance HDM. NF-κB signalling is regulated by ubiquitination. K48-linked polyubiquitin chains targets proteins for proteasomal degradation, whereas K63-linked polyubiquitin chains can induce the recruitment of downstream signalling molecules. To ensure proper regulation of NF-κB signalling, special enzymes are involved in the synthesis, recognition and degradation of various types of ubiquitin chains. The ubiquitin-modifying enzyme TNFAIP3 restricts NF-κB signalling downstream of TLR, NOD-like receptors (NLRs), different cytokine-receptors, and CD40 signalling, of which some are implicated in the recognition of HDM **(Figure 3)** (144). Specific deletion of TNFAIP3 in DCs, using *Tnfaip3*$^{fl/fl}$ mice crossed to *Cd11c*-cre mice has shown to increase DC activation(154). TNFAIP3 levels in different cell types has been shown to influence the Th2 cell response as TNFAIP3 levels in epithelial cells protect against development of Th2 immune responses upon HDM activation (155), whereas absence of TNFAIP3 in myeloid cells induces development of Th17 cells and arthritis (156). Lack of TNFAIP3 in mast cells controls the activation state, causing increased plasma extravasation and features of eosinophilic asthma upon allergen challenge (157).

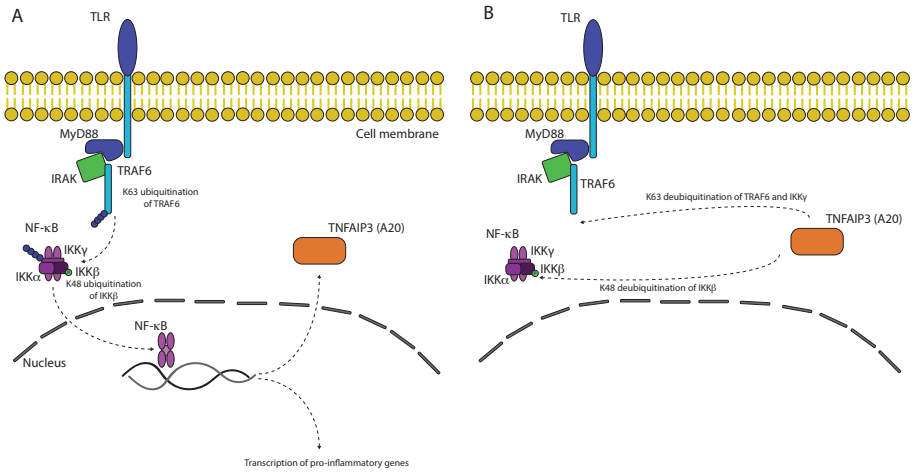

Figure 3: Negative regulation of NF-kB signalling by TNFAIP3. Upon TLR activation, a signalling cascade will be activated inducing K63 ubiquitination of TRAF6. TRAF6 K63 ubiquitination will induce K48 ubiquitination of IKKβ, leading to proteasomal degradation of IKKβ. Upon degradation of IKKβ, NF-kB translocates to the nucleus and start transcription of pro-inflammatory genes but also negative regulators as TNFAIP3/A20 (A). The deubiquitinating enzyme TNFAIP3 deubiquitinates the ubiquitin proteins on TRAF6, IKKβ and IKKγ and thereby abrogates NF-kB signalling (B).

As we found in chapter IV that TNFAIP3 levels in lung DCs controlled development of eosinophilic and neutrophilic airway inflammation, we investigated in **Chapter 5**, whether the observed severe neutrophilic asthma phenotype as observed in mice harboring *Tnfaip3*-deficient DCs was dependent on IL-17R signalling by depleting IL-17RA.

cDC1s were recently described to dampen Th2 immune responses in the case of helminth infections through IL-12 expression (158). Furthermore, cDC1s have also been shown to specifically induce Treg differentiation upon HDM exposure (64), and CD103-deficient mice

develop exaggerated Th2-mediated eosinophilic inflammation upon HDM exposure, indicating the Th2-suppressive capacity of pulmonary cDC1s (153). Therefore, we investigated in **Chapter 6** whether increasing the activation status of specifically cDC1s in the lung would increase their Th2 suppressive capacity in a HDM-driven asthma mouse model.

Finally, in **Chapter 7**, we wondered what the immunological differences in circulating immune cells would be between healthy controls, steroid controlled, partially controlled and uncontrolled asthma patients. These differences were determined based on an automated clustering method, FlowSOM, enabling cluster different cell populations with similar marker expressions.

The implications of our studies and potential directions for future research are presented in the general discussion in **Chapter 8**.

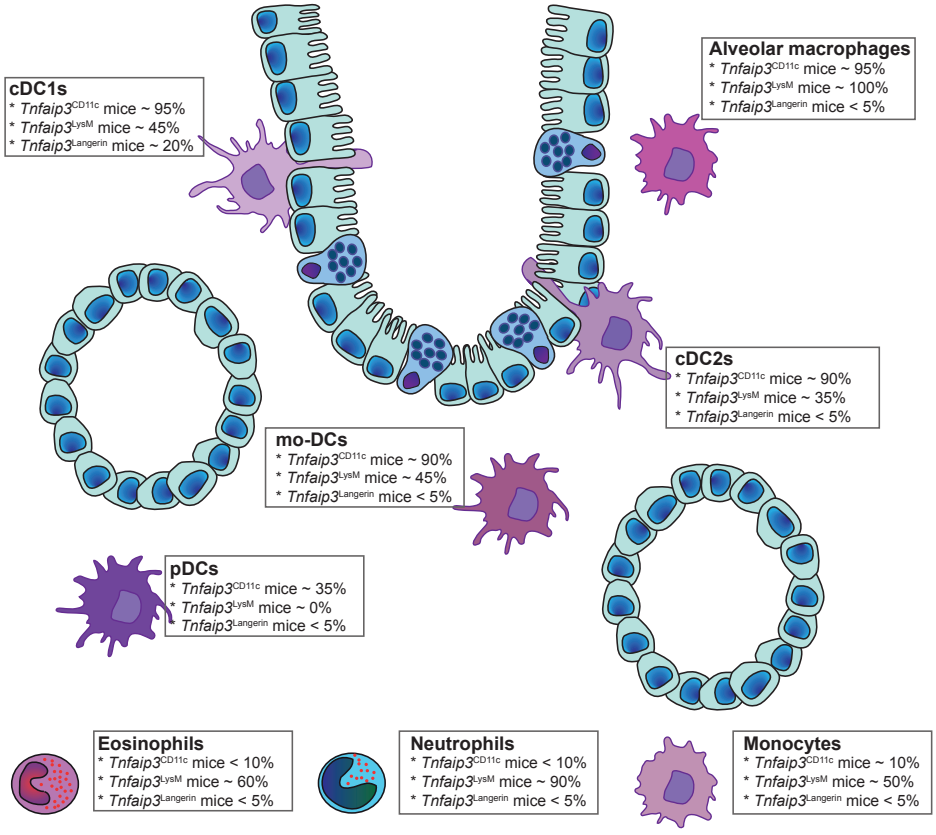

Figure 4: Activation of different DC subsets by targeting TNFAIP3. Graphical representation of the percentage *Tnfaip3*-deficient DC subsets and other myeloid populations in the lung, using either *Lyz2*CRE (*Tnfaip3*^{LysM} mice), *Cd11c*CRE (*Tnfaip3*^{CD11c} mice), or *Langerin*CRE (*Tnfaip3*^{Langerin}).

In this thesis, we made use of different transgene mouse lines that target TNFAIP3 to activate different DC subsets in the lungs and thereby determine the effect on Th cell differentiation upon HDM exposure **(Figure 4)**. The function of these different DC subsets is summarized in **Chapter III.**

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Development of eosinophilic inflammation is independent of B-T cell interaction in a chronic house dust mite-driven asthma model

Clin Exp Allergy 2017; Apr;47(4):551-564

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ABSTRACT

Background: Chronic exposure to environmental triggers, such as house dust mite (HDM), drives T helper 2 (Th2) cell-mediated asthma. Recent evidence has shown that B-T cell interaction, and in particular germinal centre reactions and follicular T helper (Tfh) cells are required for the development of eosinophilic airway inflammation in HDM-driven models containing a sensitization and challenge phase. Whether B-T cell interactions are essential for pulmonary eosinophilic inflammation following chronic allergen provocation remains unknown.

Aims: In this study, we investigated the importance of B-T cell interaction in the development of eosinophilic airway inflammation and pulmonary remodelling in a chronic HDM-driven asthma model.

Methods: We exposed C57BL/6, *Cd40l*$^{-/-}$, and *Mb1*$^{-/-}$ mice to HDM three times a week for five consecutive weeks.

Results: Chronic HDM exposure induced a pronounced eosinophilic allergic airway inflammation in broncho-alveolar lavage fluid (BALf) and lung tissue, associated with the formation of immunologically active inducible bronchus-associated lymphoid tissue (iBALT) in the lungs. The absence of B cells, or lack of CD40L signalling did not hamper eosinophilic inflammation in the airways, although the number of Tfh and Th2 cells was substantially reduced in the lungs. Importantly, type 2 innate lymphoid cells (ILC2) numbers in BALf and lung were not affected by the absence of B cells or B-T cell interaction. Furthermore, eosinophilic airway inflammation is not sufficient to induce pulmonary remodelling and airway hyperresponsiveness.

Conclusion and Clinical Relevance: From these findings, we conclude that B-T cell interaction is required for robust Tfh and Th2 cell induction, but not essential for eosinophilic airway inflammation during a chronic HDM-driven asthma model.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways, which is initiated by exposure to environmental factors in susceptible individuals (1). Asthma is defined by airway hyper responsiveness (AHR) of the bronchioles to various stimuli, such as house dust mite (HDM), leading to increased mucus production and airway remodelling. T helper (Th) 2 cell cytokines produced by both Th2 cells (IL-4, IL5, and IL-13) and type 2 innate lymphoid cells (ILC2s) (IL-5 and IL-13) are critically involved in allergic airway inflammation (2), whereas in non-allergic asthma also Th17 cells are implicated. Th2 cytokines facilitate classical allergic responses, such as IgE class switching by B cells (IL-4), eosinophilic infiltration (IL-5), and goblet cell hyperplasia (IL-13) (2,3). Th17 cytokines can also contribute to asthma symptoms as they increase smooth muscle cell proliferation (IL-17A/IL-22) and collagen deposition (IL-17A) (4). Tregs on the contrary will dampen these pro-inflammatory Th2 and Th17 cells (3).

In allergic asthma, B cells are well known for their allergen-specific IgE secretion, which exacerbates airway inflammation and induces airway narrowing through mast cell activation (5). However, beyond their antibody secretion capacities, B cells can also present antigens, produce cytokines, regulate T-cell-mediated immune responses, and induce the formation of tertiary lymphoid structures (6,7). The functional roles and importance of B cells in allergic airway inflammation have been controversial. Several studies in B-cell-deficient mice have shown that B cells are dispensable for eosinophilic airway inflammation (8-12). However, it was recently shown in a HDM-driven asthma model that B cells synergize with dendritic cells (DCs) to promote the differentiation of IL-4-commited follicular helper T (Tfh) cells into memory Th2 cells during the HDM-sensitization phase (13). Tfh cells have gained a lot of interest in HDM-driven asthma models that contain a sensitization and challenge phase, as they can either develop into effector Th2 cells (13) or promote the differentiation of effector Th2 cells (14). However, asthma patients are continuously exposed to allergens, whereby sensitization and challenge phases cannot be distinguished. Therefore, we wondered whether B cells, B-T cell interaction and Tfh cells are similarly important in an asthma model with chronic HDM provocation.

We found that exposing mice for 5 weeks with HDM induced a prominent adaptive immune response, characterized by B cell activation, development of Tfh cells and germinal centers (GC) containing inducible bronchus associated lymphoid tissue (iBALT) structures. Surprisingly, eosinophilic airway inflammation was normally present in HDM-exposed B cell-deficient *Mb1*$^{-/-}$ mice and *Cd40l*$^{-/-}$ mice, but unexpectedly had reduced pulmonary Tfh and Th2 cell numbers.

MATERIALS AND METHODS

Mice

Male and female C57/BL6, *Cd40l*$^{-/-}$, and *Mb1*$^{-/-}$ mice (15,16) were bred and housed at the animal facility of the Erasmus MC Rotterdam. Mice were housed under specific pathogen-free conditions and were ~12-16 weeks at time of analysis. All experiments were approved by the animal ethical committee of the Erasmus MC (EMC3329).

HDM-induced allergic airway inflammations

During HDM exposures, mice were anesthetized using isoflurane and sensitized intranasally with 25 µg/40 µl HDM (Greer) or 40 µl PBS as a control (GIBCO Life Technologies) three times per week, for five consecutive weeks. Mice were sacrificed for analysis at day 37 and day 65. Lung function was measured following increasing doses of nebulized methacholine (0.4 mg/ml – 25 mg/ml) using a restrained whole body plethysmograph (EMKA) under urethane sedation. BALf was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA (Sigma-Aldrich). Lungs were inflated with either PBS/OCT (1:1) solution, snap frozen in liquid nitrogen and kept at -80 °C until further processing, or placed in 4% PFA and embedded in paraffin, or homogenized for collagen measurements.

Flow cytometry procedures

Single cell suspensions were prepared from BALf, lungs, and MLN using standard procedures. Single cell suspensions were obtained from lungs by digesting the lungs using DNAse and Liberase for 30 minutes at 37 °C (17). Upon digestion, the lungs were homogenized through a 100 µm cell strainer (BD Biosciences). Lung red blood cells were lysed using osmotic lysis buffer (8.3% NH_4CL, 1% $KHCO_3$ and 0.04% NA_2EDTA in Milli-Q). Flow cytometry surface and intracellular staining procedures have been described previously (18). Monoclonal antibodies used for flow cytometric analyses are listed in Table S1. For all experiments, dead cells were excluded using Live-dead Aqua (Invitrogen). For cytokine production measured by flow cytometry, 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 hours. Data were acquired using a LSR II flow cytometer (BD Bioscience) and FACSDivaTM software (BD Bioscience) and analysed by FlowJo version 9 (Tree Star Inc software)

Lung histology

5 µm-thick paraffin embedded or snap frozen lung sections were stained with heamatoxylin/eosin or periodic acid-Schiff (PAS). For lung eosinophil staining, 5 µm-thick lung sections were fixed in acetone and incubated 1 hour with Siglef-F – PE (BD, Biosciences, clone E50-2240). After washing, slides were incubated for 30 minutes with anti-PE – PO (Rockland). Sections

were counterstained with hematoxylin. Peribronchial eosinophils were counted in 4 bronchi/slide by two independent researchers.

For confocal imaging, 10 µm-thick lung sections were fixed in acetone and incubated for 1 hour with primary antibodies (Table S1). After washing, slides were incubated for 30 minutes with secondary antibodies (Table S2). Where necessary, slides were incubated with 10% normal rat serum or 10% normal donkey serum to prevent unspecific binding of antibodies. Slides were counterstained with DAPI and analysed on a Zeiss Meta311 confocal microscope. Images were analysed using ImageJ software.

Hydroxyproline assay

Collagen quantification was performed on the right post-caval lobe by hydroxyproline assay as previously described (19). Hydroxyproline content may be converted to collagen (1 µg hydroxyproline corresponds to 6.94 µg collagen).

ELISA

Total and antigen-specific IgE were measured in serum and BALf (Opteia, BD Biosciences). KC levels were measured in BALf (DuoSet, R&D systems).

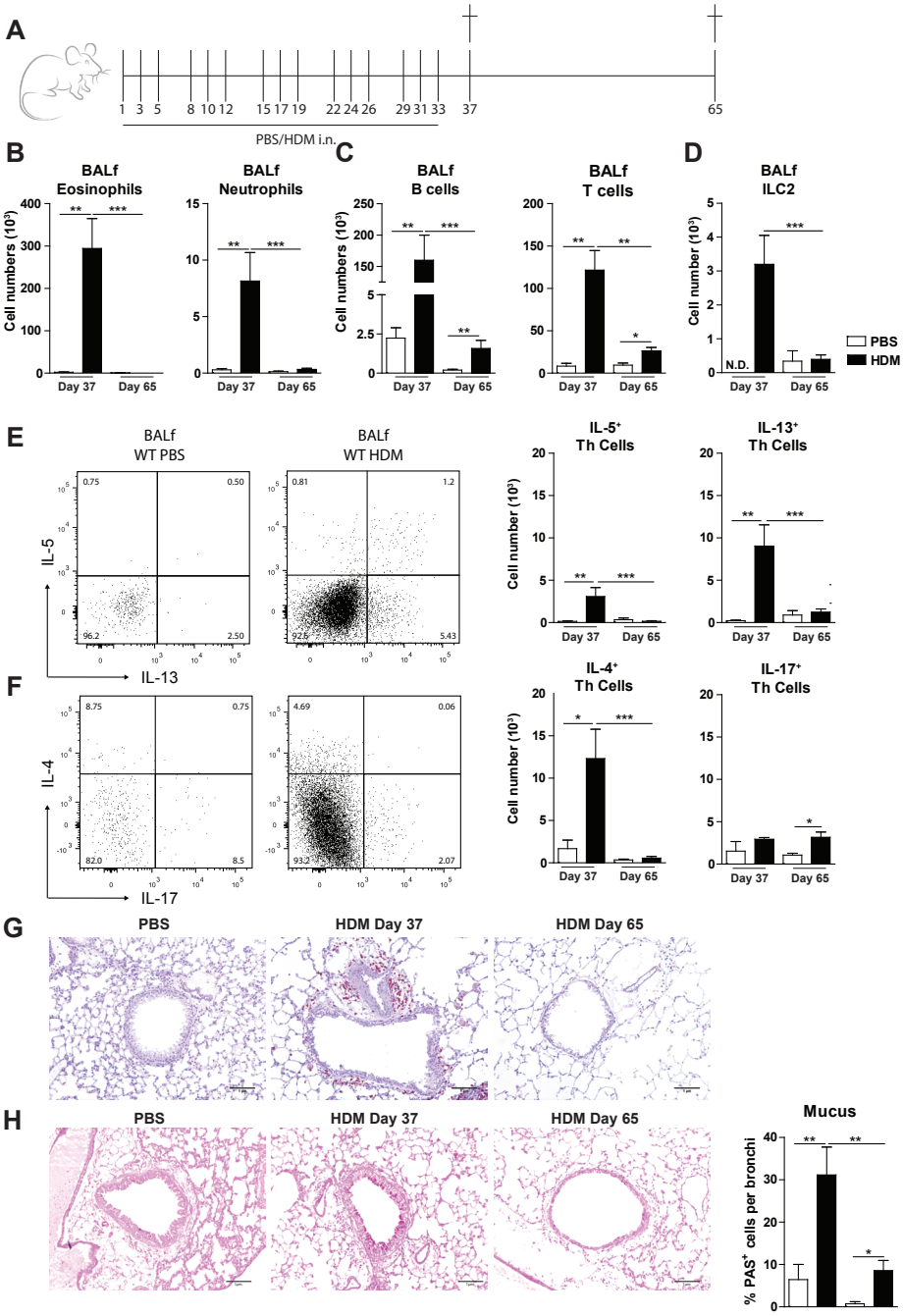
Statistical analysis

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 (GraphPad Software).

RESULTS

Chronic HDM exposure induces eosinophilic inflammation and remodelling in the airways

To determine the inflammatory kinetics in a clinically relevant chronic HDM-induced asthma model, C57BL/6 mice were treated with 25 µg HDM or PBS 3 days per week for 5 consecutive weeks and analysed 4 days (day 37) or 32 days (day 65) after the last HDM provocation (Figure 1A). HDM exposure increased eosinophil numbers in broncho-alveolar lavage fluid (BALf) and lung tissue on day 37 compared with PBS-treated controls (Figure 1B, 1F, and Figure S1). Furthermore, neutrophil, B cell, Th cell, and ILC2 numbers were also increased in the BALf on day 37 compared to PBS-treated controls (Figure 1B-D, Figure S1 – S2). IL-4-, IL-5-, and IL-13-secreting Th cells were enhanced upon HDM treatment, whereas no increase in IL-17-secreting Th cells was observed (Figure 1E).


A
1 3 5 8 10 12 15 17 19 22 24 26 29 31 33 37 65
PBS/HDM i.n.
B
BALf Eosinophils
BALf Neutrophils
C
BALf B cells
BALf T cells
D
BALf ILC2
Cell numbers (10³)
Cell number (10³)
N.D.
Day 37
Day 65
PBS
HDM
E
BALf WT PBS
BALf WT HDM
IL-5
IL-13
IL-5⁺ Th Cells
IL-13⁺ Th Cells
F
IL-4
IL-17
IL-4⁺ Th Cells
IL-17⁺ Th Cells
G
PBS
HDM Day 37
HDM Day 65
H
Mucus
% PAS⁺ cells per bronchi

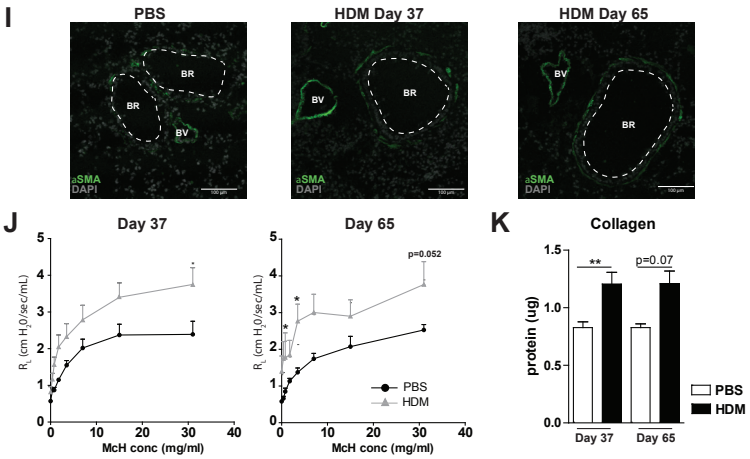


Figure 1. Chronic HDM exposure induces eosinophilic inflammation and remodelling in the airways. (A) Chronic HDM model. Mice were treated with PBS or HDM at indicated time points. Analysis was performed at day 37 and day 65. (B-D) Quantification of BALf eosinophils, neutrophils, B cells, Th cells and ILC2s by flow cytometry (E-F) Flowcytometric gating and quantification of IL-4$^+$, IL-5$^+$, IL-13$^+$ and IL-17$^+$ Th cells in BALf by flow cytometry. (G) Histologic analysis of Siglec-F$^+$ eosinophils in lung tissue. (H) Histologic analysis and quantification of mucus-producing cells in 3 bronchi per lung in paraffin-embedded lung tissue using a PAS staining. (I) Immunofluorescent staining of αSMA (green) in lungs at day 37 and day 65. (J) Airway hyper-responsiveness was measured upon increasing doses of inhaled methacholine. (K) Lung collagen content was determined by hydroxyproline assay. Results are presented as mean ±SEM of n=5-7 per group and representative of two or more independent experiments with 5-7 mice per group. * = p<0.05, ** = p<0.01, *** = p<0.001.

Remodelling of the airways is an essential feature of asthmatic disease and involves fibrosis, goblet cell hyperplasia, and smooth muscle cell hypertrophy, resulting in an increased AHR (20). Repetitive HDM exposures induced long-lasting airway remodelling, as demonstrated by the presence of mucus-producing goblet cells (Figure 1H) and smooth muscle cells (Figure 1I), AHR (Figure 1J), and increased lung collagen content (Figure 1K). On day 65, the type 2 inflammation was no longer detectable in the BALf (Figure 1B, 1E).

Together, these data show that chronic HDM provocation induces specifically robust but transient type 2 airway inflammation, as well as airway remodelling that was maintained over time.

Chronic HDM exposure induces development of iBALT in the lungs

Given the evidence that iBALT areas are increased in asthmatic patients (21), we wondered whether repetitive HDM provocations would induce iBALT formation in our mouse model.

Lungs of HDM-treated mice showed dense cellular infiltrates that were associated with bronchioles and blood vessels at day 37 (Figure 2A-B), with similarities to iBALT structures observed during influenza infection (22,23). These HDM-induced structures contained large B cell follicles adjacent to T cell areas and CD11c$^+$ antigen presenting cells (APCs) (Figure 2C-D). In contrast, in HDM-driven asthma models containing a sensitization and a short challenge phase,

iBALTs were not observed and B cell numbers in both BALf and lungs were 10 times lower as compared to chronic HDM treatment (Figure S3). These findings suggest that B cells play a more prominent role in chronic than in acute HDM-driven asthma models.

iBALTs of chronic HDM-provoked mice contained GC B cells surrounded with plasma cells. IgE-expressing GC B cells and plasma cells were found in association with B cell follicles (Figure 2E-G, and Figure S4A). Both total IgE and HDM-specific IgE were induced in BALf and serum (Figure 2H-I). T cells were also located in HDM-induced iBALT structures and could be detected inside the B cell follicles, indicative of Tfh cells (Figure 2C). An accordingly significant increase in differentiated $CXCR5^+PD1^+$ Tfh, $GATA3^+$ Th2, $ROR\gamma T^+$ Th17, and $FOXP3^+CD25^+$ Tregs was observed in the lungs of HDM-treated mice compared with control mice (Figure 2J, Fig S4B), whereas naïve $CD62L^+$ Th cells were not increased (Figure S5).

Taken together, these data show that chronic HDM-treatment induced active iBALT structures containing both Tfh cells and GC B cells, associated with IgE plasma cell formation locally in the lungs.

Germinal centre B cells and Tfh cells are induced in the MLN upon chronic HDM exposure

GC reactions and Tfh cells were shown to be important in Th2 differentiation in acute HDM-driven asthma models (13,14), therefore we investigated whether GCs and Tfh cells were also induced in the MLN of chronic HDM-exposed mice. On day 37, a strong increase in total B cells (Figure 3A), $GL7^+CD95^+$ GC B cells (Figure 3B-C), and $CD138^+$ plasma cell numbers (Figure 3D-E) was observed in the MLN of HDM-treated animals compared with PBS-treated mice. HDM treatment also increased the total number of Th cells (Figure 3F), Tfh cells (Figure 3G-H), Th2 cells, Th17 cells (Figure 3I-K), and Treg cells (Figure 3L-M). B and T cell inflammation remained enhanced on day 65 in HDM-treated mice, but GC B cells and plasma cells were significantly reduced compared with day 37 of HDM-treated mice (Figure 3A-M).

Thus, chronic HDM-exposures also induce robust GC reactions in the lung-draining MLN.

Eosinophilic inflammation is not dependent on B-T cell interactions

Given that HDM provocations induced a strong GC response and iBALT formation, we wondered whether iBALT formation and B-T cell interactions within GCs are essential for the development of eosinophilic airway inflammation. Therefore, we exposed B cell-deficient mice (*Mb1*$^{-/-}$), and mice deficient for CD40L signalling (*Cd40l*$^{-/-}$) to our chronic HDM-driven asthma model. *Mb1*$^{-/-}$ mice are deficient in the expression of Iga/CD79a molecule, an essential transducer of B cell receptor signals. As a result, B cell differentiation is abrogated in the bone marrow, and mature B cells are lacking (15). *Cd40l*$^{-/-}$ mice lack expression of the CD40L molecule, thus CD40L-CD40 interaction between T and B cells is hampered and as a consequence these mice fail to develop GCs, and class switch recombination is absent (16,24).

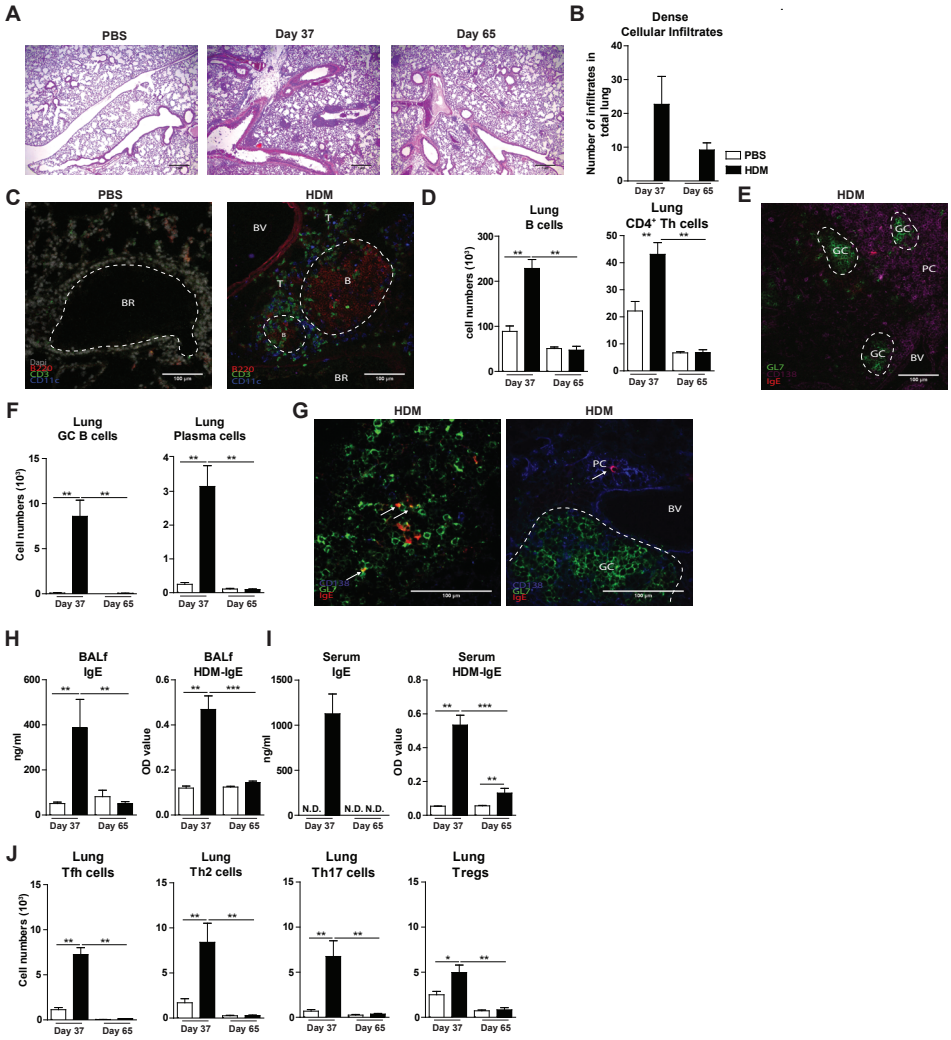


Figure 2. Chronic HDM exposure induces development of iBALT in the lungs. (A) Histological overview images showing dense cellular infiltrates in the lungs, bars represent 100 µm. (B) Number of dense cellular infiltrates in total lungs counted double-blinded. (C) Immunofluorescent staining of $CD3^+$ T cells (green), $B220^+$ B cells (red), and $CD11c^+$ APCs (blue) in lungs at day 37. (D) B and Th cells in lung tissue. (E) Immunofluorescent staining of $GL7^+$ GC B cells (green), $CD138^+$ plasma cells (blue), and IgE^+ cells (red) in lungs at day 37. (F) Quantification of GC B cells and plasma cells in lung tissue. (G) Immunofluorescent staining of IgE^+ GC B cells (yellow), and IgE^+ plasma cells (purple) in lung tissue at day 37. (H) IgE and HDM-IgE in BALf. (I) IgE and HDM-IgE in serum. (J) Quantification of Tfh ($CXCR5^+$, $PD1^+$), Th2 ($GATA3^+$), Th17 ($ROR\gamma T^+$) and Treg ($CD25^+FoxP3^+$) cells in lung tissue. Results are presented as mean ±SEM of n=5-7 per group representative of two or more independent experiments with 5-7 mice per group. * = p<0.05, ** = p<0.01, *** = p<0.001.

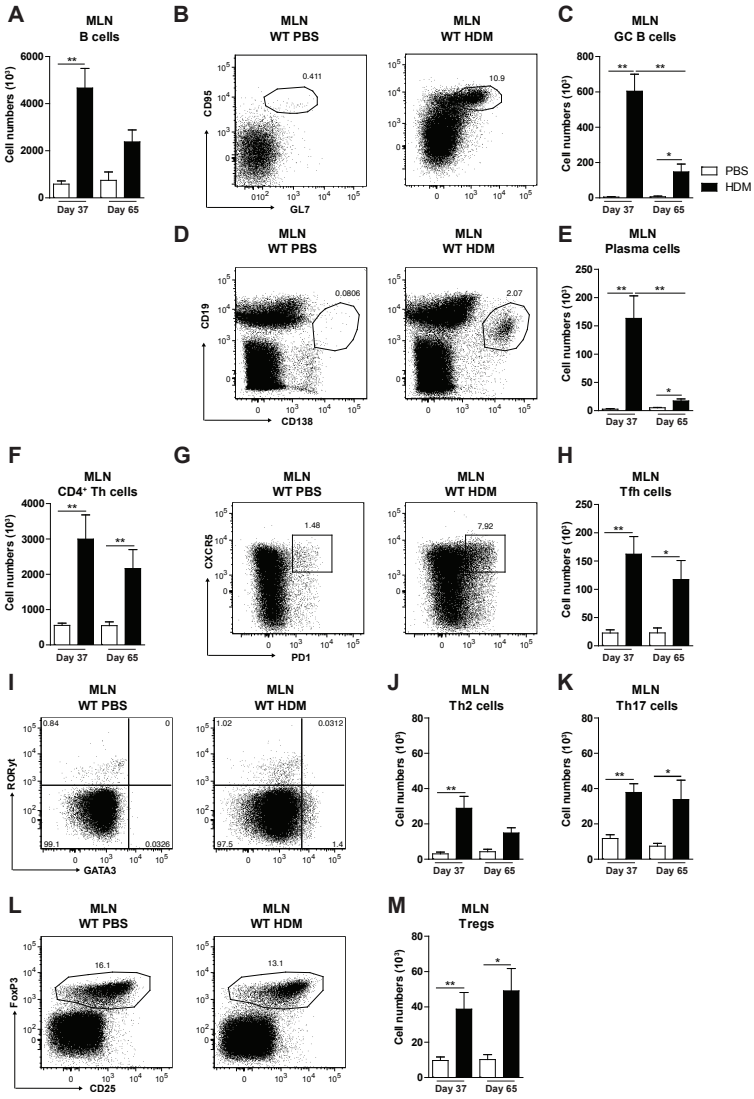


Figure 3. Germinal centre B cells and Tfh cells are induced in the MLN upon chronic HDM exposure. (A) Quantification of B cells by flow cytometry. (B) Flow cytometric gating of GC B cells from CD19^{+}B220^{+} B cells in MLN at day 37. (C) Enumeration of GC B cells by flow cytometry. (D) Flow cytometric gating of plasma cells from live cells at day 37. (E) Enumeration of plasma cells by flow cytometry. (F) Enumeration of Th cells by flow cytometry in MLN. (G) Flow cytometric gating of Tfh cells from CD4^{+} T cells in MLN at day 37. (H) Quantification of Tfh cells by flow cytometry. (I) Flow cytometric gating of RORγt^{+} Th17 and GATA3^{+} Th2 cells from CD4^{+} T cells in MLN at day 37. (J-K) Enumeration of Th2 and Th17 cells in MLN by flow cytometry. (L) Flow cytometric gating of FoxP3^{+}CD25^{+} Tregs cells from CD4^{+} T cells in MLN at day 37. (M) Quantification of Treg cell numbers in MLN by flow cytometry. Results are presented as mean ±SEM of n=5-7 per group and representative of two or more independent experiments with 5-7 mice per group. * = p<0.05, ** = p<0.01.

Surprisingly, both HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice developed BALf eosinophilic inflammation comparable to HDM-exposed WT controls as assessed at day 37 (Figure 4A). Neutrophil numbers in HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice were also not different from HDM-treated WT mice; *Mb1*$^{-/-}$ mice even showed increased neutrophil numbers compared with *Cd40l*$^{-/-}$ mice (Figure 4A). B cells were reduced in HDM-exposed *Cd40l*$^{-/-}$ mice and as expected completely absent in *Mb1*$^{-/-}$ mice (Figure 4B). BALf Th cell and ILC2 numbers were not different in HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice from HDM-treated controls (Figure 4C). Strikingly, the numbers of BALf IL-4^{+}, IL-5^{+}, IL-13^{+}, and IL-17^{+} Th cells and ILC2s in HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice were similar to WT controls (Figure 4D and Figure S6). Eosinophilic inflammation in lung tissue in HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice was also not different from HDM-treated WT mice (Figure 4E-4F).

These data show that eosinophilic airway inflammation and Th2 cytokine production can develop in the absence of B-T cell interaction upon chronic HDM exposure.

Reduced Tfh and Th2 cell numbers but normal ILC2 numbers in the absence of iBALT structures in the lung

Since Th2 inflammation in the BALf was similar between WT and *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice, we investigated whether lung inflammation was affected by the absence of B-T cell interactions or B cells. Total lung B cells of HDM-treated *Cd40l*$^{-/-}$ mice were reduced and completely absent in HDM-treated *Mb1*$^{-/-}$ mice as compared with HDM-treated controls. As expected, GC B cells and plasma cells were completely absent in both HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice (Figure 5A). In contrast to our findings in BALf, total pulmonary Th and Tfh cells of HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice were significantly reduced compared to HDM-treated controls; Th2 cell numbers in the lungs of *Cd40l*$^{-/-}$ mice were also reduced (Figure 5B). Lung Th17 and Treg cell numbers of HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice were similar to those in HDM-treated control mice (Figure 5B). Surprisingly, pulmonary ILC2 numbers were similar between HDM-treated WT and *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice (Figure 5C).

As the inflammation in the lungs is largely reduced, we wondered whether the remaining T cells would still cluster together in the absence of B cells and B-T cell interaction. HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice exhibited no iBALT structures, although T cells were located in close proximity to the bronchioles in all HDM-treated mice (Figure 5D).

Therefore, these data show that as expected B-T cell interaction is essential for iBALT development. However, ILC2 numbers are not affected by the absence of iBALT structures.

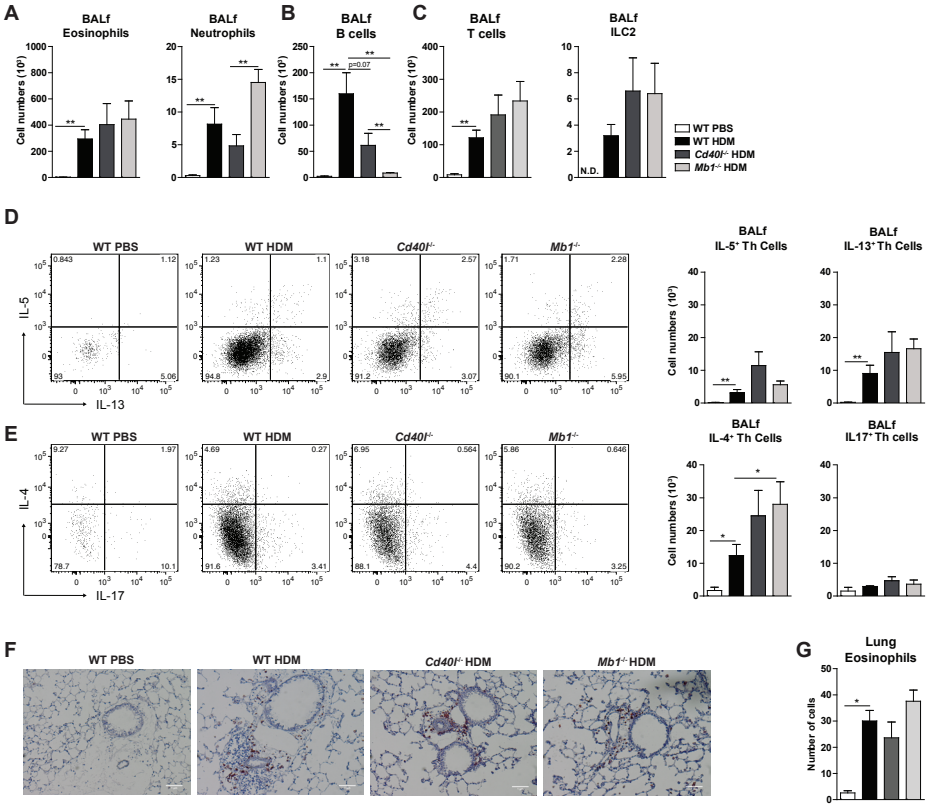


Figure 4. Eosinophilic inflammation is not dependent on B-T cell interactions. (A-C) Quantification of eosinophil, neutrophil, B cell, Th cell, and ILC2 cell numbers in BALf by flow cytometry. (D-E) Flow cytometric gating and quantification of IL-4$^+$, IL-5$^+$, IL-13$^+$ and IL-17$^+$ producing Th cells in BALf by flow cytometry. (F) Histologic analysis of Siglec-F$^+$ eosinophils in lung tissue. (G) Number of eosinophils in the lung tissue. Results are presented as mean ±SEM (n=4 and 7 in PBS and HDM, respectively). *, p<0.05, ** p<0.01, *** = p<0.001.

Tfh development in MLN in the absence of CD40L signalling upon chronic HDM-treatment

Since eosinophilic airway inflammation developed normally in the absence of B-T cell interaction, we wondered whether Tfh and Th2 cell differentiation would be hampered in the MLN in the absence of CD40L signalling and B cells upon chronic HDM exposure.

As expected, MLN from *Cd40l*$^{-/-}$ mice had reduced B and T cell numbers, compared with HDM-treated WT mice (Figure 6A-B), whereas B cells were absent in *Mb1*$^{-/-}$ mice (Figure 6A). GC B cells and plasma cells were completely absent in HDM-treated *Cd40l*$^{-/-}$ mice, indicating that GC B cell formation is dependent on CD40L signalling in a chronic HDM-induced asthma model (Figure 6C). Surprisingly, Tfh cell numbers were not significantly reduced in *Cd40l*$^{-/-}$ mice; Tfh differentiation was lacking in *Mb1*$^{-/-}$ mice (Figure 6D-E). In PBS-treated *Cd40l*$^{-/-}$ mice,

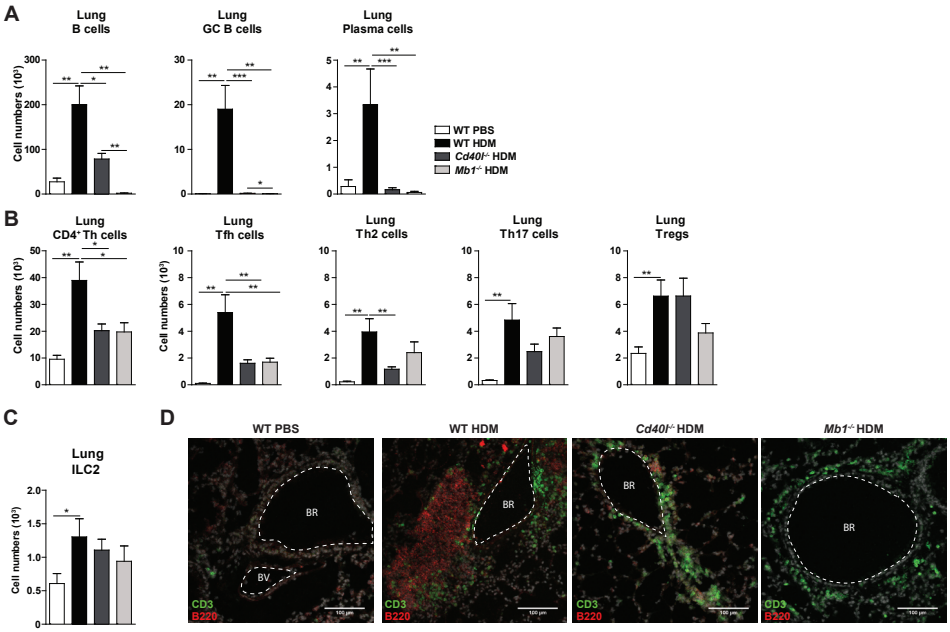


Figure 5. Reduced Tfh and Th2 cell numbers but normal ILC2 numbers in the absence of iBALT structures in the lung. (A) Quantification of B cell, GC B cell, and plasma cell numbers in lung tissue by flow cytometry. (B) Quantification of Th, Tfh, Th2, Th17, and Treg cell numbers in lung tissue by flow cytometry. (C) Immunofluorescent staining of $CD3^+$ T cells (green) and $B220^+$ B cells (red) in lungs. (D) Results are presented as mean ±SEM (n=4 and 7 in PBS and HDM, respectively). *, p<0.05, ** p<0.01, *** = p<0.001.

Tfh cells were reduced compared with WT mice, as reported before (Figure S7) (25). Tfh cells are characterized by the expression of CXCR5, a chemokine receptor that enables the migration towards the GC (26), and PD1, a potent inhibitory receptor inhibiting excessive Th cell proliferation (27). PD1 expression was reduced on Tfh cells of both HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice, compared with HDM-treated WT Tfh cells. Nevertheless, PD1 expression on Tfh cells of HDM-treated *Cd40l*$^{-/-}$ mice was still elevated, compared with PBS-treated WT Tfh cells. CXCR5 expression was only reduced in Tfh cells of *Mb1*$^{-/-}$ mice (Figure 6D, quantified in Figure 6F). In contrast to Tfh cells, Th2 cells were strongly reduced in MLN of both HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice, compared with HDM-treated WT mice. Tregs were reduced in both HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice, and Th17 cell numbers were only significantly reduced in *Mb1*$^{-/-}$ mice (Figure 6G).

In summary, these data confirm that GC B cell formation in the MLN is dependent on CD40L signalling. Importantly, in the MLN, Tfh cell induction during chronic HDM-exposure does not require CD40L, but is dependent on B cells. In contrast, Th2 cell development in the MLN is dependent on both CD40L signalling and B cells.

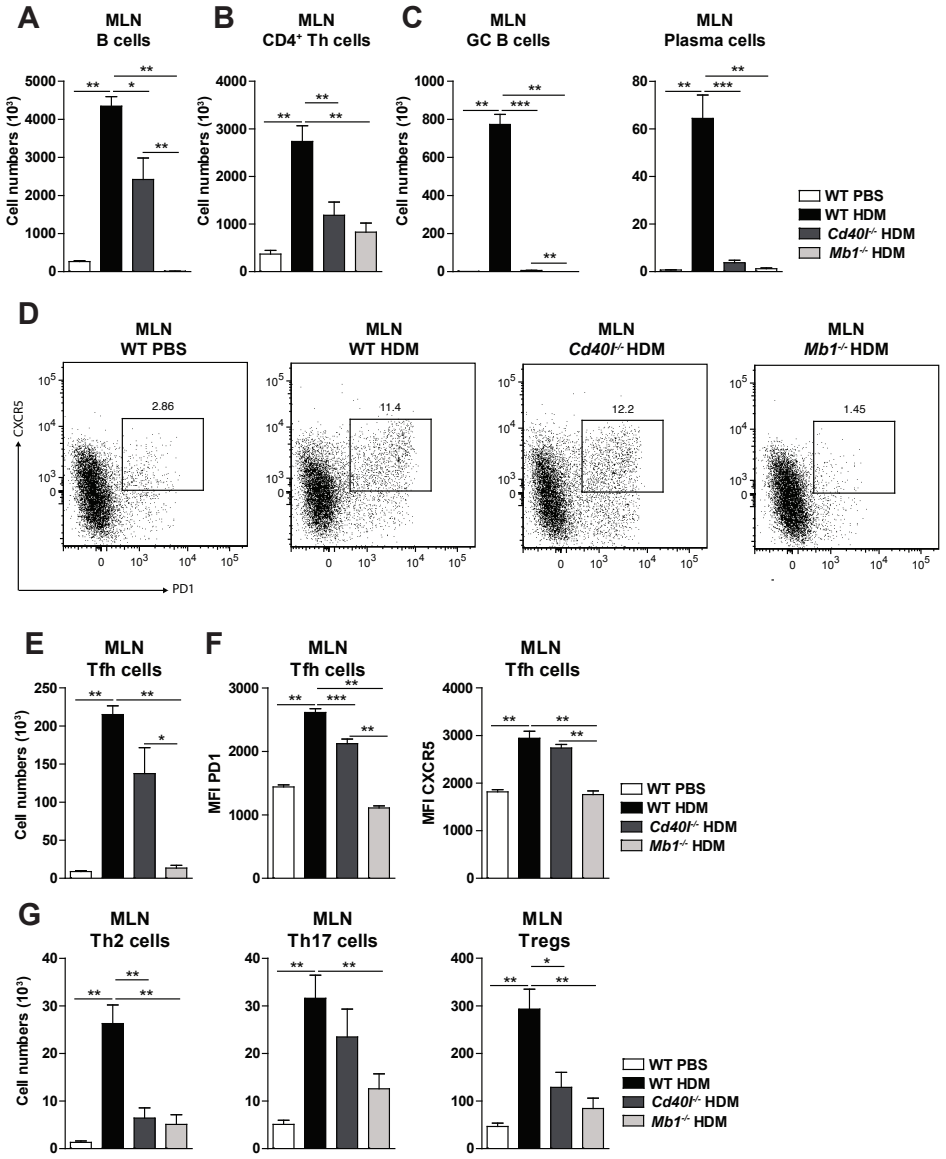


Figure 6. Tfh development in MLN requires B cells but not CD40L signalling upon chronic HDM-treatment. (A-C) Quantification of B cell, Th cell, GC B cell, and plasma cell numbers in MLN by flow cytometry. (D) Flow cytometric gating of Tfh cells. (E) Quantification of Tfh cells in MLN by flow cytometry. (F) MFI of PD1 and CXCR5 of Tfh cells by flow cytometry. (G) Quantification of Th2, Th17 and Treg cells by flow cytometry. Results are presented as mean ±SEM of n=5-7 per group. * = p<0.05, ** = p<0.01, *** = p<0.001.

B cells and B-T cell interaction are essential for pulmonary remodelling

Both cytokine production by Th2 cells, as well as IgE secretion by B cells are known to contribute to pulmonary remodelling (28), therefore we wondered whether remodelling is dependent on the presence of B cells or B-T cell interaction. HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice had a reduced area of α-smooth muscle actin (αSMA) surrounding the bronchioles and mucus-producing goblet cells compared to HDM-treated WT mice (Figure 7A-D). A trend towards reduced AHR was observed at lower methacholine concentrations in both HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice, compared to HDM-treated WT mice (Figure 7C).

These data demonstrate that the presence of B cells and CD40L signalling is essential for airway remodelling that accompanies chronic airway inflammation.

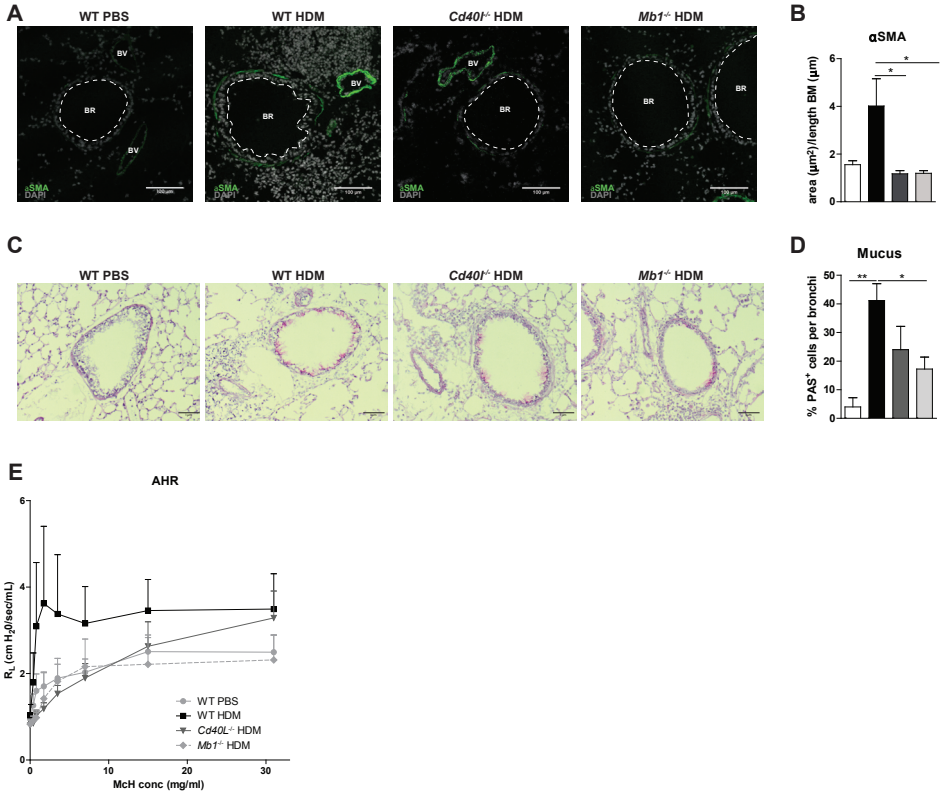


Figure 7. B cells and CD40L are essential for the development of pulmonary remodelling. (A) Immunofluorescent staining of αSMA (green) in lungs at day 37. (B) Quantification of the area αSMA per basal membrane of 3 bronchioles per mice. (C-D) Histologic analysis and quantification of mucus-producing cells in snap frozen lung tissue PAS staining. (E) Airway hyper-responsiveness was measured upon increasing doses of methacholine at day 37. Results are presented as mean ±SEM (n=4 and 7 in PBS and HDM, respectively). * = p<0.05.

DISCUSSION

B cells are essential for the maintenance and final differentiation of Tfh cells (29). Tfh cells have recently been reported to contribute to Th2-mediated inflammation in asthma, either by the differentiation into effector Th2 cells or by promoting Th2 cell differentiation (13,14). However, whether B cells and B-T cell interaction are also important for eosinophilic airway inflammation in a clinically relevant chronic asthma model characterized by prolonged allergen exposure remains unknown. Chronic HDM exposures induced transient type-2 airway inflammation, whereas structural changes such as airway remodelling and airway hyperresponsiveness sustained over time. Our study demonstrates that in a chronic HDM-mediated asthma model eosinophilic inflammation in the BALf and lung tissue can develop in the absence of B-T cell interaction and iBALT structures. The accumulation of ILC2s in BALf and lung tissue was not affected by the absence of iBALT tissue. In particular, we have found that the absence of B cells precluded the formation of Tfh cells in the MLN upon chronic HDM exposure, but nevertheless eosinophilic inflammation was induced. Thus, in contrast to published findings in acute HDM models characterized by sensitization and challenge phases (13,14), our findings in a chronic HDM asthma model demonstrate that pulmonary eosinophilic inflammation is independent of B-T cell communication and iBALT formation. Nevertheless, our chronic HDM model revealed that pulmonary Th2 inflammation and airway remodelling require B cells and B-T cell interaction.

Although CD40L is mostly studied because of its prominent role on activated T cells in the context of B-T cell interaction, CD40L is also expressed on several other cell types, including mast cells, eosinophils, monocytes, B cells, and DCs (30). It is therefore conceivable that additional immunological responses may be hampered in *Cd40l*$^{-/-}$ mice, next to B-T cell interaction and communication. For instance, CD40-CD40L signalling in DCs is also involved in Th2 cell differentiation (31). Furthermore, upon CD40-CD40L interaction OX-40L, a Th2 cell promoting co-stimulatory molecule, is induced on DCs (32). However, it is not likely that decreased Th2 differentiation in the MLN of *Cd40l*$^{-/-}$ mice is mainly a result of insufficient DC-T cell interaction, rather than abrogated B-T cell communication, because *Mb1*$^{-/-}$ mice – in which CD40-CD40L signalling between DC and T cells is still intact – also showed a similar reduction of the numbers of Th2 cells in MLN as *Cd40l*$^{-/-}$ mice.

Eosinophils were also reported to be important for airway remodelling in OVA-induced asthma models (33). In this context, however, our data indicate that a reduction in airway remodelling is observed, although similar eosinophilic inflammation is present in *Mb1*$^{-/-}$, *Cd40l*$^{-/-}$ and WT mice. This is consistent with other reports that investigated the role of eosinophils in airway remodelling upon HDM-driven asthma models (34). Recently, eosinophil derived IL-4 was also described to induce both proliferation and IL-5 and IL-13 secretion by ILC2s, suggesting a positive feedback loop between ILC2s and eosinophils (35). This would complement our

data, as we found normal levels of both eosinophils and ILC2s in the airways of mice deficient of B cells or CD40L signalling.

B cells can contribute to various aspects of asthma pathology through the secretion of cytokines, antigen presentation and their production of antigen-specific IgE (36). Our data demonstrates that during chronic HDM exposure B cells are dispensable for pulmonary eosinophilic influx, but are essential for the development of airway remodelling. Other studies have also found similar numbers of eosinophilic inflammation in B cell-deficient mice (8,11,37), whereas especially allergen-specific IgE secretion by plasma cells was shown to be sufficient to induce AHR (38,39), smooth muscle cell proliferation, and collagen deposition (40,41). In contrast, Drake *et al*, found that B cells are important for Th2-mediated asthmatic inflammation, however in contrast to our study, mice were exposed to different allergens such as, ovalbumin, *Alternaria*, *Aspergillus*, and HDM (42).

It has been reported in a model containing a sensitization and challenge phase, that HDM-specific $CD4^+$ T cells are first primed by lung-migratory DCs at the B cell follicle border where they proliferate and acquire a Tfh like-signature (13). IL-4-committed pre-Tfh cells subsequently encounter allergen-bearing B cells that acquired allergen from lung-migratory DCs. These allergen-presenting activated B cells, replace DCs as the primary APCs in the B cell follicle and provide Th cell survival and differentiation signals (13). We also found significantly lower numbers of Th2 and Tfh cells in the MLN and lung, whereas the numbers of Th17 and Tregs were similar in the absence of B cells. However similar numbers of Th cells were found in association with the bronchioles. This suggests that the decrease in both total Th cells as in Th2 cells in the lung is likely caused by the absence of iBALT structures. This is in contrast to the BALf, where we found normal Th2 levels in both HDM-treated *Mb1*$^{-/-}$ and *Cd40l*$^{-/-}$ mice as compared to WT mice, indicating that the lungs could contain two separately controlled Th2 compartments.

It is well possible that the observed role for both B cells and Tfh cells is dependent on the experimental model, allergen type, and kinetics of exposure. Clearly, our data demonstrate that in a chronic HDM-driven asthma model normal lung eosinophilic inflammation can be initiated in the absence of B-T cell interactions and iBALT structures. This finding indicates that chronic HDM exposure elicits a very robust and/or long lasting pulmonary inflammatory response that overcomes the need for Tfh cells during Th2 cell differentiation that was observed in acute HDM models (12,13). Unlike in acute models, chronic allergen exposure could result in prolonged activation of ILC2s that may support or induce eosinophilic inflammation, independently of Tfh cells or GC formation in the MLN and lungs.

Collectively our data shows that upon chronic allergen exposure, B-T cell interactions are required for robust Tfh and Th2 cell induction, however are not essential for eosinophilic airway inflammation. Eosinophilic airway inflammation alone is not sufficient to induce pulmonary

remodelling and airway hyperresponsiveness, which is likely dependent on the outcome of B-T cell interactions.

ACKNOWLEDGEMENTS

These studies were partially supported by the Netherlands Lung Foundation (grants 4.2.13.054, 3.2.12.067 and 3.2.12.087). We would like to thank Martijn Nawijn (UMC Groningen), Boudewijn van der Wel, Menno van Nimwegen (Erasmus MC), and the Erasmus MC Animal Facility (EDC) staff for their assistance during the project.

AUTHOR CONTRIBUTIONS

HV, BL, RH, MK designed the experiments. HV, IB, BL, JvH, ML, DU performed experiments and analysed data. HV, RH and MK wrote the manuscript. All authors red and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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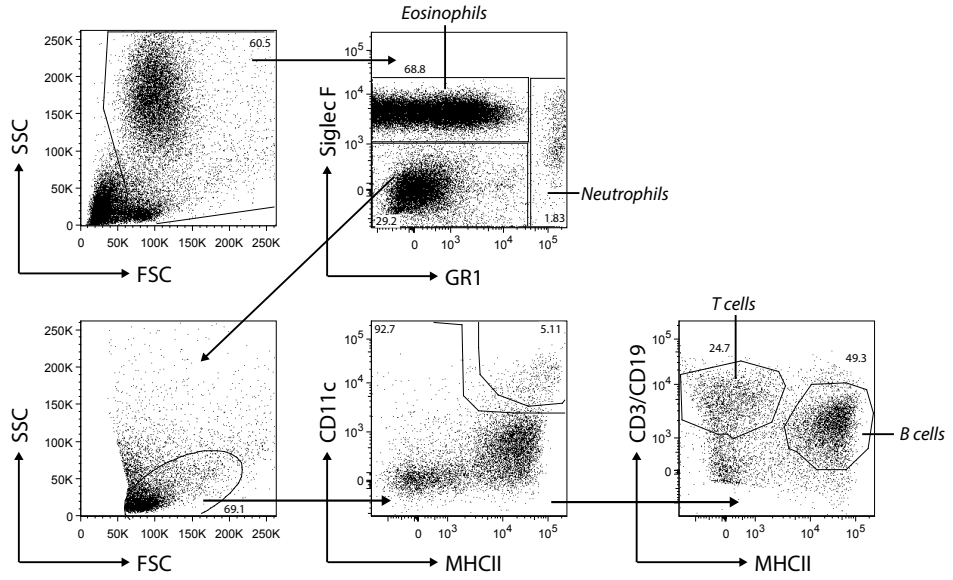

Figure S1. Flow cytometric gating of eosinophils, neutrophils, B cells, and T cells in BALf. Eosinophils were gated as Siglec F^+, GR-1^-, Neutrophils were gated as GR1+, Siglec F^{int}, T cells were gated as $CD3^+MHCII^-$, and B cells were gated as $CD19^+MHCII^+$. Due to the limited amount of fluorochromes available, CD19 and CD3 were stained with the same fluorochrome, enabling the distinction between B and T cells based on the expression of MHCII.

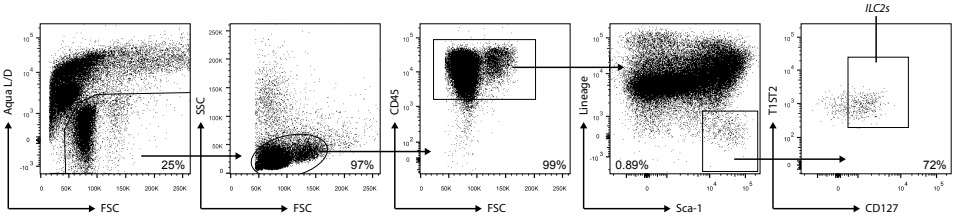

Figure S2. Flow cytometric gating of ILCs in BALf. ILC2s were gated as Lineage negative (CD3, CD5, CD8α, CD11b, CD11c, CD19, B220, NK1.1, GR-1, Ter-119, FcεRIα, and CD14) and $CD45^+$, Sca-1 $(Ly6A/E)^+$, $T1ST2^+$, and $CD127^+$.

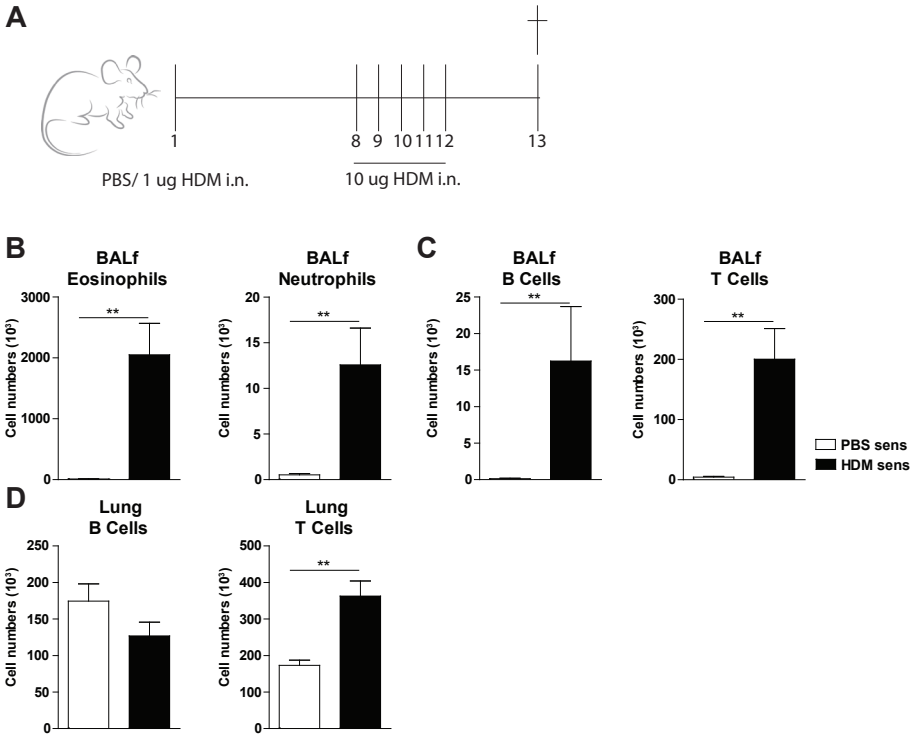

Figure S3. B cell inflammation is less prominent in the BAL and lungs of mice exposed to an acute HDM-driven asthma model. (A) Scheme for acute intranasal HDM treatment of C57BL/6 mice to induce airway inflammation. Mice were sensitized with PBS or 1 µg HDM at day 1, and challenged with 10 µg of HDM at day 8-12. Analysis was performed at day 13. (B) Quantification of eosinophil and neutrophil cell numbers in BALf by flow cytometry. (C) Quantification of B and T cell numbers in BALf by flow cytometry. (D) Quantification of B and T cell numbers in lung by flow cytometry. Results are presented as mean ±SEM (n=6 in PBS and HDM). *, $p<0.05$, ** $p<0.01$.

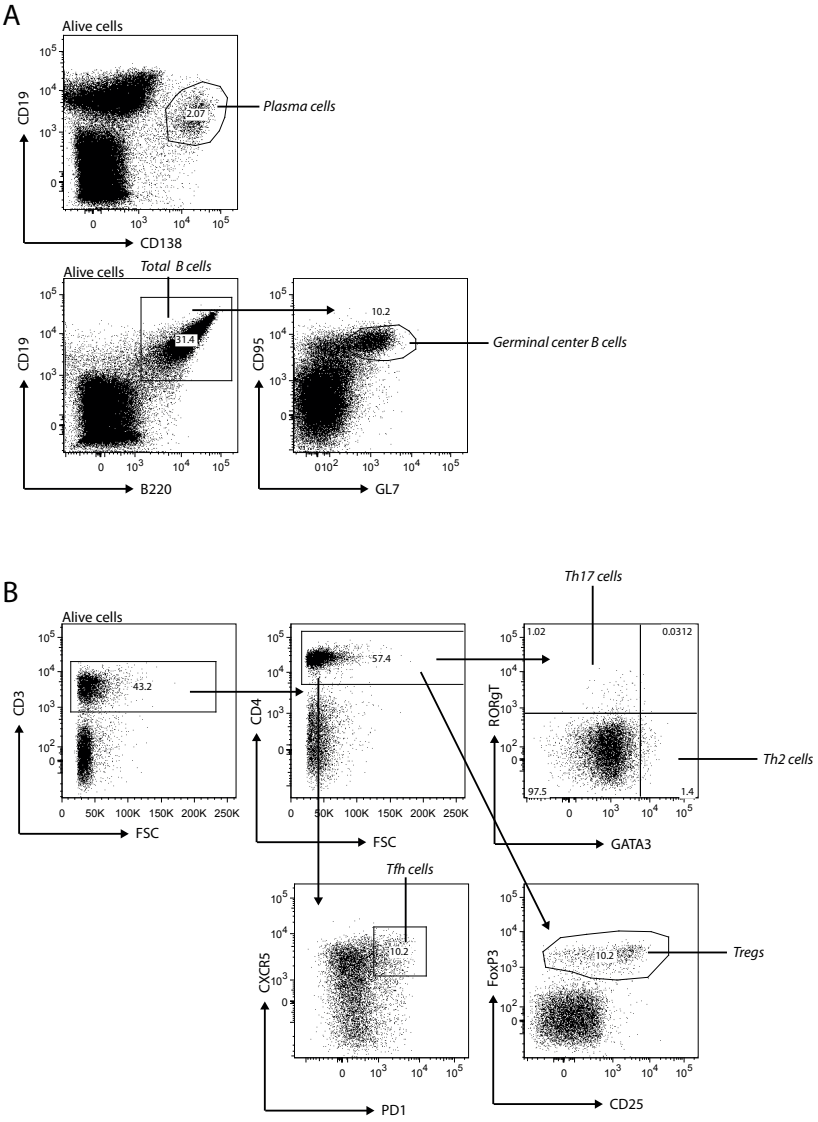

Figure S4. Flow cytometric gating of B and Th cell subsets. (A) Flow cytometric gating of total B cells, germinal center B cells, and plasma cells. (B) Flow cytometric gating of total Th cells, Tfh, Th2, Th17, and Treg cells.

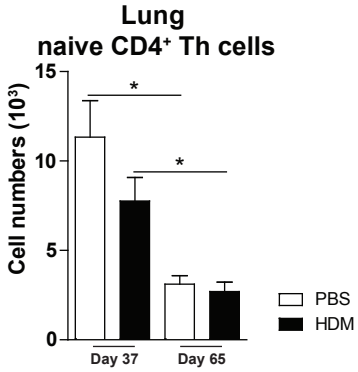

Figure S5. Naïve T cell numbers in the lung are not increased upon chronic HDM-treatment. Quantification of naïve CD62L^{+} Th cell numbers in the lung by flow cytometry. Results are presented as mean ±SEM of n=5-7 per group and all panels are representative of two or more independent experiments with 5-7 mice per group. * = p<0.05.

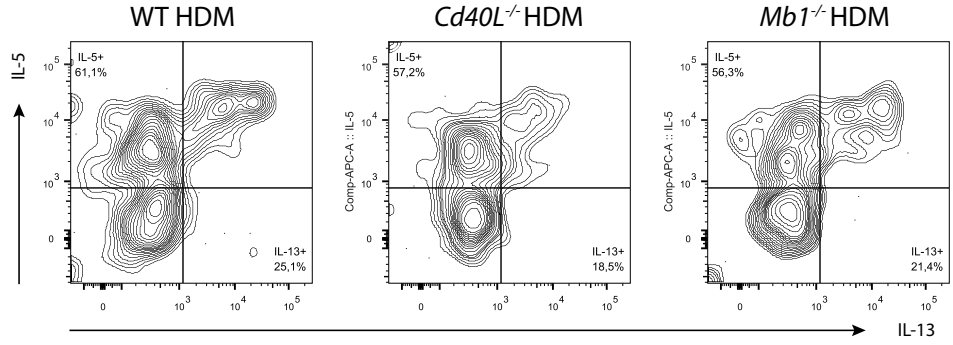

Figure S6. Cytokine production by ILC2 in the BALf of HDM-treated *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice is similar to HDM-treated WT mice. Flow cytometric gating of IL-5 and IL-13 producing ILC2 cells in the BALf of HDM-treated WT, *Cd40l*$^{-/-}$ and *Mb1*$^{-/-}$ mice at day 37.

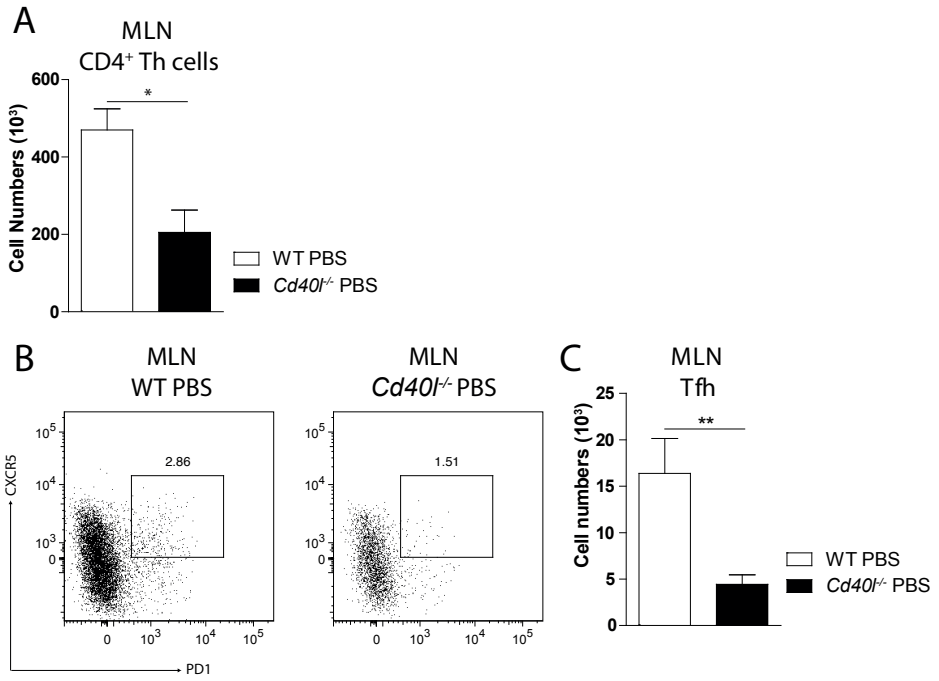

Figure S7. Total CD4⁺ T and Tfh cell numbers are reduced in MLN of *Cd40l*$^{-/-}$ mice. (A) Quantification of Th cell numbers in the lung by flow cytometry. (B) Flow cytometric gating of Tfh cells in MLN. (C) Quantification of Tfh cells in the MLN by flow cytometry. Results are presented as mean ±SEM of n=5-7 per group. * = $p<0.05$, ** = $p<0.01$.

Table S1: Antibodies used for flowcytometry

Antibody	Conjugate	Clone	Company
B220	PE	RA3-6B2	eBiosciences
CD11b	PE	M1/70	eBiosciences
CD11c	PE	N418	eBiosciences
CD127	PE-Cy7	SB/14	BD Biosciences
CD138	APC	281-2	eBiosciences
CD19	APC-eFluor 780	ID3	eBiosciences
CD19	PE	ID3	BD Biosciences
CD25	PerCP-Cy5.5	PC61	BD Biosciences
CD3	PE-CF594	145-2C11	BD Biosciences
CD3	APC-eF780	17A2	eBiosciences
CD3	PE	145-2C11	eBiosciences
CD4	Brilliant Violet 605	RM4-5	BD Biosciences
CD45	Pe-CF594	I3/2.3	Abcam
CD5	PE	53-7.3	eBiosciences
CD8a	PE	53-6.7	eBiosciences
CD62L	APC-eFluor 780	MEL-14	eBiosciences
CD95	PE-CF594	Jo2	BD Biosciences
CXCR5	Biotin	2G8	BD Biosciences
FceRIa	PE	MAR-1	eBiosciences
FoxP3	AF488	FJK-16s	eBiosciences
GATA3	eFluor 660	TWAJ-14	eBiosciences
GL7	FITC	GL7	BD Biosciences
Gr-1	PE	RB6-8C5	eBiosciences
IL-13	eFluor 450	eBio13A	eBiosciences
IL-4	Brilliant Violet 711	11B11	BD Biosciences
IL-5	APC	TRFK-5	BD Biosciences
Ly6A/E	Brilliant Violet 786	D7	BD Biosciences
Ly6C/Ly6G	Pe-Cy7	RB6-8C5	eBiosciences
MHCII	Alexa Fluor 700	M5/114.15.2	eBiosciences
NK1.1	PE	PK136	eBiosciences
PD1	Brilliant Violet 421	J43	BD Biosciences
RORyt	PE	Q31-378	BD Biosciences
Siglec-F	PE	E50-2440	BD Biosciences
Streptavidin	Brilliant Violet 786		BD Biosciences
T1ST2	FITC	DJ8	mdbioproducts
Ter-119	PE	TER119	eBiosciences

Table S2: Primary and secondary antibodies used for confocal microscopy

Primary antibodies	**Host IgG**	**Producer**	**Cat#**
a-SMA	Mouse IgG2a	R&D systems	IC1420G
B220	Rat IgG2a	eBioscience	56-0452-82
CD11c	Armenian Hamster IgG1	eBioscience	12-0114-83
CD138	Rat IgG2a	BD biosciences	553713
CD3	Armenian Hamster IgG1	BD biosciences	553062
collagen	Rabbit IgG	Merck Millipore	ABT123
GL7	Rat IgM	BD biosciences	553666
IgE	Goat IgG	Novus Biologicals	NB7530
Secundary antibodies	**Fluorchrome**	**Producer**	
donkey anti-goat IgG	Cy-3	Jackson Immunoresearch	
donkey anti-rabbit IgG	Cy-3	Jackson Immunoresearch	
goat anti-armenian hamster IgG	Cy-3	Jackson Immunoresearch	
donkey anti-rat IgG	Cy-5	Jackson Immunoresearch	
mouse anti-FITC	AF488	Jackson Immunoresearch	

Introduction II

3

DENDRITIC CELL SUBSETS IN ASTHMA
Impaired tolerance or exaggerated inflammation?

Allergen-activated dendritic cells are essential for the induction of Th cell differentiation from naïve T cells in the lung draining lymph node, but also drive the maintenance of pulmonary inflammation during continuous allergen exposure (**Chapter 1**) (1). Lung DCs are a heterogeneous cell population that consists of two types of conventional DCs (cDCs), e.g. cDCs type 1 (cDC1s) and cDC2s. Next to cDCs, the lungs also contain plasmacytoid DCs (pDCs) and under inflammatory conditions, monocyte derived DCs (moDCs) (2-4). DCs can become activated by allergens exposure and by cytokines secreted by the airway epithelium (5,6). Activation of DCs requires induction of the pro-inflammatory transcription factor NF-kB, which can be negatively regulated by the deubiquinating enzyme TNFAIP3/A20 (7). In this thesis, we have made use of transgene mouse strains, enabling the activation of different DC subsets and myeloid cells (summarized **Chapter 1, Figure 4**). In the Introduction II, an overview of the ontogeny and pulmonary localization of DC subsets and their function in asthma both in mouse as in human will be given.

DC ontogeny

DCs originate from hematopoietic stem cells (HSCs) in the bone marrow. These HSCs differentiate into the macrophage DC progenitor (MDP) (8), which give rise to both the common monocyte progenitor (cMoP) and the common DC progenitor (CDP). Whether CDPs also develop without the intermediate MDP stage and can develop directly from HSC is currently unknown. CDPs give rise to pre-cDCs and pDCs (9). Recently, two studies showed that bone marrow pre-cDCs can be divided into pre-cDC1s and pre-cDC2s that are committed to cDC1 and cDC2 development. This indicates that the decision to become cDC1s or cDC2s already occurs in the bone marrow and not in the periphery (10,11). MoDCs develop from the cMoPs (12,13).

Location of pulmonary DC subsets

As the number of pulmonary DCs is low and until recently multiple markers were needed to specify DC subsets, the number of current studies that investigated the location of specific DC subsets in the lung during steady state are rather limited. It is known that cDC1s are located in close proximity to the airway epithelium, and as they express CD103 (alpha integrin) and beta7 integrin, they can interact with E-cadherin expressed by epithelial cells. Compared to other DC subsets, cDC1s have higher expression of tight junction proteins, which facilitates the sampling of antigen by extending their dendrites into the airway lumen. However, cDC1s can also be found in the proximity of vascular endothelial cells (14). Most studies that investigated the localization of pulmonary DC subsets used CD11b as marker for cDC2s (14-16), however CD11b is not exclusively expressed by cDC2s and is also highly expressed by moDCs (1). A recent study

could distinguish moDCs and cDCs by crossing MacBlue mice (*Csf1r*-EGFP$^{tg/+}$) mice to *Itgax*-YFP or *Cx3cr1*$^{gfp/+}$ mice, in which cDCs can be distinguished by YFP expression and monocytes and macrophages by ECFP expression. This study indicated that cDCs are located near the large airways, whereas monocytes and alveolar macrophages are localized in the alveolar space (17). Using MacBlue mice, *in situ* traveling of monocytes and monocytes-derived cells could be investigated, and revealed that monocyte-derived cells in the lungs are located at the interface of blood vessels and the airways (17,18). During steady state, the majority of pulmonary pDCs are located in the alveolar interstitium (14,19), however pDCs are also found in pulmonary infiltrates upon an ovalbumin (OVA)-mediated asthma model.

Due to recent published research, investigating the localization of DC subset can now be performed with fewer markers, as Guilliams *et al*, showed that interferon regulatory factor 4 (IRF4) and IRF8-expression is exclusive for respectively cDC2s and cDC1s across different organs and species (3). Combining these markers with a universal DC marker such CD11c should therefore be sufficient to visualize cDC1 and cDC2 subsets and ease localization studies.

CONVENTIONAL TYPE 1 DENDRITIC CELLS

Development of cDC1

cDC1 development is highly dependent on the transcription factor IRF8, as IRF8 drives DC precursor generation (11), development of pre-cDCs in the bone marrow and promotes survival of terminally differentiated cDC1s (20). Basic Leucine Zipper ATF-Like Transcription Factor 3 (BATF3) is also implicated during cDC development (21), whereas DNA-binding protein inhibitor (ID2) is essential for the terminal differentiation of cDC1s (22). Ontogeny of cDC1s is also regulated by cytokines, as Fms-like tyrosine kinase receptor 3-ligand (Flt3L)-deficient mice completely lack cDC1s in the lungs (1,23). Development of cDC1s is similar to CD8a cDCs in lymphoid organs (23,24).

Function of cDC1s in airway inflammation

Conventional DC1s are well appreciated for their superior cross-presentation of antigens to CD8$^+$ T cells, essential for the induction of virus-specific CD8$^+$ T cells and anti-tumor immune responses (21,25,26). However, cDC1s have an inferior capacity to take up allergens compared to other DC subsets (27). Whether cDC1s are also implicated in Th2 skewing in response to allergen exposure remains controversial, as cDC1s are reported to promote, inhibit or be redundant for Th2 immune responses (27-29). These differences may be explained by the allergen used, amount of allergen, or the mouse strain used to deplete cDC1s. Next to promoting CD8$^+$ T cell responses, cDC1s are often associated with a tolerogenic function. cDC1s can induce differentiation of Tregs upon HDM exposure through the induction of retinoic acid and PPARγ

(28,30). During OVA or HDM-mediated airway inflammation (31) and helminth infections (32). cDC1s can limit Th2 inflammatory responses, emphasizing their tolerogenic potential **(Figure 1)**. Conventional DC1s are also crucial for the function of products known to dampen allergic airway disease, such as *Helicobacter pylori* treatment (33). Furthermore, CD103-deficient mice exposed to a OVA-mediated asthma protocol involving five OVA aerosol challenges, developed a more pronounced eosinophilic inflammation indicating their tolerogenic role during Th2-mediated immune responses (31). In contrast, Fear *et al*, showed that $CD103^{-/-}$ mice that received only a single OVA challenge had decreased eosinophilic inflammation, arguing the tolerogenic properties of cDC1s. Absence of CD103 did not affect DC migration, but decreased the percentage of allergen-loaded migratory DCs in the lung draining lymph node (34). As CD103 can be expressed on both T cells as cDC1s (35) it is hard to determine which effects are caused by the DCs, and which by the T cells. However, it is conceivable that cDC1s are essential for the uptake of antigens through the epithelial barrier at low antigen concentrations. This could explain the decrease in allergen-loaded DCs and absence of Th2 cell immune responses with only a single OVA challenge. By increasing allergen concentrations or frequent allergen exposures this can be overcome by protease activity or passive leakage, enabling other DC subsets to access the allergens and migrate towards the draining lymph node.

In addition to their capabilities to suppress Th2 cell differentiation, cDC1s also control Th17 immune responses upon Aspergillus infections through secretion of IL-2 (36), indicating that cDC1s are essential for maintaining homeostasis in the airways. Furthermore, cDC1s are also important for the removal of apoptotic cells, as resolution of airway inflammation is reduced in CD103-deficient mice (31), and cDC1s have been shown to remove apoptotic cells (37).

As it was described that pulmonary cDC1s express Langerin (14), some studies that investigated pulmonary cDC1 function, depleted pulmonary cDC1s using Langerin- diphtheria toxin receptor mice (1,27). However, flow cytometric analysis showed that only a minority of the pulmonary cDC1s expressed Langerin (38), indicating heterogeneity within the pulmonary cDC1 population.

CONVENTIONAL TYPE 2 DENDRITIC CELLS

Development of cDC2

In contrast to knowledge on cDC1 development, the transcriptional control of cDC2s is not well characterized. Differentiation of cDC2s from pre-cDCs is regulated by RELB (39), PU.1 (40), RBPJ (41-43) and IRF4 (44-46). However, it is unknown during which cDC2 developmental stage these transcription factors are important. Also, the role of the cytokine Flt3L in cDC2 development is controversial, as it has been shown that cDC2 development is independent of Flt3L (23), whereas others found a complete absence of cDC2s in Flt3L-deficient mice (1).

These different findings are likely caused by the dissimilar protocols to distinguish cDC2s from moDCs, as moDCs develop independently of Flt3L (1). The newly proposed universal gating strategy using IRF4 and IRF8 (3) makes the distinction between these DC subsets easier and will help future studies investigating the role of specific transcription factors or cytokines on the development of different DC subsets.

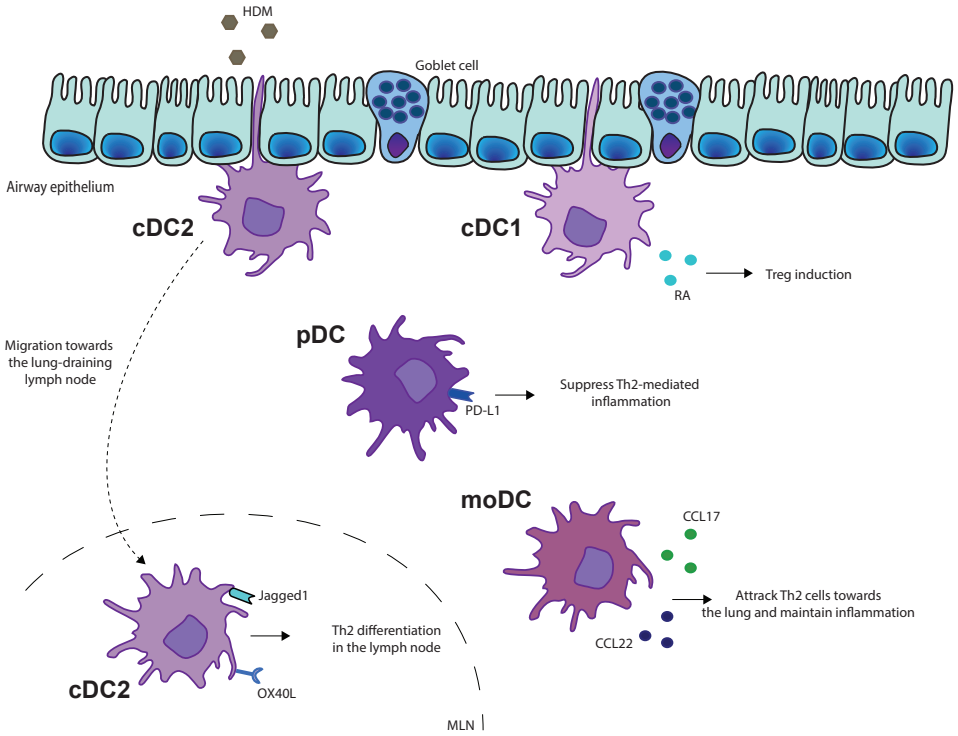

Figure 1: Dendritic Cell functions upon allergen exposure. Type 2 cDCs are essential for the migration and induction of differentiation of Th2 cells in the lung draining lymph node upon allergen Exposure. MoDCs are important for the chemotaxis of effector Th2 cells towards the lungs by secretion of chemokines CCL17 and CCL22. In asthmatic disease, plasmacytoid DCs suppress Th2-mediated inflammation via PD-L1 expression, whereas cDC1s induce Tregs via expression of retinoic acid (RA).

Function of cDC2s in asthma

Conventional DC2s can take up allergen very efficiently (1,47), migrate well to the lung draining lymph node, and induce proper T cell proliferation (1) **(Figure 1)**. Conventional DC2s are essential for the induction of Th2 cell differentiation in allergen-exposed lungs (1,47,48) and even possess the capability to induce Th17 cell differentiation in the gut (44,49). In an HDM-mediated asthma model, cDC2s can induce both Th2 and Th17 differentiation (50). HDM can be recognized by various innate receptors on the cell membrane of DCs, including C-type lectin re-

ceptors, such as Dectin-2 (51). Both HDM-mediated Th2 and Th17 differentiation is dependent on Dectin-2-mediated recognition and/or allergen uptake, as T cells of Dectin-2-deficient mice have reduced expression of both Th2 and Th17 cytokines (50). cDC2-deficient mice through IRF4 deficiency have reduced Th2 immune responses in the airways upon sensitization in the airways (52) or in the skin (53). Similarly, no eosinophilic inflammation and Th2 differentiation was induced in mice in which IRF4 was depleted in mature DCs, using a different *CD11c*Cre, which did not affect cDC2 cell development (54). This indicates the importance of cDC2s for the induction of Th2 differentiation. Dectin-1 expression on DCs appears to be important for migration, as Dectin-1-deficient cDC2s display lower levels of CCR7, and have lower numbers of migratory cDC2s in the lung draining lymph node. Furthermore, Dectin-1$^{-/-}$ mice did not develop eosinophilic inflammation and did not show induction of Th2 or Th17 cytokines in an HDM-mediated asthma model (55). Indicating that Dectin-1 is required for the induction of chemokines and chemokine receptors on cDC2s, needed to migrate towards the lung draining lymph node and induce T cell differentiation. cDC2s exclusively express OX-40L (56), essential for Th2 cell differentiation, implicating the importance of cDC2s for Th2 differentiation.

MONOCYTE-DERIVED DENDRITIC CELLS

Development of moDCs

As their name implicates and stated above, moDCs derive from monocytes. There are two types of monocytes, Ly6C^{hi} and Ly6C^{low} monocytes (57). Ly6C^{hi} monocytes migrate towards inflammatory sites and give rise to Ly6C^{hi} moDCs, Ly6C^{low} moDCs (58) and Ly6C^{low} monocytes (59). MoDCs downregulate Ly6C, upon differentiation from monocytes (1). Ly6C^{low} monocytes patrol the vasculature (57,60) and can differentiate into more long-lived Ly6C^{low} moDCs (58). It is even suggested that monocytes or moDCs can serve as a cDC precursor, in which cDC1s can arise from Ly6C^{hi} CCR2^{hi} monocytes, and cDC2s develop from Ly6C^{low} CCR2^{low} monocytes (61).

Function of moDCs in asthma

After a primary high dose HDM in the airways, moDCs accumulate within 48 hours in the lungs and peak at 72 hours in the lung draining lymph node (1). HDM and other environmental factors trigger the airway epithelium to secrete chemokines and cytokines (62). Secretion of CCL2 will drive migration of monocytes towards the lungs (63), where they will differentiate into moDCs under the regulation of both CCL2 (1) and CSF-1 (23). MoDCs are efficient in antigen uptake, however their capacity to drive T cell proliferation is inferior to cDC2s. Instead, moDCs produce vast amounts of cytokines and chemokines essential for the recruitment and activation of Th2 cells upon HDM exposure (1) **(Figure 1)**. This indicates that moDCs are dispensable for Th2 differentiation but essential during the effector phase of asthma models,

as depletion of CD11b$^+$ myeloid cells during allergen challenge drastically reduces eosinophilia (64). Nevertheless, upon high antigen dose, moDCs migrate towards the lymph nodes and induce Th2 differentiation in the absence of cDCs upon exposure to HDM (1) or cockroach extract (65). Depletion of migratory cDCs enhances Th2 cell-mediated immune responses in an OVA-alum model (66). Furthermore, absence of Th2 cell-mediated immunity, due to the absence of DCs can be reverted by adoptive transfer of monocytes, which then differentiate into moDCs (67). These data are in agreement with recent results indicating that systemic administration of CD11b$^+$ cells from the bone marrow, likely being monocytes, are efficient inducers of Th2-mediated eosinophilic airway inflammation (68). This implicates that at high allergen concentration, moDCs can acquire migratory capacities and are capable of inducing Th2 differentiation and thereby drive Th2-mediated immune responses.

PLASMACYTOID DENDRITIC CELLS

Development of pDCs

Plasmacytoid DCs differentiate directly in the bone marrow from CDPs (69). Differentiation of pDCs depends on Flt3L and STAT3 signalling, in combination with various transcription factors, such as E2-2, IRF8, Ikaros, and PU.1, of which E2-2 is highly specific for pDC development (70-72).

Function of pDCs in asthma

Plasmacytoid DCs are essential for anti-viral immune responses as they produce large amounts of type I interferons (IFN-a) after toll-like receptor 7 (TLR7) activation (73,74). In comparison to other DC subsets, pDCs have a limited capacity to take up, and present antigens (1,19,75,76). pDCs are described to have a tolerogenic function in asthma, as pDCs induce Treg cell differentiation (77,78), and depletion of pDCs in Siglec-H-DTR mice increased the proliferation of antigen-specific CD4$^+$ T cells (79). Increasing pDC numbers by Flt3L treatment alleviates eosinophilic inflammation, which is reversed upon pDC depletion (80). Programmed death-ligand 1 (PD-L1) expression on pDCs is essential for their suppressive effect, as PD-L1-deficient pDCs could not alleviate allergic airway inflammation, whereas indoleamine-pyrrole 2,3-dioxygenase or inducible T cell costimulator ligand (ICOSL)-deficient pDCs could (80) **(Figure 1)**. Development of HDM-driven allergic asthma can be inhibited by the adoptive transfer of pDCs of sensitized donors (81). Different pulmonary pDC subsets are described, e.g. CD8a$^-$b$^-$, CD8a$^+$b$^-$, and CD8a$^+$b$^+$ pDCs (82). Only CD8a$^+$b$^-$ pDCs and CD8a$^+$b$^+$ pDCs have tolerogenic capacities, whereas CD8a$^-$b$^-$ pDCs display more pro-inflammatory functions upon TLR7 and TLR9 stimulation (82). Specifically, CD8a$^+$b$^+$ pDCs CD8a$^+$b$^-$ pDCs have increased expression of retinal dehydrogenase leading to retinoic acid production resulting in increased Treg differentiation (82).

PDCs are also essential for the beneficial effects observed in immunotherapy via the complement subunit C1q. Administration of C1q reduces airway hyperresponsiveness (AHR) and eosinophilia as efficiently as dexamethasone administration, and pDC depletion abrogates the protective effect of C1q (83).

Viral infections are often a trigger for asthma exacerbations. Viral particles activate DC subsets via TLR7, and its expression was decreased in pDCs by allergic inflammation. TLR7-deficient mice displayed reduced IFN secretion, increased virus replication and increased eosinophilic inflammation and AHR, implicating that impaired TLR7 expression on pDCs by allergic inflammation could exaggerate asthma exacerbations (84). Furthermore, pDCs transferred from donors with respiratory tract syncytial virus (RSV) did not provide protection from Th2-mediated inflammation as transferred pDCs from naïve mice did (81). However, CpG maturated pDCs are well capable of protecting from eosinophilic inflammation (80), suggesting that the mode of activation of pDCs can alter their function.

CONCLUDING REMARKS

Taken together, different DC subsets exert different functions. Next to differences in $CD4^+$ or $CD8^+$ T cell activation, it appears that in allergic airway inflammation cDC2s and moDCs are essential for the induction and maintenance of Th2 and/or Th17-mediated inflammation, whereas cDC1s and pDCs are in general suppressive. The exact function of cDC1s during Th2 inflammation remains controversial, as cDC1s are ideally situated to sample incoming allergens, however in allergic asthmatic patients, cDC1s are unable to control inflammation to these harmless antigens. Newly developed stainings and technologies will therefore in the future provide more extensive insight into the similarities and differences of DC functions in asthma.

HUMAN PULMONARY DENDRITIC CELL SUBSETS

Transcriptional development of human DCs

In human lungs, three different DC subsets have been described, human DC subset 1 (DC1) that expresses BDCA1 (CD1c), DC2s, that expresses BDCA3 (CD141) and pDCs, that express BDCA2 (CD123) (3,4,85). Gene-expression profiles of human DC1 and DC2 revealed that human DC subsets resembled mouse cDC1s and cDC2s respectively (3,86-89). Development of human DCs is also highly dependent on Flt3L, as Flt3L injection drastically increases the number of DCs in blood of healthy volunteers (90). Similar to the differentiation of mouse DC subsets, the differentiation of human pDCs is mediated by E2-2 (91), whereas differentiation of cDC1s and cDC2s is controlled by transcription factors BATF3 (92) and IRF4 (93,94) respectively.

Location of human DC subsets in the lungs

BDCA1$^+$ cDC2s were increased in the airway epithelium of asthma patients that display a Th2 phenotype, whereas this was not observed in patients without a Th2 profile (95). Strikingly, DCs are increased in the outer wall of the large airways in patients that suffer from fatal asthma, which are likely moDCs, as they express XIIIa (96). XIIIa is a coagulation factor that is also expressed on macrophages in the skin (97), which are also derived from monocytes and points to a similar precursor cell. Unfortunately, the lack of lung material consisting of airway epithelium as well as airway interstitium of both healthy controls and asthma patients makes it hard to interpret the localization of different DC subset during steady state and in the asthmatic lung. Recent consensus about universal markers that can identify DC subsets will facilitate the visualization of DC subsets in human organs (3).

Function of human DCs in asthma

Until recently most studies investigating human DC function compared pDCs to myeloid DCs (mDCs) that include both cDC1s and cDC2s. Allergic asthmatics have increased frequencies of cDC1s and cDC2s in peripheral blood (98), induced sputum and BAL upon allergen inhalation compared to controls (99-101). After allergen inhalation, only cDC2s migrated towards the bronchial tissue (101). Allergen exposure increased the expression of TSLP receptor but not IL-33 receptor on cultured cDCs from CD34$^+$ precursors of the bone marrow, which are implicated as Th2 instructive cytokine receptors (101). Inhalation of allergens induced expression of IL-25 receptor on both cDCs and pDC (102) **(Figure 2)**. The co-stimulatory molecule OX-40L and the expression of the Th2 chemoattractant CCL17 was higher on cDCs of patients with mild asthma than on cDCs of healthy controls (103). In patients that displayed high Th2 cell numbers, a large number of airway mucosal cDC2s expressed FceRIa compared to Th2-cell low asthma patients (95). This is likely, as IgE levels are increased in patients that exhibit high Th2 cell numbers, indicating that IgE increases the expression of FceRIa. IgE-bound antigens are rapidly internalized, processed and presented by DCs to antigen-specific CD4$^+$ T cells (104,105). cDC2s loaded with Der p1 allergen-IgE immune complexes induced IL-4 and lowered IFNg-expression upon co-culture with naïve T cells (106), indicating that allergen-IgE immune complexes promote Th2 differentiation (107).

cDCs of allergic asthmatics induced increased Th2 differentiation upon stimulation with TSLP and Der p compared to cDCs of controls. TSLP-stimulated cDCs of allergic asthmatics and not of controls, induced IL-9 production and PU.1 expression. When the Th2 priming capacity of cDC1 and cDC2s from human blood and lungs was compared, it was found that both lung as blood cDC1s are superior in Th2 differentiation (109). However, in this study, live attenuated influenza virus was used to activate cDC1s and cDC2s, which primarily will activate cDC1 as they are essential for the induction of anti-viral immune responses (25). During acute asthma exacerbations, the expression of CD141, a marker for cDC1 is increased in blood leukocytes on

moDCs, but surprisingly not on cDC1s (110). This could indicate that cDC1s or CD141 expression plays an important role in the pathogenesis of asthma. $CD1c^+$ cDC2s from allergic rhinitis patients efficiently prime Th2 differentiation (111) and express lower levels of ICOSL as compared to controls **(Figure 2)**. Blockade of ICOSL in cDC2s of controls increases the production of Th2 cytokines, indicating that decreased ICOSL expression on cDC2s could promote Th2 differentiation (112). Both human cDC1s and cDC2s have been reported to induce Th2 cytokines, however Th2 differentiation by cDC1s was observed upon exposure to live-attenuated viral particles (111). This may implicate that during virus infections, cDC1s in asthmatics shift from promoting Th1 immune responses or maintaining tolerance towards a more Th2-promoting phenotype.

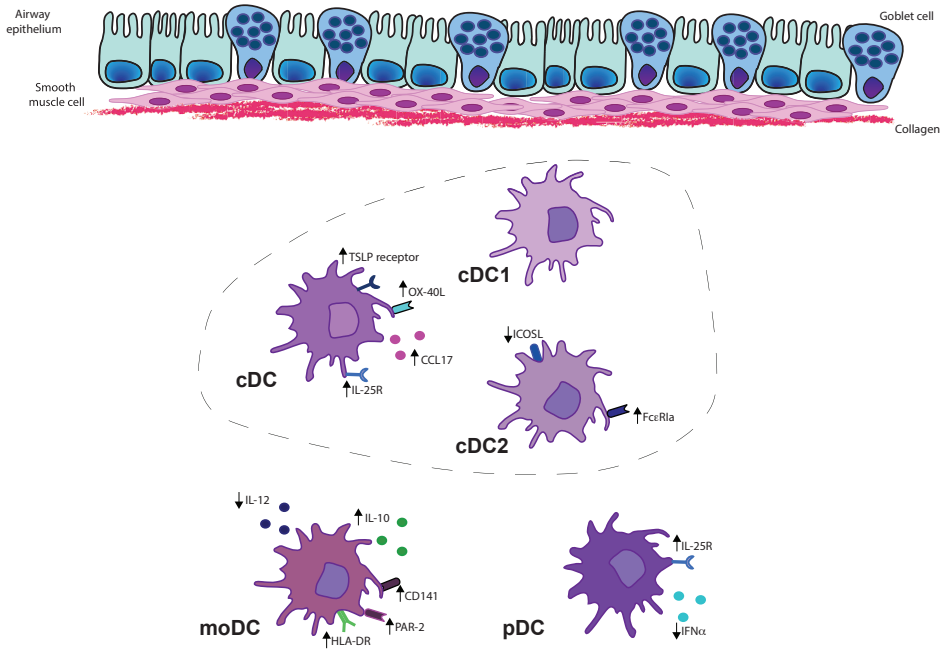

Figure 2: Dendritic cell subset alterations in asthmatic patients. Conventional DCs, including both cDC1s and cDC2s of asthma patients display higher levels of IL-25R, TSLP receptor, OX-40L and secretion of CCL17. Especially ICOSL expression in cDC2s of asthmatics is reduced whereas FceRIa expression is increased in asthmatics that display a Th2 high phenotype. MoDCs of asthmatics display increased expression of HLA-DR, CD141 and PAR-2, and the anti-inflammatory cytokine IL-10, whereas IL-12 production is reduced. Plasmacytoid DCs of asthmatics show increased expression of the IL-25R, whereas IFNa secretion was reduced.

Allergen inhalation increased pDCs numbers in the airway lumen (99,113), however variable results exist whether circulating pDCs differ between asthmatics and healthy individuals (114,115). In a birth cohort, numbers of circulating pDCs predicted respiratory tract infections,

wheezing and asthma diagnosis by 5 years of age (116). It was also shown that pDCs of severe asthmatics produced less IFNa following influenza infection, than pDCs of healthy controls (117) **(Figure 2)**.

HDM activation of cultured monocyte-derived DCs of HDM-allergic asthma patients induced more T cell proliferation (118) and Th2 differentiation than control moDCs (119). This could be due to a higher HLA-DR expression of moDCs from allergic asthmatics compared to non-asthmatic subjects (118) **(Figure 2)**. When examining the frequency of intermediate monocytes, expressing both CD14 and CD16, conflicting results exist in severe asthmatics (120,121). Monocytes of allergic patients showed increased IL-10 and decreased IL-12 production upon HDM and Der p1 stimulation, which enhanced Th2 differentiation (122). Intermediate monocytes of severe asthma patients display higher expression of Protease-Activated Receptor-2 (PAR-2), a G-coupled receptor activated by proteases as compared to mild/moderate asthma patients (121). PAR-2-mediated activation of monocytes induces secretion of IL-1b, IL-6 and IL-8 (123), indicating that activation via PAR-2 facilitates secretion of cytokines important for Th17 cell differentiation and neutrophil activation and attraction, which does not occur in mild/moderate asthmatics.

CLINICAL IMPLICATIONS

In conclusion, whereas in mice the function of different DC subsets in asthma pathogenesis is becoming more and more clear, there are no studies at present that compared the Th2 or Th17-priming capacity of different human DC subsets in response to allergens. The limited number of DCs in peripheral blood and the difficulty to obtain lung or lung-draining lymph nodes, hamper these studies. Current possibilities in single cell analysis will enable analysis of these different DC subsets, and provide insights in differences in DC characteristics in asthmatics that display either Th2, mixed Th2/Th17 or Th17-mediated asthma.

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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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4

ABSTRACT

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-κB activation. Ablation of TNFAIP3, one of the crucial negative regulators of NF-κB 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-1ß, 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.

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-1β, 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-κB, and thereby initiates transcription of pro-inflammatory cytokines, such as IL-1 and IL-12 (22,23). The activation threshold of DCs and NF-κB levels is carefully regulated, as overt activation can lead to immune responses to self-antigens in addition to allergens. NF-κB activation is negatively regulated by TNFAIP3 (TNFa-induced protein 3, also known as A20), a ubiquitin modifying enzyme that deubiquitinates several key intermediate NF-κB signalling molecules, and thus acts as a brake on DC activation (24,25). Genetic polymorphisms in *TNFAIP3* and TNFAIP3 interacting protein (*TNIP*) have been associated with risk of developing asthma and allergies (26,27). 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 (26). 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 (28). 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 (29). 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}EGFP 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 analysed 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 (35). 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 the innate effect of HDM exposure on cytokine production by pulmonary DCs, mice were treated with 100 µg of HDM extract intratracheally. 24 hours later, single cell

suspensions were obtained from lungs by digesting the lungs using DNAse and Liberase for 30 minutes at 37°C(3). Upon digesting, the lungs were homogenized through a 100 µm cell strainer (BD Biosciences). Red blood cells were lysed using osmotic lysis buffer (8.3% NH_4CL, 1% $KHCO_3$ and 0.04% NA_2EDTA in Milli-Q).

To examine Th-cell proliferation and differentiation in naïve HDM-specific T-cells, we adoptively transferred $2x10^6$ CFSE-labelled 1-DER T-cells, specific to the Der p 1WAFSGVAAT peptide (35) 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) (52), as earlier described by Nota et al. (53) on TIGR multiexperiment viewer (MEV) software (54). MeV software was used to generate heatmaps and principal component analysis.

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 (55). 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 FACSDivaTM software (Beckton Dickinson) and analysed by FlowJo version 9 (Tree Star Inc software).

Lung histology

5 um-thick paraffin embedded lung sections were stained with heamatoxylin/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 the manufacturers protocol (Opteia, BD Biosciences). IL-5 (Ready Set Go, eBioscience), IL-13, IL-17 (R&D systems), and IFNγ (Opteia, BD Biosciences) levels were measured in supernatant of HDM restimulated MLN cells. IL-1β (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 analysis 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 (30), we crossed *Tnfaip3*^{LysM} mice to Rosa26-stop^{fl}EGFP mice, to visualize TNFAIP3 deletion in various myeloid cell types. As described before, GFP expression was found in pulmonary macrophages, neutrophils, monocytes, and DCs (30) (Figure 1A). LysM-driven cre-recombinase activity in DCs has been reported previously (30), but is exceptional as cDCs seem to derive from a dedicated pre-cDC progenitor (31,32). Pulmonary DCs can be subdivided into cDCs (CD103$^+$ cDC1s and CD11b$^+$ cDC2) and CD64$^+$ moDCs (32). Both moDCs, cDC2s and cDC1s showed similar GFP expression as pulmonary monocytes (Figure 1B-C). To determine whether TNFAIP3-deletion would alter the proportions of GFP$^+$ cells, GFP expression was determined in *Tnfaip3*$^{LysM\text{-}WT}$, *Tnfaip3*$^{LysM\text{-}HZ}$ and

Tnfaip3$^{LysM\text{-}KO}$ mice. The proportions of GFP-expressing cDC2s, moDCs, and monocytes was enhanced in *Tnfaip3*$^{LysM\text{-}KO}$xROSA26^{fl}EGFP mice compared with *Tnfaip3*$^{LysM\text{-}WT}$xROSA26^{fl}EGFP mice (Figure 1A/1C). Not only the proportions of GFP^{+} cells increased, also total DCs, cDC1s, and moDC numbers were elevated in naïve *Tnfaip3*$^{LysM\text{-}KO}$ mice compared with both *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}HZ}$ mice (Figure 1D).

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.

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 (29) and exposed them to an acute HDM-driven asthma model (Figure 2A). As expected, HDM-sensitized *Tnfaip3*$^{LysM\text{-}WT}$ mice had increased eosinophils in the broncho-alveolar lavage (BAL) compared to PBS-treated controls (Figure 2C). In contrast, HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice developed only a very mild eosinophilic inflammation, but neutrophilic inflammation was strongly increased compared to WT controls. Moreover, using *Tnfaip3*$^{LysM\text{-}HZ}$ mice, a gene dose effect was observed, as these mice developed a mixed eosinophilic/neutrophilic inflammation (Figure 2C). T-cells, B-cells, DC, and macrophages influx did not differ between *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}KO}$ mice, yet B cells were increased in HDM-sensitized *Tnfaip3*$^{LysM\text{-}HZ}$ mice compared to HDM-sensitized *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}KO}$ mice (Figure 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*$^{LysM\text{-}WT}$ mice the numbers of IL-13-expressing Th2-cells were increased, *Tnfaip3*$^{LysM\text{-}KO}$ mice mainly showed an increase in IL17-expressing Th17 and interferon γ (IFNg)-expressing Th1-cells.

IL-17/IFNg-double-expressing CD4 T-cells were not induced (data not shown). *Tnfaip3*$^{LysM\text{-}HZ}$ mice had elevated numbers of Th2-cells compared to *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}KO}$ mice, and similar numbers of Th17-cells compared to *Tnfaip3*$^{LysM\text{-}KO}$ mice (Figure 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 IFNγ secretion were also increased in *in vitro* HDM-restimulated MLN cells of HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice compared with both *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}HZ}$ mice. IL-13 levels were elevated in *in vitro* HDM-restimulated MLN cells of HDM-sensitized *Tnfaip3*$^{LysM\text{-}HZ}$ mice compared with HDM-sensitized *Tnfaip3*$^{LysM\text{-}WT}$ mice (Figure 2G).

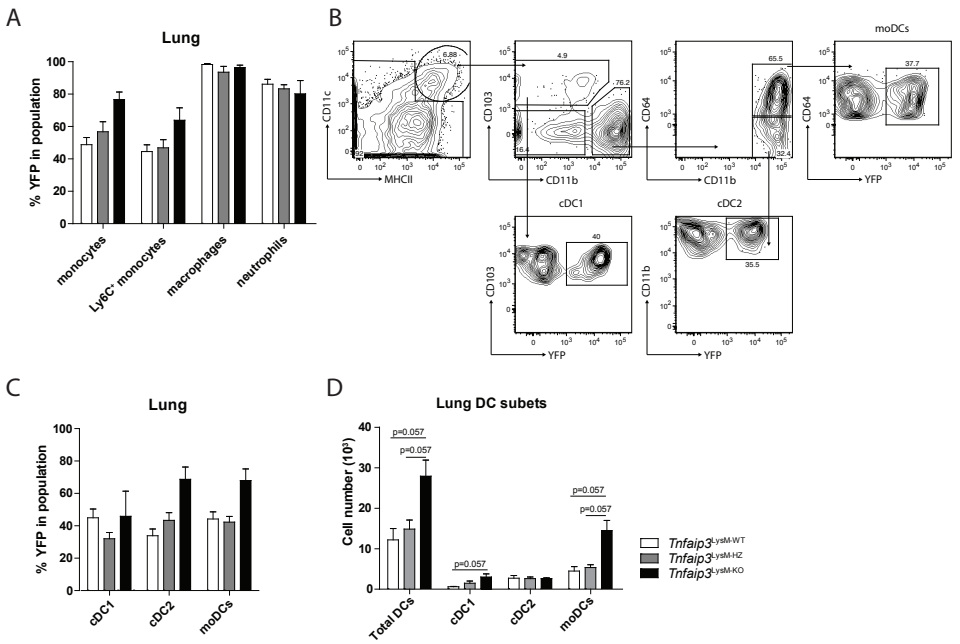

Figure 1: LysM expression targets all pulmonary DC subsets. (A) Gating strategy for different pulmonary DC subsets using flow cytometry. (B) Percentage GFP positive cells within the gated population. (C) Percentage GFP positive cells within the gated population in *Tnfaip3*$^{LysM\text{-}WT}$, *Tnfaip3*$^{LysM\text{-}HZ}$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

In summary, ablation of TNFAIP3 levels in myeloid cells dose dependently induces a neutrophilic, Th17-mediated airway inflammation rather than a Th2-mediated eosinophilic airway inflammation in an HDM-driven asthma model.

Tnfaip3$^{LysM\text{-}KO}$ 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 (33). 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 (Figure 3A) (34). Upon chronic HDM exposure, eosinophilic and B-cell inflammation was increased in both *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}KO}$ mice compared to PBS controls. Neutrophils and total T-cell numbers were higher in HDM-treated *Tnfaip3*$^{LysM\text{-}KO}$ mice compared to HDM-treated *Tnfaip3*$^{LysM\text{-}WT}$ mice (Figure 3C-D). No differences were found Th2-cell numbers in BAL fluid of *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}KO}$ mice, whereas Th17 and Th1-cell numbers were strongly increased in *Tnfaip3*$^{LysM\text{-}KO}$ mice compared with HDM-treated *Tnfaip3*$^{LysM\text{-}WT}$ (Figure 3E). Serum levels of HDM-specific IgE were similar between HMD-treated *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}KO}$ mice (Figure 3F). HDM-treated *Tnfaip3*$^{LysM\text{-}KO}$ mice showed

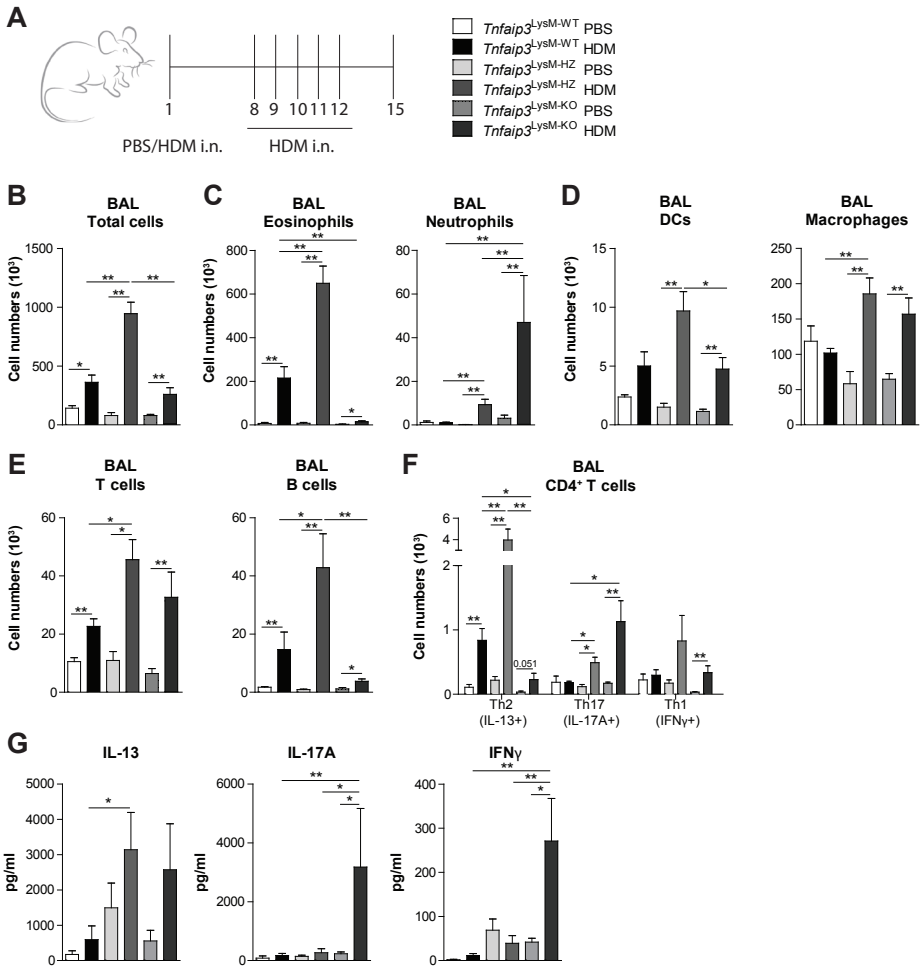

Figure 2: Myeloid specific deletion of *Tnfaip3* induces Th17 neutrophilic asthma upon an acute HDM-driven asthma protocol. (A) Mice were sensitized i.n. with PBS or 1 µg HDM on day 0 and challenged i.n. with 10 µg HDM daily between days 7 and 11 to induce allergic asthma. Analysis was performed at day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

denser pulmonary inflammatory sites compared to HDM-treated *Tnfaip3*$^{LysM\text{-}WT}$ mice (Figure 3G). Despite the absence of clear differences in the airway hyperresponiveness (AHR) causing cytokine IL-13-expressing CD4$^+$ T-cells, lung resistance was surprisingly higher in HDM-treated *Tnfaip3*$^{LysM\text{-}KO}$ mice compared to HDM-exposed *Tnfaip3*$^{LysM\text{-}WT}$ mice (Figure 3H).

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

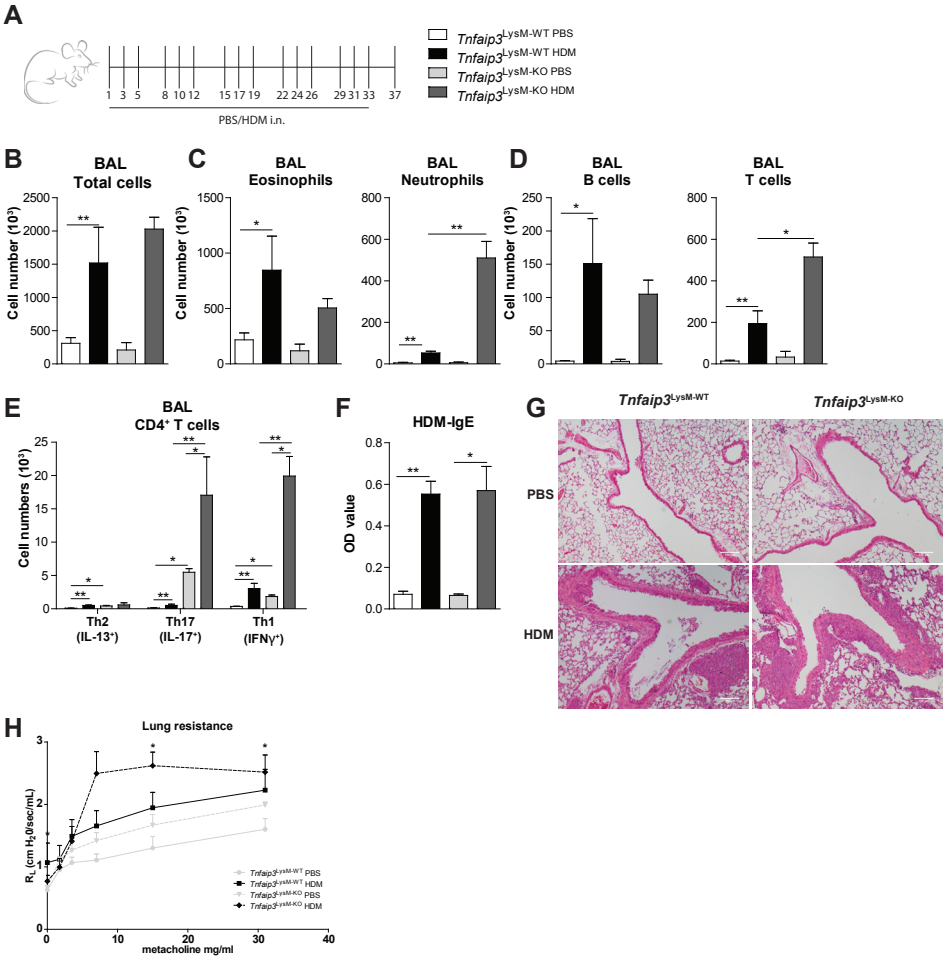

Figure 3: *Tnfaip3*$^{LysM-KO}$ mice develop severe corticosteroid resistant asthma upon chronic HDM exposure. (A) Mice were exposed to a chronic HDM model, and treated with PBS or HDM at indicated time points. Analysis was performed at day 37. (B-D) Quantification of total BAL inflammation, eosinophils, neutrophils, B-cells and T-cells by flow cytometry. (E) Number of IL-13$^+$, IL-17$^+$ and IFNγ$^+$ CD4$^+$ T-cells in the BAL were analysed by flow cytometry. (F) Histologic analysis of lung inflammation by H&E staining. (G). Serum HDM-specific IgE levels. (H) Bronchial hyper-responsiveness was measured upon increasing doses of inhaled methacholine. Results are presented as mean ±SEM of n=3-5 per group and representative of two or more independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001.

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*$^{LysM\text{-}KO}$ 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. In these mice, *Tnfaip3* is specifically deleted in all DC subsets and alveolar macrophages as previously shown by Kool *et al* (25). HDM-sensitized *Tnfaip3*$^{CD11c\text{-}KO}$ mice were resistant to development of eosinophilic inflammation, yet a strong neutrophilic inflammation was induced compared with HDM-sensitized WT controls. Like the *Tnfaip3*$^{LysM\text{-}HZ}$ mice, HDM-treated *Tnfaip3*$^{CD11c\text{-}HZ}$ mice developed a mixed eosinophilic/neutrophilic inflammation (Figure 4A-B). Numbers of T-cells and macrophages in BAL were not

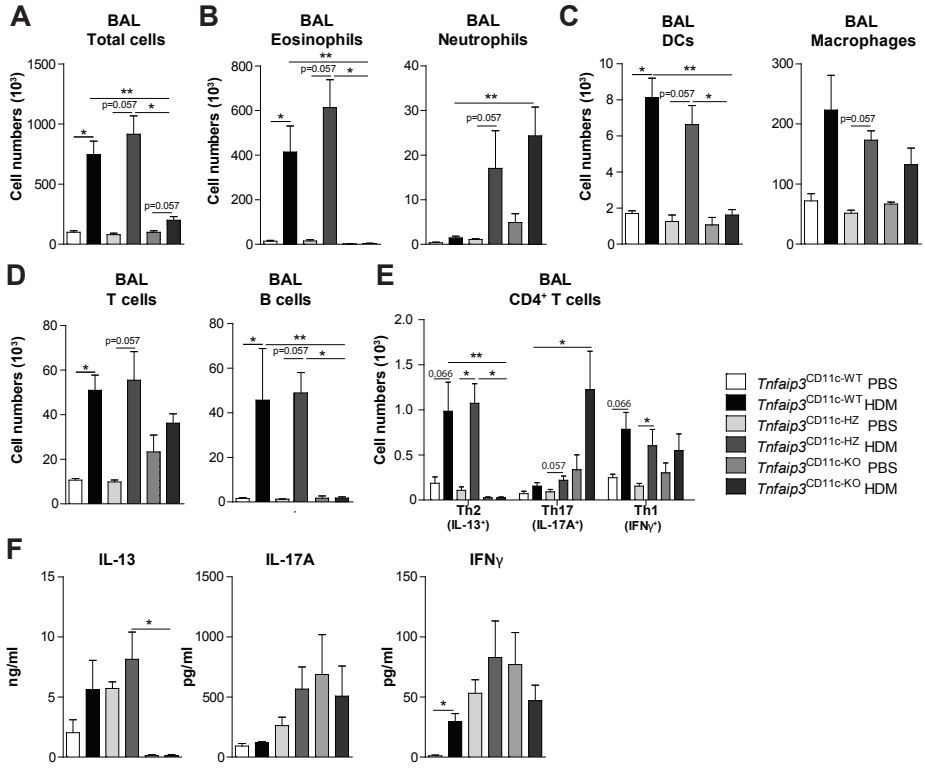

Figure 4: DC specific deletion of Tnfaip3 also induces Th17 mediated neutrophilic asthma. (A-D) Quantification of total BAL inflammation, eosinophils, neutrophils, DCs, macrophages, B cells and T-cells by flow cytometry. (E) Number of IL-13$^+$, IL-17$^+$ and IFNγ$^+$ CD4$^+$ T-cells in the BAL were analysed by flow cytometry. (F) Quantification of IL-13, IL-17 and IFNγ in supernatant of HDM-restimulated MLN cells by ELISA. Results are presented as mean ±SEM of n=3-7 per group and representative of two or more independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001.

significantly different between HDM-sensitized *Tnfaip3*$^{CD11c\text{-}WT}$, *Tnfaip3*$^{CD11c\text{-}HZ}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice, but B cells and DCs were reduced in HDM-sensitized *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared with both HDM-sensitized *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}HZ}$ mice (Figure 4C-D). Th2-cell numbers in BAL fluid of HDM-sensitized *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}HZ}$ mice were increased compared to HDM-sensitized *Tnfaip3*$^{CD11c\text{-}KO}$ mice. HDM-sensitized *Tnfaip3*$^{CD11c\text{-}KO}$ mice showed increased Th17-cells in BAL fluid compared to HDM-sensitized *Tnfaip3*$^{CD11c\text{-}WT}$ mice (Figure 4E). MLN cells of both HDM-sensitized *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}HZ}$ 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*$^{CD11c\text{-}KO}$ mice. IL-17A levels were higher in HDM-restimulated MLN cells of HDM-sensitized *Tnfaip3*$^{CD11c\text{-}KO}$ mice than of HDM-sensitized *Tnfaip3*$^{CD11c\text{-}WT}$ mice (Figure 4F).

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

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

To investigate more directly whether *Tnfaip3*$^{CD11c\text{-}KO}$ 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 transgenitically express TCRs encoding a Vβ4 and Vα8 chain, leading to a precursor frequency of 60-70% of Der p1-reactive CD4$^+$ T cells (35).

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

The expression of lineage defining transcription factors like RORgT, GATA3 and T-bet were used to study differentiation of Th17, Th2 and Th1-cells, respectively. Strikingly, the percentage of RORgT$^+$ 1-DER T-cells was increased in both undivided and divided 1-DER T-cells in *Tnfaip3*$^{CD11c\text{-}KO}$ mice compared to both *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}HZ}$ mice (Figure 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*$^{CD11c\text{-}KO}$ mice had decreased levels of GATA3 compared with *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}HZ}$ mice (Figure 5F). No differences were found in the expression levels of T-bet in 1-DER T-cells in *Tnfaip3*$^{CD11c\text{-}WT}$, *Tnfaip3*$^{CD11c\text{-}HZ}$, and *Tnfaip3*$^{CD11c\text{-}KO}$ mice (Figure 5F).

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

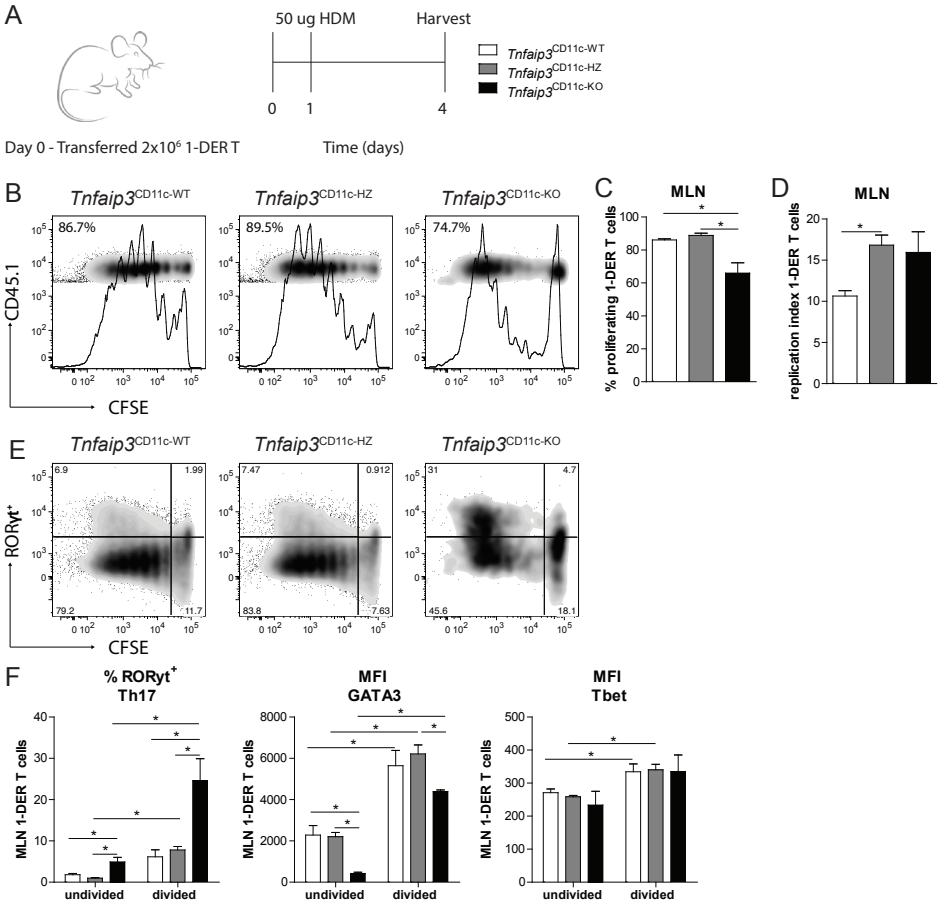

Figure 5: HDM specific differentiation of Th17-cells by *Tnfaip3* deficient DCs. (A) Scheme for intravenous transfer of CFSE labelled 1-DER T-cells into *Tnfaip3*^{CD11c} mice and subsequent exposure to HDM intranasal at indicated time points. Analysis was performed at day 5. (B) Flow cytometric gating of proliferating 1-DER T-cells in the MLN. (C) Quantification of the % proliferating 1-DER T-cells in the MLN by flow cytometry. (D) Flow cytometric gating of RORγT^{+} dividing 1-DER T-cells in the MLN. (E) Percentage of RORγt^{+} Th17-cells within the undivided and divided 1-DER T-cell population in the MLN by flow cytometry. (F) Quantification of the 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 ±SEM of n=4 per group. * = p<0.05, ** = p<0.01, *** = p<0.001.

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 (36). We therefore resorted to a model of intra-tracheal adoptive transfer of bone marrow (BM)-derived DCs to induce asthma (37). 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 (Figure S1) (38). 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* (Figure S2). We intra-tracheally sensitized C57BL/6 mice by adoptive transfer of HDM or PBS-stimulated BM-derived DCs from *Tnfaip3*$^{CD11c\text{-}WT}$ and *Tnfaip3*$^{CD11c\text{-}KO}$ mice, followed by 5 HDM challenges (Figure 6A). As previously reported in this model when mice were sensitized with ovalbumin-stimulated DCs, C57BL/6 mice sensitized with HDM-stimulated *Tnfaip3*^{WT} DCs developed an increased eosinophilic airway inflammation both in absolute numbers as in percentages compared to mice sensitized with PBS-stimulated *Tnfaip3*^{WT} DCs (Figure 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 HDM-stimulated *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs, but was not different between mice sensitized with HDM-stimulated *Tnfaip3*^{WT} or *Tnfaip3*^{KO} DCs (Figure 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} DC-sensitized mice. The percentage of IFNg-expressing CD4^{+} T-cells was not different between all mice (Figure 6D).

In conclusion, HDM-stimulated *Tnfaip3*^{KO} DCs specifically induce Th17-cell differentiation above Th2-cell differentiation, resulting in an absence of eosinophilic inflammation.

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

To gain insight into the mechanism by which *Tnfaip3*^{KO} DCs induced Th17-cell differentiation, we performed gene expression profiling of PBS or HDM-stimulated GM-CSF BM-cultured *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs. Both unstimulated as HDM-stimulated GM-CSF BM-cultured *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs differed in their expression profile as observed in the Principal Component Analysis (PCA) (Figure 7A). In the unstimulated situation, 934 genes were differentially expressed between *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs (Figure S3), whereas 1290 genes were differentially expressed upon HDM stimulation in either *Tnfaip3*^{WT} or *Tnfaip3*^{KO} DCs (Figure S4). Of these 1290 genes, 1054 were differentially expressed in HDM versus unstimulated *Tnfaip3*^{WT}

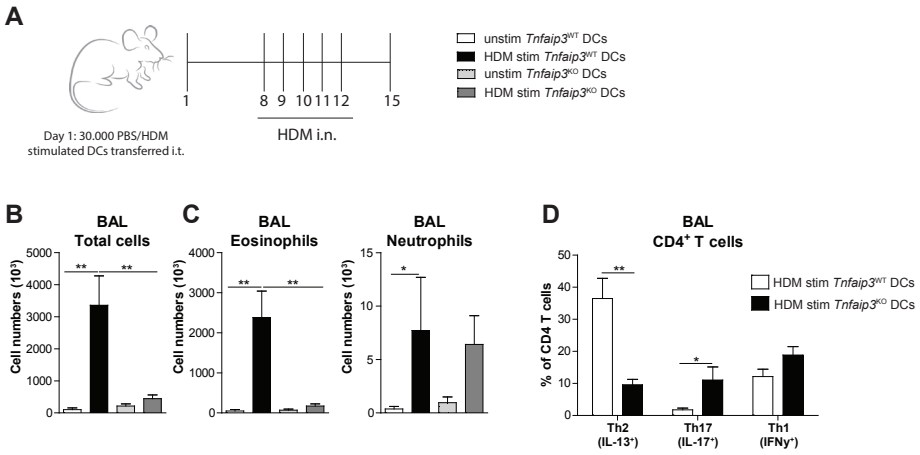

Figure 6: Adoptive transfer of *Tnfaip3*-sufficient or deficient DCs drives eosinophilic or neutrophilic asthma. (A) C57bl/6 mice were sensitized on day 1 i.n. with 30.000 PBS/HDM stimulated GM-CSF cultured DCs from bone marrow of *Tnfaip3*^{CD11c} mice. All mice were challenged i.n. with 10 µg HDM daily 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 IFNγ$^+$ CD4$^+$ T-cells in the BAL were analysed by flow cytometry. Results are presented as mean ±SEM of n=4-6 per group and are representative of two independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001.

DCs, 174 genes in both *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs, and 62 genes only in *Tnfaip3*^{KO} DCs (Figure 7B). Clustering of these 1290 genes, revealed that multiple genes involved in Th17 differentiation, such as IL-6 and IL-1β were upregulated upon HDM stimulation specifically in *Tnfaip3*^{KO} DCs. Also CXCL3, a known chemoattractant for neutrophils was increased in HDM-stimulated *Tnfaip3*^{KO} DCs (39). Furthermore, IL-12b, a cytokine known to inhibit Th2 differentiation when expressed in DCs (40) was specifically induced in HDM-stimulated *Tnfaip3*^{KO} DCs (Figure 7C-D). IL-1β, IL-23 and IL-12 protein expression in culture supernatants of BM-derived DC cultures was also increased upon HDM stimulation, whereas IL-6 protein expression was already increased in unstimulated GM-CSF BM-cultured *Tnfaip3*^{KO} DCs compared to HDM-stimulated GM-CSF BM-cultured *Tnfaip3*^{WT} DCs (Figure 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}x*Tnfaip3*^{CD11c} mice.

IL-23^{YFP}x*Tnfaip3*^{CD11c} mice received an intranasal administration of 100 mg 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}x*Tnfaip3*$^{CD11c\text{-}KO}$ mice was increased compared to IL-23^{YFP}x*Tnfaip3*$^{CD11c\text{-}WT}$ mice (Figure 8A-B). cDC2s in IL-23^{YFP}x*Tnfaip3*$^{CD11c\text{-}KO}$ mice

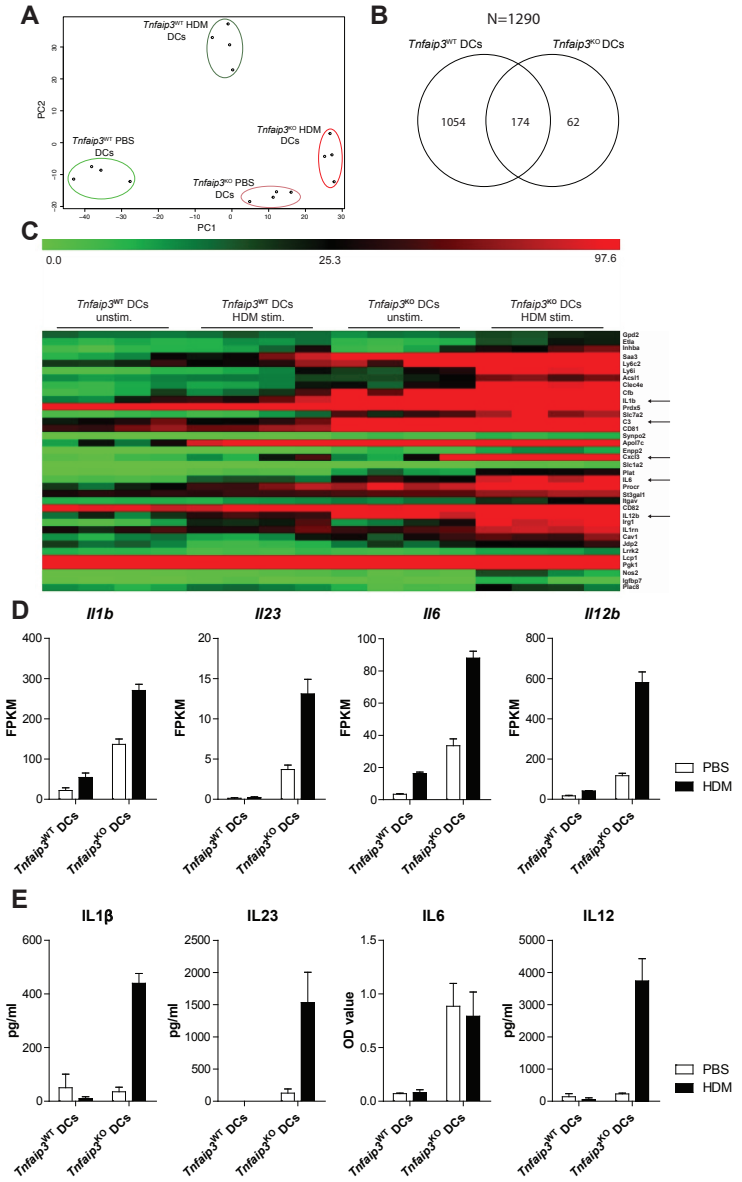

Figure 7: GM-CSF cultured *Tnfaip3*^{KO} DCs display a Th17-instructive signature after HDM stimulation. (A) Principal Component Analysis of PBS/HDM stimulated GM-CSF BM-cultured *Tnfaip3*^{WT} and *Tnfaip3*^{KO} DCs. (B) RNA sequencing analysis of total mRNA from PBS/HDM stimulated GM-CSF BM-cultured 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 IL1β, IL23, IL-6 and IL12 in supernatant of PBS/HDM stimulated GM-CSF bone marrow cultured DCs using ELISA. Results are presented as mean ±SEM of n=4-6 per group. * = p<0.05, ** = p<0.01, *** = p<0.001.

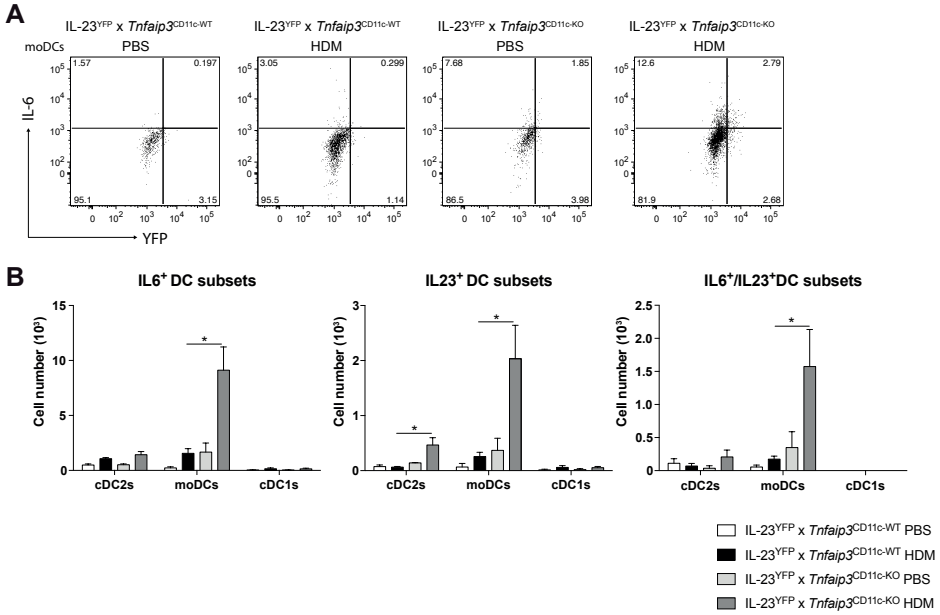

Figure 8: MoDCs and cDC2s in IL-23^{YFP}x*TNFAIP3*$^{CD11c\text{-}KO}$ mice display a pro-Th17-signature upon HDM stimulation *in vivo*. (A) Flow cytometric gating of IL-6 and IL-23 producing pulmonary DCs in IL-23^{YFP}x*Tnfaip3*^{CD11c} mice 24 hours after HDM exposure. (B) Quantification of total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

only had significantly increased IL-23 production, whereas IL-6 and IL-23 production was not different between cDC1s in IL-23^{YFP}x*Tnfaip3*$^{CD11c\text{-}WT}$ and IL-23^{YFP}x*Tnfaip3*$^{CD11c\text{-}KO}$ mice (Figure 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 (41,42). 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-κB activation driving expres-

sion of key cytokine pathways. Ablating the negative feedback regulator of NF-κB signalling, TNFAIP3, in myeloid cells and DCs was shown to be essential for Th17 differentiation (25,29).

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 specifically cDC2s and moDCs increased their potential to produce cytokines implicated in Th17-cell differentiation, such as IL-1ß, IL-6 and IL-23, whereas IL-12 is known to inhibit Th2 differentiation (43). IL-6 secretion by DCs is also known to hamper Th2 differentiation (44), either directly through inhibiting GATA3 expression, or indirectly by restricting IL-4 expression by follicular Th-cells (45). This indicates that both IL-12 as IL-6 could explain why development of Th2 immunity was hampered.

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 (46), suggesting that combined expression of Th2 and Th17-cell cytokines can further enhance AHR. Furthermore, suppression of IL-13 and IL-17 simultaneously decreases AHR more efficiently (47).

Sensitization of C57BL/6 mice by 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, 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 (41), 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-κB activation (25,48). NF-κB directly promotes transcription of pro-inflammatory Th17 differentiation target genes IL-1β and IL-6 (49), which indicates that TNFAIP3 deletion in DCs directly enables the transcription of Th17-promoting cytokines through increased NF-κB signalling.

Now that we have established that intrinsic levels of TNFAIP3 in DCs can determine the type of allergen-induced inflammation, it will be important to understand how this pathway is regulated in DCs, and can be manipulated in the future. First, SNPs in the *TNFAIP3* gene as well as its binding partner *TNIP1* are associated with asthma and allergy development in at least 2 genetic association studies, suggesting that an important aspect of this pathway might be genetically regulated (26,27). 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 (18,50). 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* (26). TNFAIP3 seems to be strongly regulated by microRNAs (mir125 family and mir221) (51), 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-κB-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.

ACKNOWLEDGEMENTS

These studies were partly supported by NWO-VENI (916.11.067), European Framework program 7 (FP7-MC-CIG grant 304221.), and Netherlands Lung Foundation (3.2.12.087 and 4.2.13.054JO). We would like to thank Tridib Das, Melanie Lukkes, Boudewijn van der Wel, Wilfred van Ijcken (Erasmus MC), and the Erasmus MC Animal Facility (EDC) staff for their assistance during the project.

AUTHOR CONTRIBUTIONS

HV, RWH, MK designed the experiments. HV, IB, JvH, MvN, DvU, SYP, MS performed experiments and analysed data. HV, BL, RWH and MK wrote the manuscript. All authors red and approved the final manuscripts.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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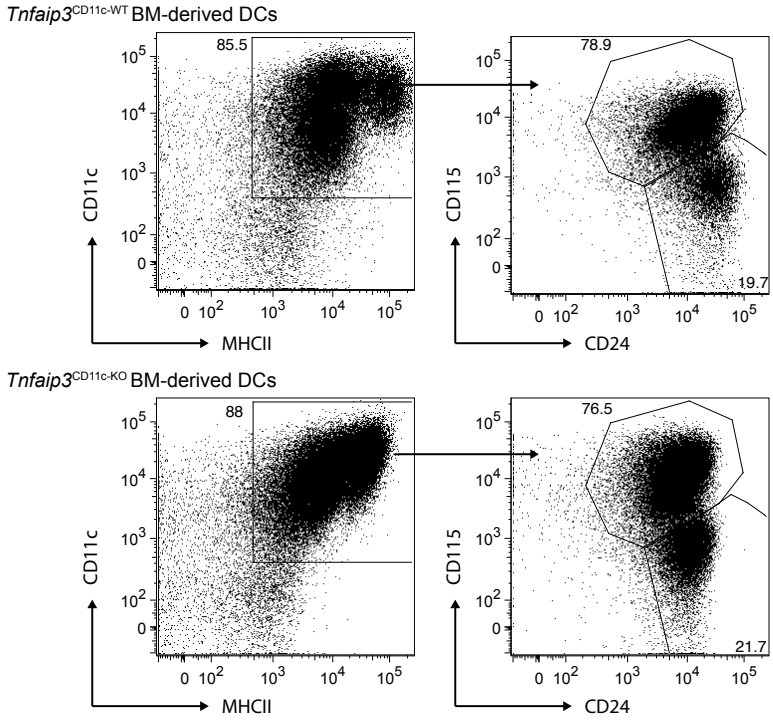
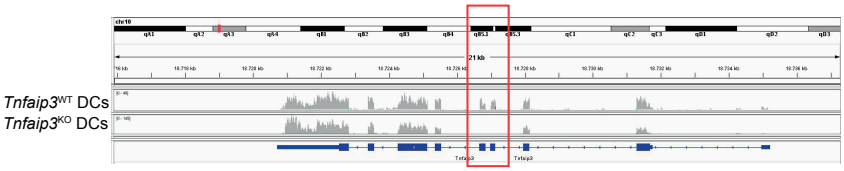
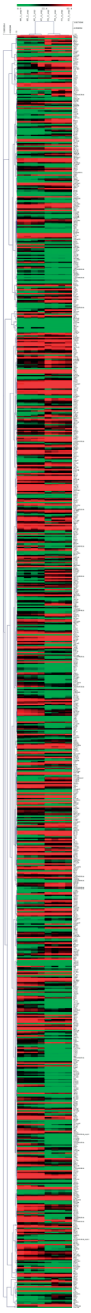


Supplementary figure 1: Composition of moDCs and cDCs in GM-CSF cultured BM-DCs. Flow cytometric analysis of the composition of moDCs and cDCs in GM-CSF cultured BM-DCs.



Supplementary figure 2: 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 3: 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 4: 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.

Table S1: Antibodies used for flowcytometry

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	TWAJ-14	eBioscience
GR-1	Pe-Cy7	RB6-8C5	eBiosciences
IFNy	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

HDM-driven neutrophilic airway inflammation in mice with TNFAIP3-deficient myeloid cells is IL-17-independent.

Manuscript in preparation

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5

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 the enzyme TNFAIP3/A20 is a crucial determinant of eosinophilic or neutrophilic airway inflammation. However, whether neutrophilic inflammation is dependent on IL-17 remains unknown.

Objective: In this study, we investigated the importance of IL-17RA-signalling in the development of 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 exposed 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 reduced but not abrogated in the absence of IL-17RA. Mucus-producing cells in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice were reduced compared to their controls. Production of IL-5, IL-13 and IFNg by CD4^{+} T cells was similar between WT and *Il17ra*^{KO} mice, whereas IFNg-expressing CD4^{+} T cells increased in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. Strikingly, a spontaneous accumulation of pulmonary Th17 and γδ-17 T cells was observed in *Tnfaip3*$^{LysM\text{-}KO}$*Il-17ra*^{KO} mice compared to all other genotypes. This coincided with increased IL-23 expression in the lungs of HDM-exposed *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice.

Conclusion/Discussion: IL-17RA-signalling is dispensable for the development of HDM-induced eosinophilic or neutrophilic airway inflammation, however not for mucus production. Strikingly, defective IL-17RA-signalling combined with activated *Tnfaip3*-deficient myeloid cells leads to spontaneous accumulation of pulmonary Th17 and γδ-17 T cells, probably due to high local IL-23 production. This model could be exploited to study spontaneous differentiation and accumulation of pulmonary Th17 and γδ-17 T cells.

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 as it induces airway remodelling by promoting fibroblast proliferation, reduces apoptosis of smooth muscle cells, and increases the expression of mucin genes in airway epithelial cells (5-7). Th17 cells primarily produce IL-17 and Th17-mediated 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 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). Recently, we found that increasing the activation status of DCs by ablation of the *Tnfaip3/A20* gene in myeloid cells induced a neutrophilic inflammation in house dust mite (HDM)-mediated asthma protocols **(Chapter 4)**. TNFAIP3 (TNFa-induced protein 3, also known as A20) is a ubiquitin modifying enzyme that deubiquitinates several key intermediate NF-κB signalling molecules, and thereby controls NF-κB-mediated cell activation (15). TNFAIP3 expression in myeloid cells controls development of rheumatoid arthritis (RA), as TNFAIP3 depletion in myeloid cells induces spontaneous development of RA (16).

To gain better insight into the role of IL-17 in HDM-driven neutrophilic airway inflammation, we crossed myeloid-specific *Tnfaip3* knockout mice (*Tnfaip3*^{LysM} mice) (16) to *Il17ra*^{KO} mice, generating *Tnfaip3*^{LysM}*Il17ra*^{KO} mice, in which IL-17A, IL-17E and IL-17F-signalling is disabled (17). We found that the absence of IL-17RA-signalling does not significantly affect eosinophilic or neutrophilic inflammation, whereas it does hamper hyperplasia of mucus-producing goblet cells. Strikingly defective IL-17RA-signalling in combination with increased activation of myeloid cells by TNFAIP3/A20 depletion induces a spontaneous accumulation of Th17 cells and γδ-17 T cells in the airways.

MATERIALS AND METHODS

Mice

Male and female C57BL/6 mice harbouring a conditional *Tnfaip3* allele between LoxP-flanked sites (18) were crossed to transgenic mice expressing the Cre recombinase under the LysM promotor (19), generating *Tnfaip3*$^{fl/fl}$x*Lyz2*$^{+/cre}$ mice[16] (*Tnfaip3*$^{LysM\text{-}KO}$ mice). *Tnfaip3*$^{fl/fl}$*Lyz2*$^{+/+}$ littermates (wild type (WT) mice) were used as controls. *Tnfaip3*^{LysM} mice were crossed with *Il17ra*^{KO} mice (4), creating *Tnfaip3*$^{fl/fl}$x*Lyz2*$^{+/cre}$x*Il17ra*$^{-/-}$ (*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.

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 confocal analysis and 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 (20). 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 by 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. For confocal imaging, 10-µm-thick cryo-sections were fixed in acetone and incubated for 1 h with primary antibodies anti-CD3 (Clone KT3, Bioceros, Utrecht, The Netherlands), anti-TCRγδ (FITC, Clone GL3, BioLegend, San Diego, CA, USA) and anti-Ki67 (FITC, Clone SolA15, Ebioscience). After washing, slides were incubated for 30 min with secondary antibodies anti-Rat Cy-5 (Jackson Immunoresearch, West Grove, PA, USA), anti-Hamster Cy3 (Jackson Immunoresearch) and anti-FITC Alexa Fluor 488 (Jackson Immunoresearch). To prevent unspecific binding of antibodies, an intermediate blocking step with 10% normal rat serum was performed. Lastly, slides were counterstained with DAPI and images were acquired on a Zeiss Meta311 confocal microscope. Images were analysed using ImageJ software[21].

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, 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 normalized to the internal control, and data were determined as copies of mRNA/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-17 T cells with progressing age

To investigate the role of IL-17R-signalling in HDM-driven neutrophilic airway inflammation responses, we crossed *Tnfaip3*^{LysM} mice (16) with *Il17ra*^{KO} mice (4). It has been demonstrated that aged *Tnfaip3*$^{LysM\text{-}KO}$ mice develop arthritis (16) and that *Il17ra*^{KO} mice have altered monocyte homeostasis (22). 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 comparison to WT and *Il17ra*^{KO} control mice (Figure 1A), which did not correlate with splenic cell counts (Figure 1B). Monocytes and neutrophils (gated as shown in Figure S1) were significantly increased in 8-week-old *Tnfaip3*$^{LysM\text{-}KO}$ mice 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 (16). 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 increased 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 (Figure S2).

As IL-17 controls its own expression, and regulates IL-17 expression in both CD4^{+} as γδ T cells (23), we assessed conventional TCRαβ T cells and γδ T cell in the spleen (gating shown in Figure 1E). Total CD4^{+} T helper (Th) cells were not altered between each mouse genotype 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 increased in 8-week old *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to *Tnfaip3*$^{LysM\text{-}KO}$ mice, but were not significantly altered between 18-week old *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} and *Tnfaip3*$^{LysM\text{-}KO}$ mice (Figure 1G). No significant differences were observed in splenic γδ T cell numbers at 8 weeks, while 18-week old *IL17ra*^{KO} mice had increased splenic γδ T cell numbers compared to WT mice. Splenic γδ T cell numbers further enhanced in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to both *Tnfaip3*$^{LysM\text{-}KO}$ and *IL17ra*^{KO} mice (Figure 1H). RORγt^{+} γδ (γδ-17) T cells were increased in spleens of *Il17ra*^{KO} mice and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice at both ages, compared to WT and *Tnfaip3*$^{LysM\text{-}KO}$ mice respectively (Figure 1I). At 18 weeks, splenic γδ-17 T cells further amplified in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to *IL17ra*^{KO} littermates (Figure 1I).

Taken together, these data show that loss of IL-17RA-signalling in combination with TNFAIP3 deficiency in myeloid cells induces accumulation of monocytes and neutrophils upon aging. In contrast, splenic Th17 cells and γδ-17 T cells were already increased in young *Tnfaip3*$^{LysM\text{-}KO}$*Il-17ra*^{KO} mice, and their numbers were amplified at older age.

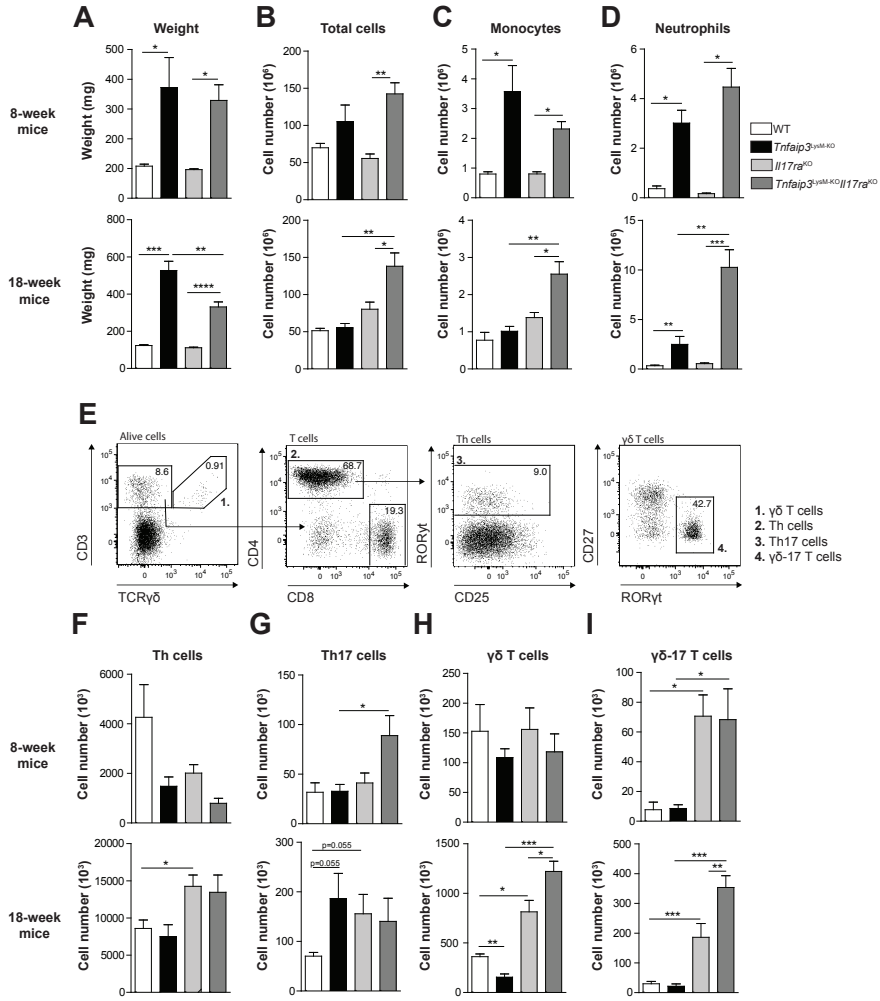

Figure 1: Loss of IL-17RA-signalling in combination with myeloid TNFAIP3 deficiency increases splenic monocytes, neutrophils and γδ-17 T cells with progressing age. *Tnfaip3*^{LysM}*IL17ra* mice were analysed at 8 weeks and 18 weeks of age. (A-B) Quantification of spleen weight (A) and total cell numbers (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)and γδ-17 T cells (I) in spleen cell suspensions by flow cytometry. Results are presented as mean ± SEM of n = 4-10 per group. *P < 0.05, **P < 0.01, ***P < 0.001.

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

We have previously shown that *Tnfaip3*$^{LysM-KO}$ mice develop Th17-associated neutrophilia upon HDM-treatment, whereas WT mice develop Th2-mediated eosinophilic inflammation **(Chapter**

4). As neutrophil numbers were not affected by the loss of IL-17RA-signalling in *Tnfaip3*$^{LysM\text{-}KO}$ mice at young age, we exposed young *Tnfaip3*^{LysM}*Il17ra* mice to an HDM-driven allergic airway inflammation model (Figure 2A), to investigate the role of IL-17RA-signalling on neutrophilic airway inflammation. As previously shown **(Chapter 4)**, HDM-sensitization and challenge induced a predominant eosinophilic inflammation in WT mice, whereas *Tnfaip3*$^{LysM\text{-}KO}$ mice developed a neutrophilic inflammation in the bronchoalveolar lavage (BAL) compared to PBS-sensitized WT littermates (Figure 2B). Absence of IL-17RA-signalling lowered eosinophilic or neutrophilic inflammation, but this did not reach statistical significance (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 strikingly 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 increased 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 compared to HDM-sensitized WT mice (Figure 2C). Remarkably, with additional loss of IL-17RA-signalling, the amount of goblet cells and *Muc5a* mRNA levels were severely reduced in the airways of HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-sensitized *Il17ra*^{KO} mice (Figure 2C-D).

In HDM-sensitized WT mice, total BAL T cells and CD4^{+} T cells increased compared to PBS-sensitized WT mice (Figure 2E). All BAL T cell subsets and γδ T cells were prominently increased in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-sensitized *Il17ra*^{KO} mice (Figure 2E). Total BAL T cells, CD4^{+} T cells and γδ T cells were prominently increased in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice (Figure 2E). Also, 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 (Figure S3).

Briefly, 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. Surprisingly, a distinct increase of γδ T cells was observed specifically in the airways in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice.

Loss of IL-17RA-signalling does not affect 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 (24-26). As eosinophilia and neutrophilia were not signif-

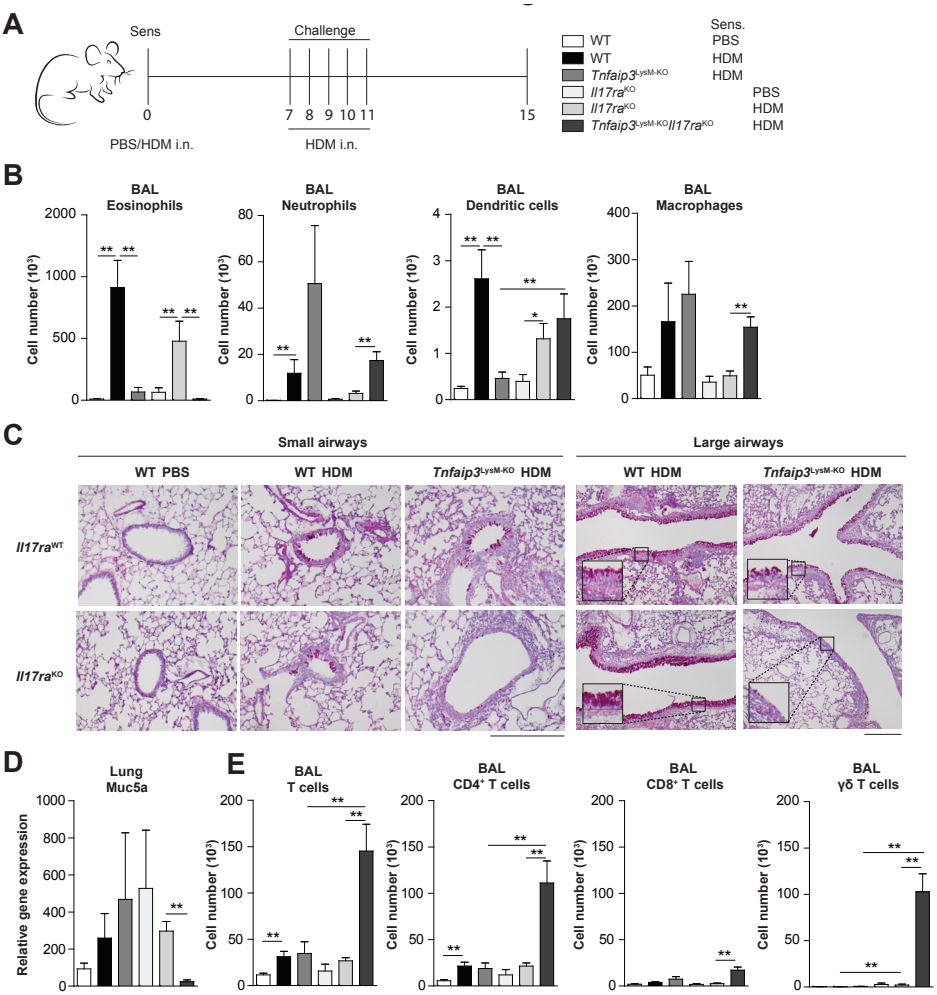

Figure 2: House dust mite induced eosinophilic and neutrophilic airway inflammation are unaltered in the absence of IL-17RA-signalling. (A) Mice were sensitized with PBS or HDM (1 µg) on day 0 and challenged with 10 µg HDM from day 7-11. Analysis was performed at day 15. (B) Quantification of bronchoalveolar 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$^+$ T cells, CD8$^+$ T cells and γδ T cells in BAL by flow cytometry. Results are presented as mean ± SEM of $n = 6$ per group. $*P < 0.05$, $**P < 0.01$.

icantly affected by the loss of IL-17RA-signalling in HDM-sensitized WT and *Tnfaip3*$^{LysM-KO}$ 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).

HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice did not show significant differences in IL-13^{+} or IL-5^{+} Th cells compared to WT HDM-sensitized controls (Figure 3B). Also, 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*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice had reduced IL-13^{+} and IL-5^{+} Th cells compared to HDM-sensitized *Il17ra*^{KO} mice (Figure 3B). As previously shown in **Chapter 4**, BAL IL-17^{+} Th cells increased in HDM-sensitized *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 *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to either HDM-sensitized *Il17ra*^{KO} or *Tnfaip3*$^{LysM\text{-}KO}$ mice (Figure 3B).

Since IL-17 can also be produced by γδ-17 T cells (27), we evaluated the cytokine expression from γδ T cells upon HDM antigen exposure (Figure 3C). HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice had increased IL-17^{+} γδ T cells in BAL in comparison to HDM-sensitized WT mice (Figure 3D). Further loss of IL-17RA-signalling in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice resulted in remarkably elevated IL-17^{+} γδ T cells in BAL (Figure 3D). In the MLN, IL-17-producing Th cells and γδ T cells were increased in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to their controls, although not as prominent as in the BAL (Figure 3E). Th17 and γδ T cells are known to produce other cytokines than IL-17, such as GM-CSF (28) and IL-22 (29). We quantified mRNA expression levels of these cytokines in the lungs. Strikingly, both *Csf2* and *Il22* gene expression were increased in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-sensitized *Il17ra*^{KO} mice and *Tnfaip3*$^{LysM\text{-}KO}$ mice (Figure 3F).

In conclusion, lack of IL-17RA-signalling did not alter Th2 cytokines in HDM-sensitized mice, while significantly increasing IL-17-production by Th and γδ cells in mice lacking myeloid TNFAIP3. Next to IL-17, also GM-CSF and IL-22 levels were increased in lung tissue of *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice.

IL-17RA-deficiency combined with activated myeloid cells directs local proliferation of T cells and γδ T cells in the lungs

As specifically *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice had elevated numbers of pulmonary Th17 and γδ-17 T cells in response to HDM exposure, we wondered whether these cells are induced by HDM or already present in lungs and MLN of naïve mice. Total Th cell numbers were similar between lung and MLN of naïve mice of each genotype (Figure 4A). However, pulmonary Th17 cells were increased in *Il17ra*^{KO} mice compared to WT mice (Figure 4B). Th17 cell numbers were further enhanced in lung and MLN of *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to *Tnfaip3*$^{LysM\text{-}KO}$ and *Il17ra*^{KO} mice (Figure 4B). Both total pulmonary γδ T cells and γδ-17 T cells were increased in *Il17ra*^{KO} mice and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to respectively WT and *Tnfaip3*$^{LysM\text{-}KO}$

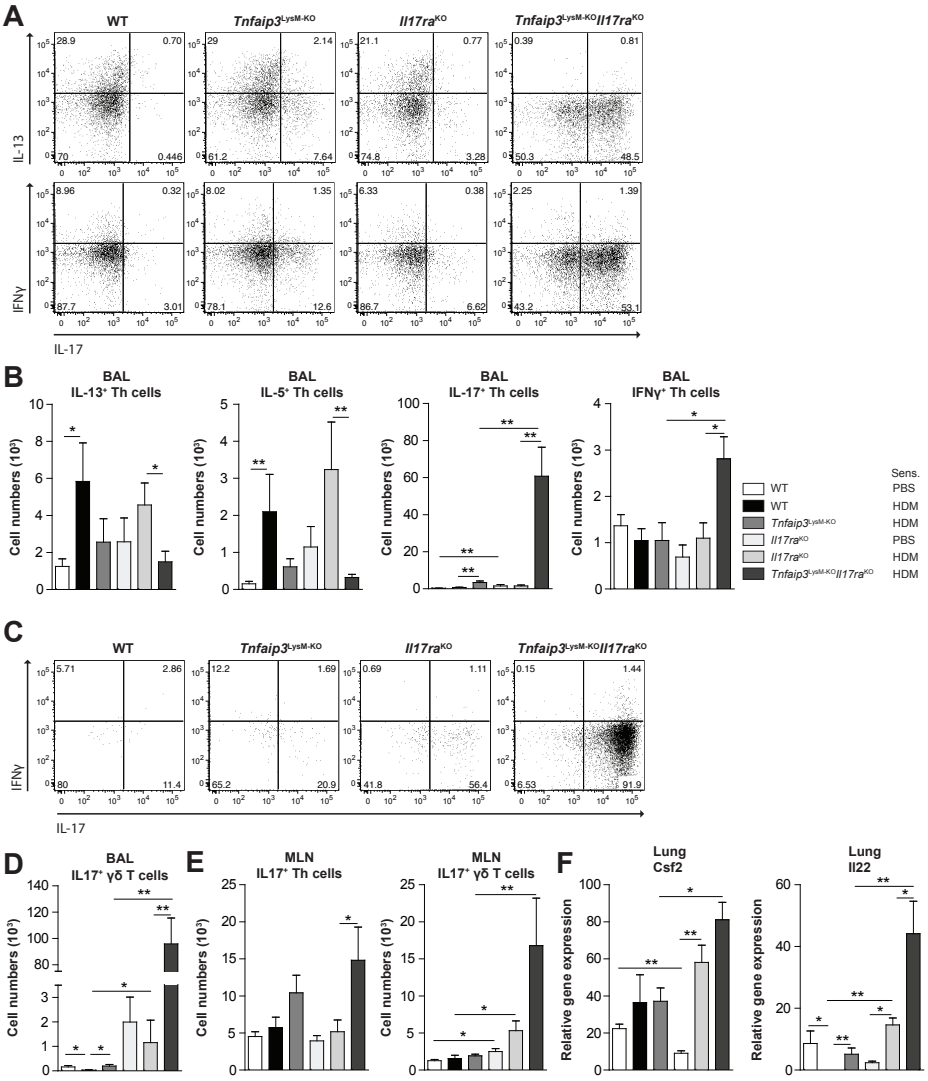

Figure 3: Loss of IL-17RA-signalling does not affect lung Th2 cytokines in a HDM-sensitized model, but increase IL-17 production. *Tnfaip3*^{LysM}*Il17ra* mice were analysed after completion of the HDM exposure protocol. (A) Flow cytometry data is shown of intracellular cytokine expression within Th cells of representative HDM exposed mice. (B) Quantification of BAL Th cell cytokines IL-13, IL-5, IL-17 and IFNγ as determined by flow cytometry. (C) Flow cytometry data is exemplified of BAL γδ T cells intracellular cytokine expression of representative HDM exposed mice. (D) Enumeration of IL-17^{+} γδ T cells in BAL by flow cytometry. (E) Quantification of IL-17^{+} Th cells and γδ T cells in cell suspensions of the MLN by flow cytometry. (F) Plotting Th17 produced cytokine *Csf2* and *Il22* mRNA levels within lung homogenates of PBS and HDM challenged *Tnfaip3*^{LysM}*Il17ra* mice. Results are presented as mean ± SEM of $n = 6$ per group. *$P < 0.05$, **$P < 0.01$.

controls (Figure 4C-D). Similar to Th17 cells, MLN γδ-17 T cells showed a marked increase in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to *Il17ra*^{KO} mice (Figure 4D).

The increase in pulmonary Th17 cells and γδ T cells could be a consequence of increased thymic output, as both T cells and γδ T cells are generated in the thymus (30). Therefore, we evaluated thymic T cell development and observed no differences in thymus cell counts, proposing normal ab T cell development (Figure S4A). However, only thymic γδ-17 T cells were increased in *Tnfaip3*$^{LysM\text{-}WT}$*Il17ra*^{KO} and *Il17ra*^{KO} mice compared to their respective IL-17RA-sufficient littermates (Figure S4B-C).

Next, the increased presence of pulmonary T cells and γδ T cells could also be a consequence of local proliferation. We thus performed confocal microscopy for CD3 and the proliferation marker Ki-67. *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice had cellular infiltrates consisting of CD3^{+}Ki-67^{+} cells present around the bronchi, which was not observed in the lung parenchyma (Figure 4A). In the cellular infiltrates of *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice both Ki-67^{+} T cells (white arrows) and Ki-67^{+} γδ T cells (blue arrows) were present (Figure 4B).

These findings show that Th17 cells and γδ-17 T cells are increased in lungs and MLN of naïve mice that harbour both *Tnfaip3*-deficient myeloid cells and have deficient IL-17RA-signalling, due to local proliferation and elevated thymic output of γδ-17 T cells.

Increase in Th17 and γδ-17 T cell-instructive cytokines in the lungs of *Tnfaip3*LysM-KO*Il17ra*-KO mice

Both Th17 and γδ-17 T cell differentiation depend on the cytokine milieu, whereby IL-1β, IL-6, and IL-23 play a predominant role (31, 32). And especially activated type 2 conventional DCs (cDC2s) and monocyte-derived DCs are a dominant source of these cytokines (31), therefore we characterized the number and activation status of DC subsets (Figure 5A). The number of migratory cDC1s or migratory cDC2s in the MLN did not alter within the four genotypes after HDM provocation (Figure 5B). In contrast, HDM-exposed *Tnfaip3*$^{LysM\text{-}KO}$ mice had significantly higher numbers of moDCs compared to WT controls (Figure 5B). The expression level of the co-stimulatory molecule CD86 on migratory DCs in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice was increased compared to *Il17ra*^{KO} mice (Figure 5C). Especially the migratory cDC2s were responsible for the enhanced CD86 expression in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice, as migratory cDC1s had unaltered CD86 expression. MoDCs of both *Tnfaip3*$^{LysM\text{-}KO}$ mice and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice exhibited increased CD86 expression compared to their respective littermate controls (Figure 5C).

Next, we examined mRNA expression of cytokines implicated in Th17 and γδ-17 T cell differentiation and accumulation within lungs of mice after HDM sensitization. HDM-exposed WT mice exhibited lower *Il1b* expression compared to PBS-exposed WT mice (Figure 5D). In contrast, HDM-treated *Tnfaip3*$^{LysM\text{-}KO}$ mice and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice demonstrated elevated IL-1β and IL-6 expression in the lungs compared to HDM-treated WT and *Il17ra*^{KO} littermate controls (Figure 5D). In contrast, IL-23 expression was markedly increased in HDM-exposed

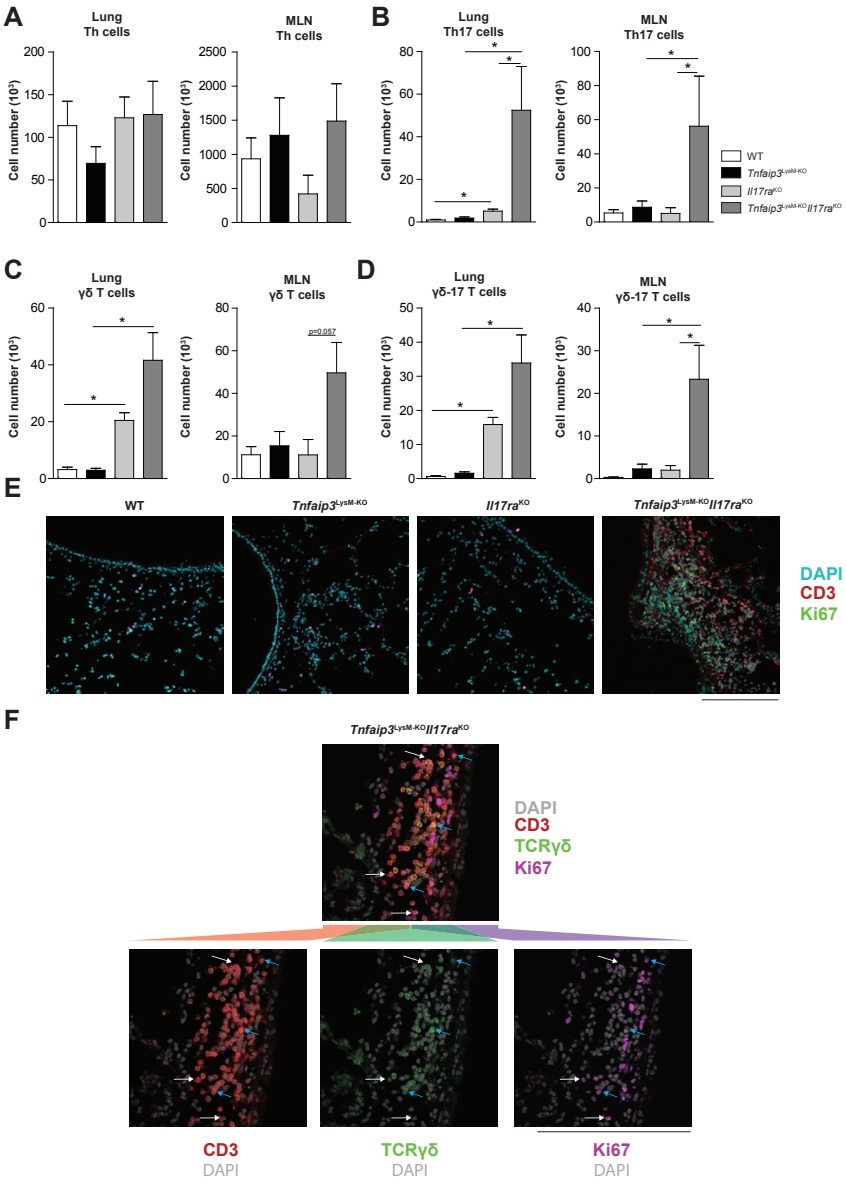

Figure 4: IL-17RA-deficiency in combination with activated myeloid cells directs local proliferation of T cells and γδ T cells in the lungs. (A-D) Cell numbers are depicted of Th cells (A), Th17 cells (B), γδ T cells (C) and γδ-17 T cells (D) in lung and mediastinal lymph node (MLN) by flow cytometry. (E) Immunofluorescent staining of $CD3^+$ T cells (red), Ki-67^+ (green) and DAPI nucleus stain (blue) in lungs of representative naive *Tnfaip3*^{LysM}*IL17ra* mice. The area near the large airways is shown in each image. (F) A lung infiltrate of *Tnfaip3*$^{Lysm\text{-}KO}$*Il17ra*^{KO} is further decomposed to separate fluorchromes $CD3^+$ (red), TCR$\gamma\delta^+$ (green) and KI-67^+ (purple) together with DAPI (gray). White arrows in (F) depict Ki67+ T cells, blue arrows depict Ki67^+ γδ T cells. Scale bars (200μm) are at the lower right corner of the microscopic images. Results are presented as mean ± SEM of $n = 4$ per group. $^*P < 0.05$.

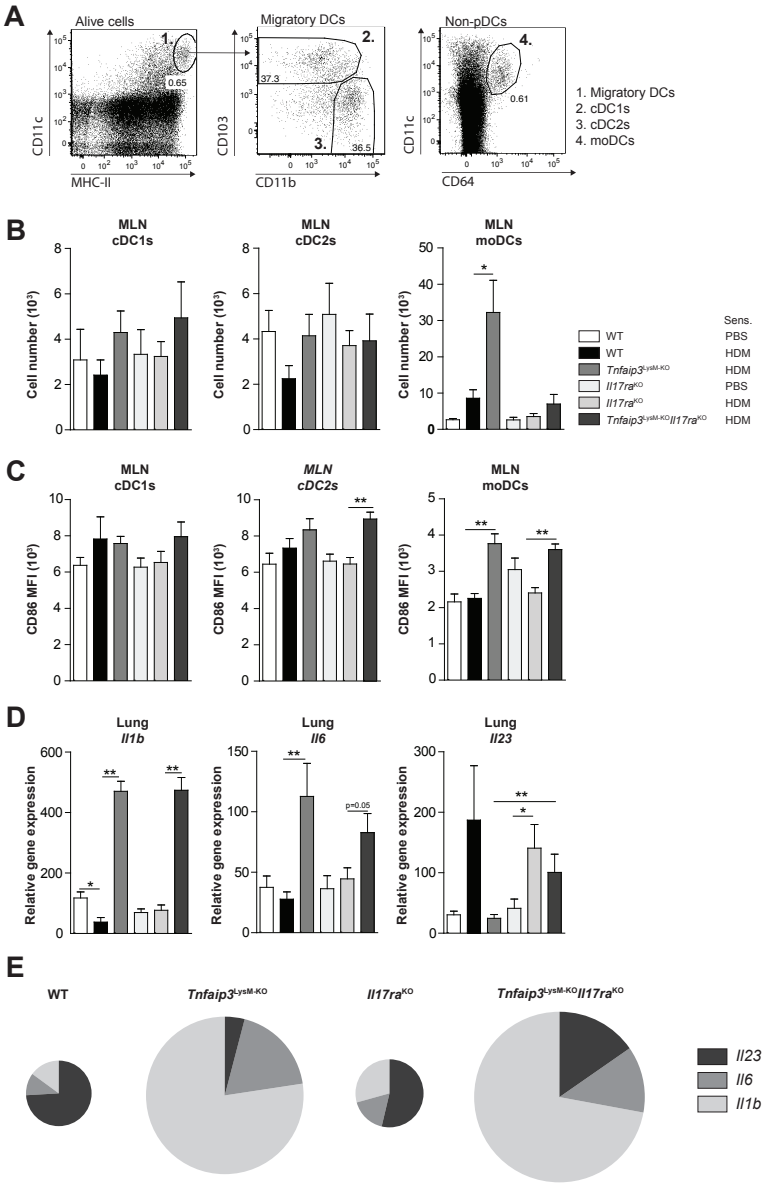

Figure 5: Increase in Th17 and γδ-17 T cell-instructive cytokines in the lungs of *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. Mice were analysed after completion of the HDM protocol at an age of 6-8 weeks (A) Flow cytometric gating of DC subsets. Example is shown of a MLN derived from a WT mouse. (B) Quantification of MLN migratory cDC1s, cDC2s and moDCs using flow cytometry. (C) Enumeration of DC activation in MLN migratory cDC1s, cDC2s and moDCs by flow cytometry. (D) Plotting of relative cytokine mRNA levels within lung homogenates of PBS and HDM challenged *Tnfaip3*^{LysM}*IL17ra* mice. All values were relative to mRNA expression of the household GAPDH gene. (E) Representation of cytokines that drive Th17 and γδ-17 T cell development and survival in pie chart format. Results are presented as mean ± SEM of $n = 4$ per group. *$P < 0.05$, **$P < 0.01$.

Tnfaip3$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to *Tnfaip3*$^{LysM\text{-}KO}$ controls (Figure 5D). In order to improve visualization of the relative contribution to Th17 and γδ-17 T cells differentiating cytokines, IL-1β, IL-23 and IL-6 were plotted in pie chart format (Figure 5E). This suggests that an optimal cytokine mix with high IL-23, IL-1β and IL-6 was only present in the lungs of *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice (Figure 5E).

In conclusion, however cDC numbers and activation states were similar between *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} and *Tnfaip3*$^{LysM\text{-}KO}$ mice, the increase in moDCs in *Tnfaip3*$^{LysM\text{-}KO}$ mice, was not observed in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. HDM-exposure in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice induces an IL-17 promoting environment with high levels of IL-23, IL-1β and IL-6. This increase in IL-23 together with the presence of IL-1β and IL-6 could contribute to the differentiation and accumulation of Th17 cell and γδ-17 T cells in the lungs of *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice.

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 (33). Recently we have shown that the presence of intrinsically activated myeloid cells, obtained through TNFAIP3/A20 ablation, induces development of Th17 cell associated neutrophilic asthma in contrast to Th2 cell driven eosinophilic inflammation induced in control mice **(Chapter 4)**. However, the exact function of IL-17 and the influence of IL-17R-signalling on neutrophilic asthma are incompletely understood. To investigate whether neutrophilic inflammation development as observed in HDM treatment *Tnfaip3*^{LysM} mice is dependent on IL-17-signalling in asthma, mice with specific deletion of TNFAIP3 in myeloid cells were crossed to IL-17RA-deficient mice.

Surprisingly, absence of IL-17RA-signalling only had limited effects on neutrophilic or eosinophilic inflammation in our HDM-driven airway inflammation mouse models. Th2 cell differentiation was not hampered by ablation of IL-17RA-signalling, whereas the presence of mucus-producing cells was reduced. Strikingly, ablation of IL-17RA in mice that harbour intrinsically activated myeloid cells resulted in a spontaneous accumulation of γδ-17 T cells and Th17 cells in the lungs. Furthermore, mice harbouring TNFAIP3-deficient myeloid cells and IL-17RA-deficiency displayed enhanced expression of IL-23 in the lungs.

IL-17RA subunit forms a heterodimer with 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) (17). 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 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 (4,9,34-36). IL-17RA-signalling in airway epithelial cells induces IL-8 production, a chemoattractant for neutrophils (4). However, certain allergens, including HDM can provoke IL-8 secretion by the airway epithelium through toll-like receptor (TLR) stimulation (37-39). Furthermore, neutrophils are also attracted by the chemokine CXCL13, which is highly expressed by *Tnfaip3*-deficient DCs **(Chapter 4)**. This implicates that neutrophilic inflammation observed in HDM-treated *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice is either induced by direct allergen-mediated epithelial activation, by CXCL13 from *Tnfaip3*-deficient DCs, or both mechanisms could be involved.

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 incriminates 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 (35). 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 (24, 25). 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, blockage of IL-17 in OVA-mediated models attenuates eosinophilic inflammation (24,25). Although IL-17 can be produced by both CD4$^+$ T cells and γδ T cells, more recent studies have showed that the regulatory effects of IL-17 on airway inflammation and remodelling in an OVA-mediated airway inflammation model are mediated by IL-17-producing γδ T cells and not by conventional CD4$^+$ T cells (40). Depletion of IL-17-producing γδ T cells during the resolution phase of OVA-mediated airway inflammation model exacerbates eosinophilia, Th2 cytokines and AHR. This implicates that particularly γδ-17 T cells are essential for the resolution of OVA-mediated airway inflammation and AHR (26). Whether γδ-17 T cells are also involved in the resolution phase of HDM-mediated airway inflammation remains to be determined.

In contrast to airway type-2 inflammation, goblet cell hyperplasia was almost completely absent in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. This suggests that the presence of Th2 cytokines in WT mice, and 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 (41-44), and Th17 cytokines IL-17A (7) and IL-17F (45). Furthermore, IL-25 (e.g. IL-17E) is also implicated in goblet cell hyperplasia (46,47). Also, the combination of OVA-specific Th2 and Th17 cells induced more mucus-producing goblet cells than OVA-specific Th2 cells alone (48). This indicates that these IL-17 family cytokines and Th2 cytokines can induce hyperplasia of mucus-producing cells separately and can evens 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.

Accumulation of Th17 cells and γδ-17 T cells was observed in the mucosa of the lungs of *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. In our experiment, ablation of IL-17RA-signalling only slightly increases the presence of IL-17-expressing T cells, however combined with *Tnfaip3*-deficient myeloid cells, pulmonary Th17 cells and γδ-17 T cells were massively enhanced, both in naïve and allergen exposed *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. Increased pulmonary IL-23-expression, high levels of IL-1b 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 drives clonal expansion of Th17 cells and γδ-17 T cells (49,50), whereas IL-17 acts as a negative feedback to control its own expression (23). Strikingly, only IL-23, but not IL-1b 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.

IL-17-secreting potential of γδ T cells is already determined in the thymus by TGF-b and Notch signals (27,51), thus they are antigen naïve. This is logical as γδ T cells are innate-like cells, preferentially populate mucosal organs and thereby act as the first line of antigen-independent defence upon infections (52).

In conclusion, our results indicate that cytokines that signal through IL-17RA reduce but not abrogate development of HDM-induced eosinophilic and neutrophilic airway inflammation, whereas they are essential for mucus production. Absence of IL-17RA signalling combined with *Tnfaip3*-deficient myeloid cells induces a massive accumulation of both Th17 and γδ-17 T cells in the lungs of naïve mice, which was more prominent upon HDM exposure. This spontaneous accumulation is likely the result of defective IL-17RA-signalling combined with increased IL-23 expression and with other IL-17-promoting cytokines, such as IL-1b and IL-6. These findings provide new insights into Th17 and γδ-17 T cells biology, and indicate that therapeutic interventions targeting IL-17 or IL-17RA can induce spontaneous accumulation of Th17 or γδ-17 T cells. This provides a new model to study Th17 and γδ-17 T cells pulmonary accumulation and survival.

ACKNOWLEDGEMENTS

These studies were partly supported by NWO-VENI (916.11.067), European Framework program 7 (FP7-MC-CIG grant 304221), Dutch Arthritis Foundation (12-2-410) and the Netherlands Lung Foundation (3.2.12.087, 4.2.13.054JO). We would like to thank Dr. Louis Boon (Bioceros), Anne Huber and the Erasmus MC Animal Facility (EDC) staff for their assistance during the project.

AUTHOR CONTRIBUTION

HV, TD, RWH, 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 red and approved the final manuscripts.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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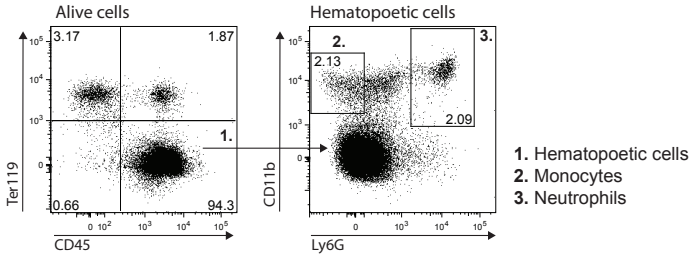
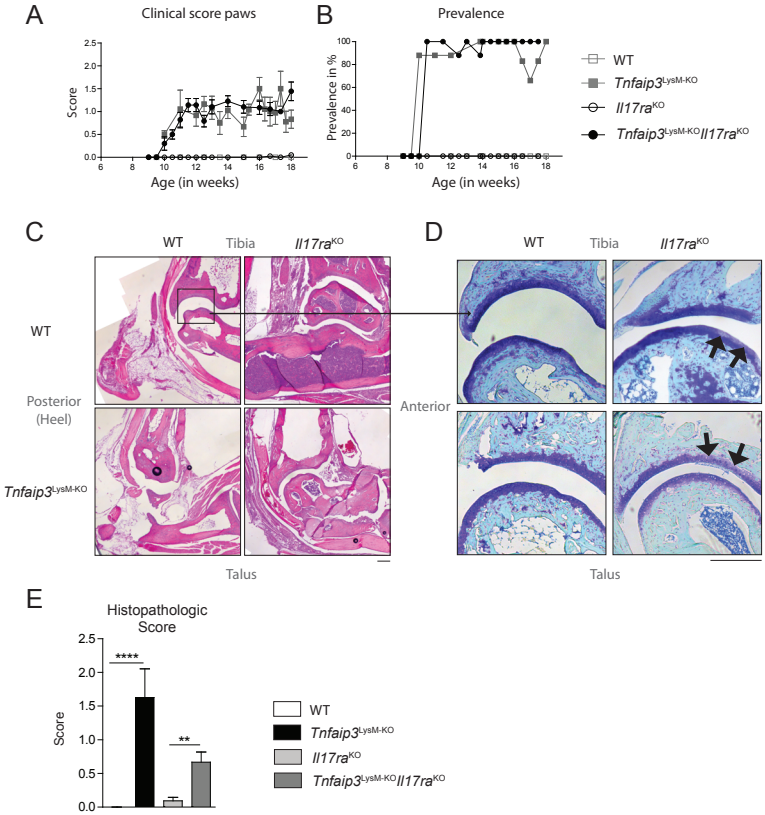
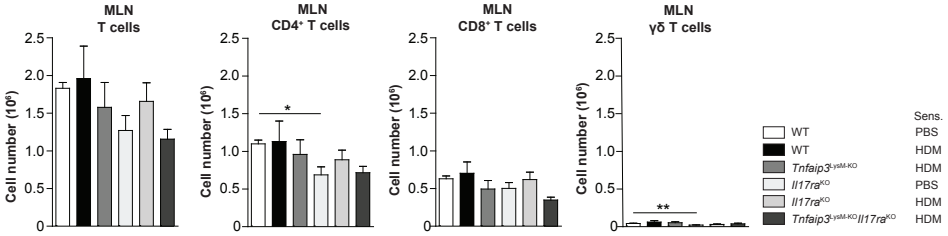


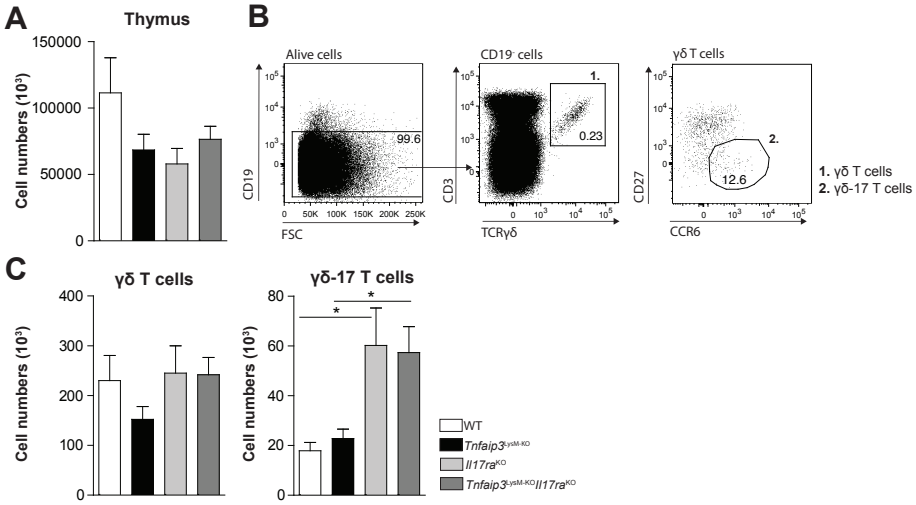
Supplementary figure 1: Flow cytometric gating strategy of myeloid cells. An example is shown of a spleen derived from a WT mouse.



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 = 6-12 per group. $^{**}P < 0.01$, $^{**}P < 0.001$, $^{****}P < 0.0001$.



Supplementary figure 3: HDM exposure does not lead to increased γδ-17 T cells in the MLN of myeloid *Tnfaip3*-deficient and IL-17RA-signalling deficient mice. Quantification of total CD3$^+$ T cells, CD4$^+$ T 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 figure 4: T cell development is normal in the thymus of *Tnfaip3*-deficient mice, regardless of IL-17RA-signalling. Naïve mice were analysed at an age of 8 weeks. (A) Quantification of thymic cell numbers. (B) Flow cytometric gating of developing γδ T cells in the thymus. Example is shown of a thymus derived from a naïve *WT* mouse. (C) Quantification of γδ T cells with programmed γδ -17 T cell subset in thymic cell suspensions using flow cytometry. Results are presented as mean ± SEM of n = 6 per group. *P < 0.05.

Table S1: Antibodies used for flowcytometry

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
FcεRI	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

Tnfaip3-deficient Langerin^{+} cDC1s have increased IL-12 and PD-L1 expression and abrogate Th2-mediated airway inflammation

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6

ABSTRACT

Background: Conventional type 1 dendritic cells (cDC1s) are critical for antiviral and antitumor immunity through the induction of antigen-specific cytotoxic $CD8^+$ T cell responses. Controversy exist concerning cDC1 function in Th2 responses, because both suppressive and supporting roles have been reported. DC activation crucially involves transcription factor NF-kB and is essential for the activation of naïve T cells and their differentiation into T cell subsets. *TNFAIP3*, a key negative regulator of NF-κB signalling has been shown to control DC activation.

Objective: In this study, we investigated whether increased activation of cDC1s, through reduced TNFAIP3 expression, would affect Th2-mediated inflammation.

Methods: Mice with conditional deletion of the *Tnfaip3* gene, using Langerin-Cre transgenic mice (*Tnfaip3*$^{Langerin\text{-}KO}$) were exposed to Th2-driven airway inflammation models, such as house dust mite (HDM) or ovalbumin (OVA)/alum adjuvant.

Results: Ablating *Tnfaip3* gene expression in Langerin-expressing cDC1s abrogated Th2-mediated inflammation in both an HDM and OVA alum-adjuvant model. The frequencies of Th2-suppressive Tregs and IL-10 and $IFN\gamma^+$ $CD8^+$ T cells were increased in *Tnfaip3*$^{Langerin\text{-}KO}$ mice, compared to *Tnfaip3*$^{Langerin\text{-}WT}$ mice in both models. Impaired Th2 immunity was associated with increased expression of IL-12 and PD-L1 in all pulmonary DC subsets, and not only in *Tnfaip3*-deficient cDC1s.

Discussion: We show that increased activation of cDC1s, through reduced TNFAIP3 expression, abolishes development of Th2-mediated inflammation. This implies that increasing the activation status of cDC1s could be a novel pharmacological intervention strategy to treat Th2-mediated diseases.

INTRODUCTION

T helper (Th) 2-mediated disease, such as asthma affects people all over the world. Th2 cytokines facilitate the classical allergic response, such as IgE class switching by B cells (IL-4), eosinophilic inflammation (IL-5), and goblet cell hyperplasia (IL-13) (1). Dendritic cells (DCs) are potent antigen-presenting cells that induce the differentiation of naïve Th cells into effector cells. Based on surface marker expression, two main conventional DC subsets can be identified: cDC1s and cDC2s (2,3). During inflammation, a third population called monocyte-derived DCs (moDCs) arises (4). cDC2s are considered to be efficient at priming $CD4^+$ T cells through MHC class II-restricted antigen (Ag) presentation (5). Upon allergen inhalation, cDC2s drive Th2 differentiation, whereas moDCs maintain Th2 inflammation through the secretion of chemokines (6). On the other hand, cDC1s have superior Ag-cross-presenting capabilities and play pivotal roles in antiviral and antitumor immunity through induction of antigen-specific cytotoxic $CD8^+$ T cell responses (7-9). Conflicting data exist concerning cDC1 function in Th2-mediated diseases, whereby cDC1s were reported to either suppress allergic airway inflammation (10), be redundant (6) or essential for Th2 immune responses (11). During experimental helminth infection, cDC1s control the amplitude of Th2 immune responses through their IL-12 production (12).

DC activation is needed for proper Th cell differentiation into Th1, Th2, Th17 and regulatory T (Treg) cells (13). Activation of DCs in Th2-mediated diseases often occurs through allergen-mediated triggering of pattern recognition receptors (PRRs) on DCs (14,15). PRR triggering on DCs activates the pro-inflammatory transcription factor NF-κB, which initiates transcription of pro-inflammatory cytokines. NF-κB activation is negatively regulated by TNFAIP3 (TNFα-induced protein 3, also known as A20), a ubiquitin-modifying enzyme that deubiquitinates several NF-κB signalling molecules (16,17). Thereby TNFAIP3 controls DC activation (18). In this study, we investigated whether TNFAIP3 depletion in cDC1s would affect their activation status and/or Th2-suppressive capacity. To determine this, we crossed floxed *Tnfaip3* mice (19) with a transgenic mouse line that expresses Cre under the control of the Langerin promotor (20), and performed house dust mite (HDM)-driven and an alum-adjuvant driven allergic airway inflammation experiments. Our data indicate that TNFAIP3 depletion in pulmonary cDC1s inhibits Th2-mediated immune responses. This was associated with an increase in the production of the Th2-suppressive cytokine IL-12 as well as increased the expression of the co-inhibitory molecule PD-L1 on various DC subsets.

MATERIALS AND METHODS

Mice

Tnfaip3$^{fl/fl}$ mice (19) were crossed to Langerin$^{CRE/+}$ mice (20) to generate *Tnfaip3*^{Langerin} mice. *Tnfaip3*^{Langerin} mice were crossed to ROSA26^{fl}EGFP mice. Experiments were performed on mice backcrossed to the C57BL/6 genetic background for at least six generations. Mice were housed and bred under SPF conditions at the Erasmus MC and analysed at 6-12 weeks of age. All experiments were performed with approval by the animal ethics committee of the Erasmus MC.

HDM-induced allergic airway inflammation

During HDM exposures, mice were anesthetized using isoflurane. Mice were sensitized intranasally (i.n.) with 1 µg/40 µl HDM (Greer) or 40 µl PBS as a control on day 1 (GIBCO Life Technologies), and challenged i.n. on days 7-11 with 10 µg/40 µl HDM (6). Mice were sacrificed on day 15. Broncho-alveolar lavage (BAL) was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA (Sigma-Aldrich). Lungs were inflated with PBS/OCT (1:1) solution and placed in 4% PFA and embedded in paraffin.

OVA Alum-adjuvant induced Th2 immune responses

Imject alum (Pierce Biochemicals) was mixed with a solution of 20 µg/ml ovalbumine (OVA) antigen (Worthington) (1:20) in saline (OVA-alum) and was stirred for at least 1 hour as described previously (21). Mice were immunized intraperitoneally (i.p.) with 500 µl OVA-Alum on day 0 and on day 6. Blood was taken on day -1 and 5. On day 13 mice were sacrificed and spleens, MLN and blood were obtained.

Depletion of Tregs

Mice were treated i.p. with 500 ug of monoclonal anti-CD25 (PC61) or with a monoclonal antibody against b-galactosidase (GL113) as isotype control. Mice were injected on day -1, 2, 7, and 10.

HDM-induced acute immune responses

To study co-stimulatory molecule expression and cytokine production by pulmonary DCs, mice were treated with 100 µg/80 µl of HDM extract intra-tracheally (i.t). 24 hours later, single cell suspensions were obtained from lungs by digesting the lungs using DNAse (Sigma) and Liberase TM (Roche) for 30 minutes at 37°C (6). After digestion, the lungs were homogenized through a 100 µm cell strainer (BD Biosciences). Red blood cells were lysed using osmotic lysis buffer (8.3% NH_4Cl, 1% $KHCO_3$ and 0.04% NA_2EDTA in Milli-Q). Cell suspensions were prepared and used for flow cytometry procedures.

Antibody treatment of mice

To study the effect of IL-12 and IFNγ on DC activation, mice were treated i.p. with 500 µg anti-IL-12p40, 500 µg anti-IFNγ antibodies, or with monoclonal antibody b-galactosidase (GL113) as isotype control, 10, 7, 4, and 1 day prior to 100 µg/80 µl HDM administration on day 0. Mice were sacrificed on day 1.

Flow cytometry

Single cell suspensions were prepared from broncho-alveolar lavage (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(22). Monoclonal antibodies used for flow cytometric analyses are listed in Table S1. For all experiments, dead cells were excluded using Fixable viability dye (eBioscience). 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 Biosciences), for 4 hours. 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 (BD Biosciences) and FACS software (BD Biosciences) and analysed by FlowJo version 9 (Tree Star Inc software).

Lung histology

5 um-thick paraffin embedded lung sections were stained with periodic acid and Schiffs reagents to visualize mucus producing cells.

ELISA

Total IgE, HDM-specific IgG1, OVA-specific IgE, IgG1, and IgG2c were measured in serum (Opteia, BD Biosciences).

Statistical analysis

Mann-Whitney U tests were used for comparison between two groups and a p-value of < 0.05 was considered statistically significant. Analysis was determined using Prism (GraphPad Software, USA).

RESULTS

Langerin$^+$ cDC1s comprise only a small proportion of all pulmonary cDC1s

Langerin (CD207) expression has been shown in Langerhans cells in the skin and in a proportion of pulmonary cDC1s (20,23). Corresponding to Zahner *et al,* we also found that Langerin was expressed by 15% of cDC1s in the lung, whereas other pulmonary DC subsets did not express

Langerin (Figure 1A-B). To determine whether Langerin-Cre targeting of cDC1s parallels CD207 expression, we crossed Langerin-Cre mice (20) to Rosa26-stop^{fl}EGFP mice (Langerin-cre x Rosa26-stop^{fl}EGFP mice). DC subsets were examined according the gating strategy as shown in Supplementary Figure 1. In the lungs and lung-draining mediastinal lymph node (MLN) of naive mice at the age of 6-8 weeks, approximately 15% of the cDC1s expressed EGFP, whereas in the broncho-alveolar lavage (BAL), 35% of cDC1s expressed EGFP. Expression of EGFP in other DC subsets in BAL, lung or MLN was below 3% (Figure 1C-D). TNFAIP3 deletion did not affect EGFP expression, because the proportions of EGFP-expressing pulmonary DCs were not different between *Tnfaip3*$^{Langerin\text{-}WT}$ and *Tnfaip3*$^{Langerin\text{-}KO}$ Rosa26-stop^{fl}EGFP mice (Figure 1E).

Since TNFAIP3 deletion affects the activation status of DCs we determined the expression of MHCII, CD86 and PD-L1. Surprisingly, MHCII and CD86 expression were decreased, whereas PD-L1 expression was increased on pulmonary cDC1s of *Tnfaip3*$^{Langerin\text{-}KO}$ mice compared to *Tnfaip3*$^{Langerin\text{-}WT}$ mice (Figure 1F). As the *Langerin* promotor targeted only a fraction of pulmonary cDC1s, we compared the expression of PD-L1 in EGFP$^+$ and EGFP$^-$ cDC1s of *Tnfaip3*^{Langerin} x Rosa26-stop^{fl}EGFP mice. PD-L1 expression was especially increased in EGFP$^+$ cDC1s compared to EGFP$^-$ cDC1s in *Tnfaip3*$^{Langerin\text{-}KO}$ x Rosa26-stop^{fl}EGFP mice and both EGFP$^-$ and EGFP$^+$ cDC1s in *Tnfaip3*$^{Langerin\text{-}WT}$ x Rosa26-stop^{fl}EGFP mice (Figure 1G-H).

In conclusion, these data show that Langerin-expressing cDC1s comprise a small proportion of lung cDC1s. Ablation of TNFAIP3 in Langerin$^+$ cDC1s induced EGFP expression in a similar proportion of cDC1s as Langerin$^+$ cDC1s, and increased the numbers of pulmonary cDC2s, moDCs and pDCs. TNFAIP3 deletion in cDC1s decreased expression of MHCII and CD86, while increasing the expression of the co-inhibitory molecule PD-L1.

Depletion of TNFAIP3 in cDC1s increases pulmonary DCs numbers, whereas T cells are unaltered

As mice with TNFAIP3 depletion in either DCs using *Cd11c*-cre, or myeloid cells using the *LysM*-cre developed auto-immune diseases upon aging (18,24), we also analysed whether *Tnfaip3*^{Langerin} mice developed auto-immunity. *Tnfaip3*^{Langerin} mice were followed until 24 weeks of age without developing any signs of auto-immunity or skin inflammation (data not shown). We next investigated whether Langerin-Cre-mediated TNFAIP3 deletion in a fraction of pulmonary cDC1s affected the numbers of pulmonary DC subsets. Numbers of total DCs, and specifically cDC2s, moDCs and plasmacytoid (pDCs) were increased in lungs of *Tnfaip3*$^{Langerin\text{-}KO}$ mice, compared to WT controls. cDC1 numbers were unaffected in *Tnfaip3*$^{Langerin\text{-}KO}$ mice (Figure 2A). As activation of DCs can affect T cells (25), we characterized T cells in the lung, mediastinal lymph nodes (MLN) and spleen. Both CD4$^+$ as CD8$^+$ T cells numbers were similar between lung, MLN and spleen of *Tnfaip3*$^{Langerin\text{-}KO}$ and *Tnfaip3*$^{Langerin\text{-}WT}$ mice (Figure 2B-C).

This indicates that activation of cDC1s increases pulmonary DC subsets, but does not affect T cell numbers both locally as systemically.

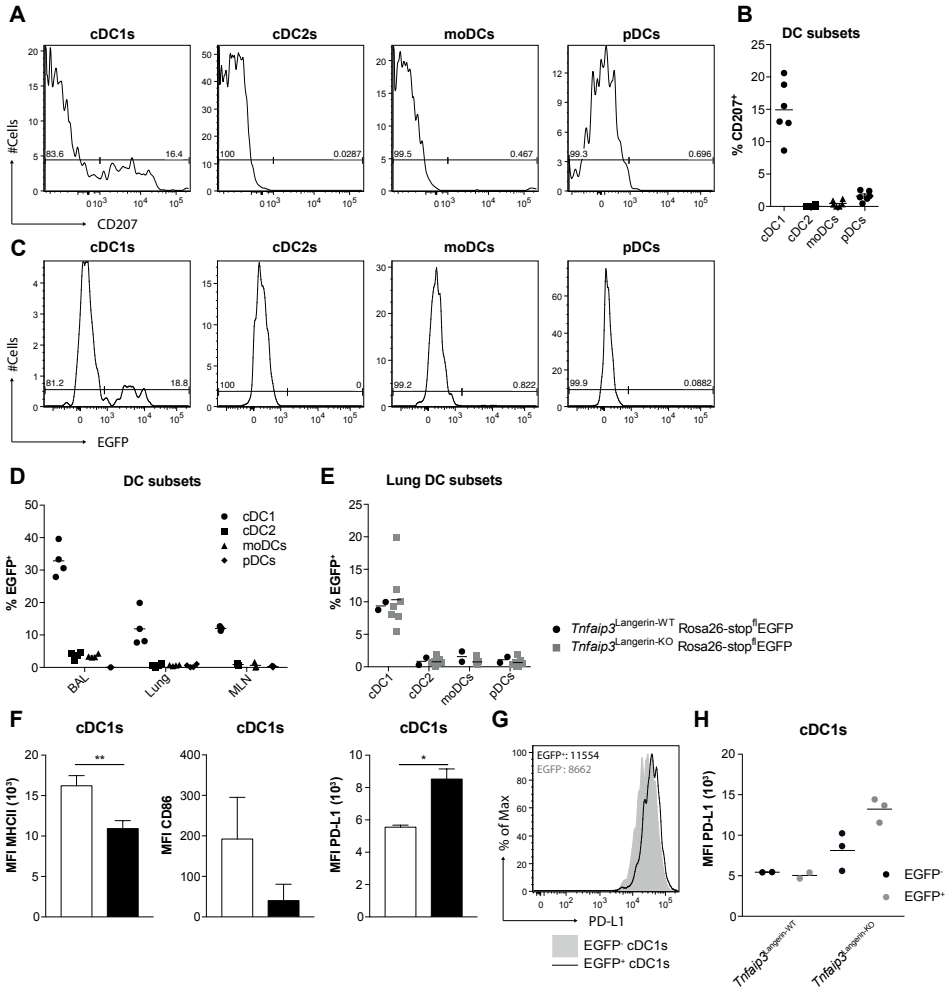

Figure 1: Langerin^{+} cDC1s comprise only a small proportion of all pulmonary cDC1s. (A) Flow cytometric gating of lung DC subsets in C57BL/6 mice. CD207 expression was evaluated in cDC1s, cDC2s, moDCs, and pDCs. (B) Quantification of the percentage CD207 expression in different DC subsets by flow cytometry. (C) Flow cytometric gating of EGFP expression in lung DC subsets of Langerin-cre x Rosa26-stop^{fl}EGFP mice. (D) Quantification of the percentage EGFP expression in different DC subsets by flow cytometry in BAL, Lung and MLN. (E) EGFP expression in cDC1s in BAL, Lung and MLN. (F) Quantification of Mean Fluorescent Intensity of MHCII, CD86, and PD-L1 in lung cDC1s by flow cytometry. (G) PD-L1 expression in EGFP^{+} and EGFP^{-} pulmonary cDC1s by flow cytometry. (H) Quantification of PD-L1 in EGFP- and EGFP+ cDC1s in the lung of *Tnfaip3*$^{Langerin\text{-}WT}$ x Rosa26-stop^{fl}EGFP and *Tnfaip3*$^{Langerin\text{-}KO}$ x Rosa26-stop^{fl}EGFP mice. Results are presented as mean ± SEM of n = 2-7 per group and representative of two independent experiments.

Tnfaip3$^{\text{Langerin-KO}}$ mice show reduced Th2-mediated inflammation, while Tregs, IL-10^{+} and IFNγ^{+} CD8^{+} T cell numbers are increased upon HDM treatment

To investigate the effect of cDC1-specific TNAIP3-deficientcy on Th2 differentiation, we exposed *Tnfaip3*$^{\text{Langerin-KO}}$ mice to inhaled HDM to induce allergic airway inflammation (Figure 3A). HDM-sensitized and challenged *Tnfaip3*$^{\text{Langerin-WT}}$ mice developed BAL eosinophilic inflammation, which was severely reduced in *Tnfaip3*$^{\text{Langerin-KO}}$ mice (Figure 3B). The numbers of B and T cells were also increased in *Tnfaip3*$^{\text{Langerin-WT}}$ mice upon HDM-sensitization, whereas a minor B and T cell infiltration was found in the BAL of HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice (Figure 3B). Th2 cytokine secreting CD4^{+} T cells were increased in HDM-sensitized *Tnfaip3*$^{\text{Langerin-WT}}$ mice compared to PBS-sensitized controls. In contrast, the total numbers of IL-5^{+} and IL-13^{+} CD4 T cells in HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ were not increased compared with PBS-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice. In this model, no induction of IL-17^{+} or IFNγ^{+} CD4^{+} T cells was observed in HDM-sensitized *Tnfaip3*$^{\text{Langerin-WT}}$ or *Tnfaip3*$^{\text{Langerin-KO}}$ mice (Figure 3C). IgE and HDM-specific IgG1 increased upon HDM sensitization in *Tnfaip3*$^{\text{Langerin-WT}}$ mice compared to PBS sensitized control mice. No significant differences were found in the concentration of total IgE and HDM-specific IgG1 in serum of HDM-sensitized *Tnfaip3*$^{\text{Langerin-WT}}$ and *Tnfaip3*$^{\text{Langerin-KO}}$ mice (Figure 3D), however, HDM-specific IgG1 in serum of HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice was slightly elevated compared to HDM-sensitized *Tnfaip3*$^{\text{Langerin-WT}}$ mice. Complementary, HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice showed no lung inflammation and mucus production, which were observed in *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 3F). As Th2-mediated airway inflammation is hampered in HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice, we wondered whether Th2-suppressive cells would be increased in HDM-treated *Tnfaip3*$^{\text{Langerin-KO}}$ mice. MLNs of both PBS and HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice had an increased percentage of Foxp3^{+}CD25^{+} Tregs compared to both PBS and HDM-sensitized *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 3G). HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice had similar number of CD8^{+} T cells in the BAL as compared to HDM-sensitized *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 3H). Strikingly, both intracellular IL-10^{+} and IFNγ^{+} CD8^{+} T cells in the BAL of HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice were increased compared to HDM-sensitized *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 3I). Strikingly the increase in Tregs in *Tnfaip3*$^{\text{Langerin-KO}}$ mice was not responsible for the reduced Th2-driven immune responses, as *Tnfaip3*$^{\text{Langerin-KO}}$ mice depleted of Tregs by an anti-CD25-depleting antibody (PC61), still did not develop Th2-driven inflammation (Figure S2A-C).

This implicates that TNFAIP3 deletion in cDC1s and Langerhans cells hampers development of Th2 cytokine production and IgE secretion upon HDM exposure, whereas antigen-specific IgG1 is not affected. Furthermore, TNFAIP3 deletion in cDC1s induces higher numbers of anti-inflammatory Tregs and IL-10^{+} and IFNγ^{+} CD8^{+} T cells after inhaled HDM. Intriguingly, depleting Tregs in *Tnfaip3*$^{\text{Langerin-KO}}$ mice does not restore Th2-mediated inflammation, indicating that Tregs are not essential for the suppression of Th2-mediated inflammation.

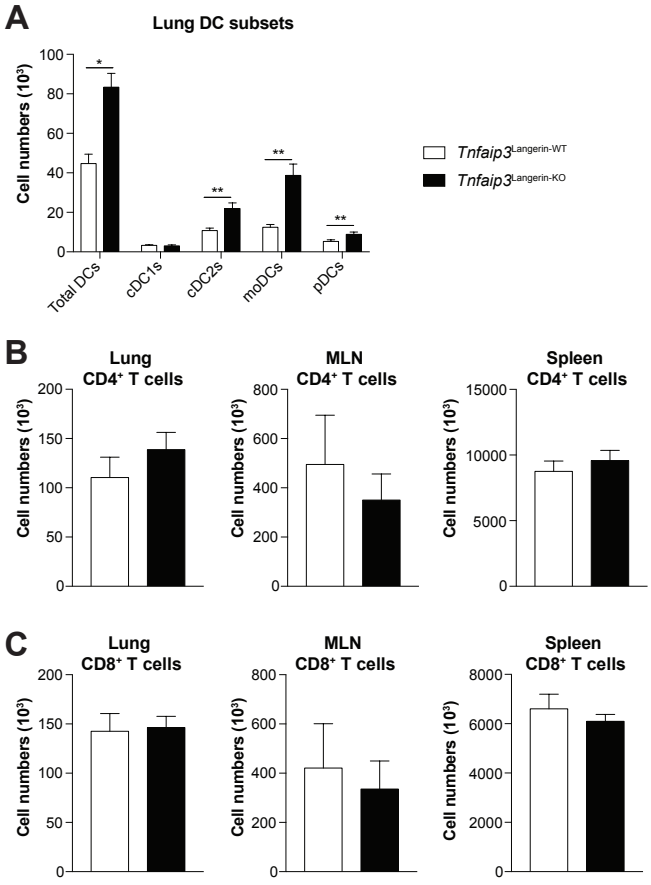

Figure 2: Depletion of TNFAIP3 in cDC1s increases pulmonary DCs numbers, whereas T cells are unaltered. (A) Quantification of pulmonary DC subsets by flow cytometry. (B) Enumeration of $CD4^+$ T cells by flow cytometry in lung, MLN and spleen by flow cytometry. (C) Quantification of $CD8^+$ T cells by flow cytometry in lung, MLN and spleen by flow cytometry. Results are presented as mean ± SEM of n = 3-4 per group and representative of two independent experiments.

Systemically induced Th2 inflammation, using OVA-alum is hampered in *Tnfaip3*$^{\text{Langerin-KO}}$ mice

As TNFAIP3 is only depleted in pulmonary cDC1s and Langerhans cells in the skin of *Tnfaip3*$^{\text{Langerin-KO}}$ mice, we determined whether *Tnfaip3*$^{\text{Langerin-KO}}$ mice would also mount decreased Th2 immune responses that are systemically induced. To induce Th2 immune responses systemically, mice were treated intra-peritoneally with the Th2-inducing adjuvant alum to which ovalbumin (OVA) (OVA-alum) was coupled, on days 0 and 6 (Figure 4A). OVA-specific IgE, IgG1 and IgG2c were induced upon OVA-Alum treatment in *Tnfaip3*$^{\text{Langerin-WT}}$ and *Tnfaip3*$^{\text{Langerin-KO}}$ mice, indicative of successful sensitization (Figure 4B). OVA-Alum treated *Tnfaip3*$^{\text{Langerin-KO}}$ mice

had decreased OVA-specific IgE levels as compared to OVA-Alum treated *Tnfaip3*$^{\text{Langerin-WT}}$ mice, however OVA-specific IgG1 was unaltered (Figure 4B). *Tnfaip3*$^{\text{Langerin-KO}}$ mice had similar numbers of CD4$^+$ T cells both in MLN and spleen as *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 4C), but the proportion of IL-5$^+$/IL-13$^+$ CD4$^+$ T cells in both MLN and spleen were reduced in *Tnfaip3*$^{\text{Langerin-KO}}$ mice compared to *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 4D). Intra-peritoneal sensitization with OVA-Alum also induced an increased proportion of Tregs in the MLN of OVA-Alum treated *Tnfaip3*$^{\text{Langerin-KO}}$ mice compared to *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 4E). No differences were observed in the total numbers of CD8$^+$ T cells (Figure 4F), but the frequency of IL-10$^+$/IFNγ$^+$ CD8$^+$ T cells was increased in MLN and spleen of *Tnfaip3*$^{\text{Langerin-KO}}$ mice as compared to *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 4G).

These data demonstrate that TNFAIP3 deletion in pulmonary and skin cDC1s even systemically can inhibit development of Th2 immune responses, and induce anti-inflammatory Tregs and IL-10$^+$ and IFNγ$^+$ CD8$^+$ T upon OVA-alum exposure.

Tregs and CD8$^+$ T cells are already increased in naive *Tnfaip3*$^{\text{Langerin-KO}}$ mice

As the percentage of effector memory Tregs and numbers of IL-10$^+$ CD8$^+$ T cells was already increased in PBS-sensitized and HDM-challenged mice, we wondered whether these cells would be altered in naive *Tnfaip3*$^{\text{Langerin-KO}}$ mice. Indeed, Treg percentages were increased in MLN of *Tnfaip3*$^{\text{Langerin-KO}}$ mice as compared to *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 5A-B). We investigated various activation markers on the Tregs, of which CD69 is an early activation marker, and CD103 is expressed on mucosal Tregs and is associated with a strong suppressive effector function (26). The frequency of CD103 and CD103/CD69-expressing Tregs in the MLN of *Tnfaip3*$^{\text{Langerin-KO}}$ mice was increased compared to *Tnfaip3*$^{\text{Langerin-WT}}$ mice, at the expense of CD69$^+$ Tregs (Figure 5C-D). The numbers of IL-10$^+$, IFNγ$^+$ and IL-10$^+$/IFNγ$^+$ double positive CD8$^+$ T cells in the lungs of naive *Tnfaip3*$^{\text{Langerin-KO}}$ mice were augmented compared to naïve *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 5E-F).

In conclusion, these data indicate that Tregs and IL-10/IFNγ-secreting CD8$^+$ T cells are already increased in naive *Tnfaip3*$^{\text{Langerin-KO}}$ mice.

TNFAIP3 deletion in cDC1s increases PD-L1 and IL-12 expression in all pulmonary DC subsets

As Tregs and IL-10 and IFNγ-secreting CD8$^+$ T cells are increased in *Tnfaip3*$^{\text{Langerin-KO}}$ mice, we examined DC subsets and changes in co-stimulatory marker expression by DCs either at baseline or upon HDM stimulation. The number of different DC subsets in the lung was followed 24 hours after a single intra-tracheal HDM exposure. The total number of DCs, cDC2s, moDCs, and pDCs were increased in naïve *Tnfaip3*$^{\text{Langerin-KO}}$ mice compared to controls, whereas strikingly the number of cDC1s was unaltered. After HDM exposure, total DC numbers and moDCs increased in *Tnfaip3*$^{\text{Langerin-WT}}$ mice, which was not observed in *Tnfaip3*$^{\text{Langerin-KO}}$ mice (Figure 6A). Since activated DCs migrate towards the MLN, we also evaluated DC numbers in the MLN. Total DC and cDC2 numbers in PBS-treated *Tnfaip3*$^{\text{Langerin-KO}}$ mice were not different from PBS-treated

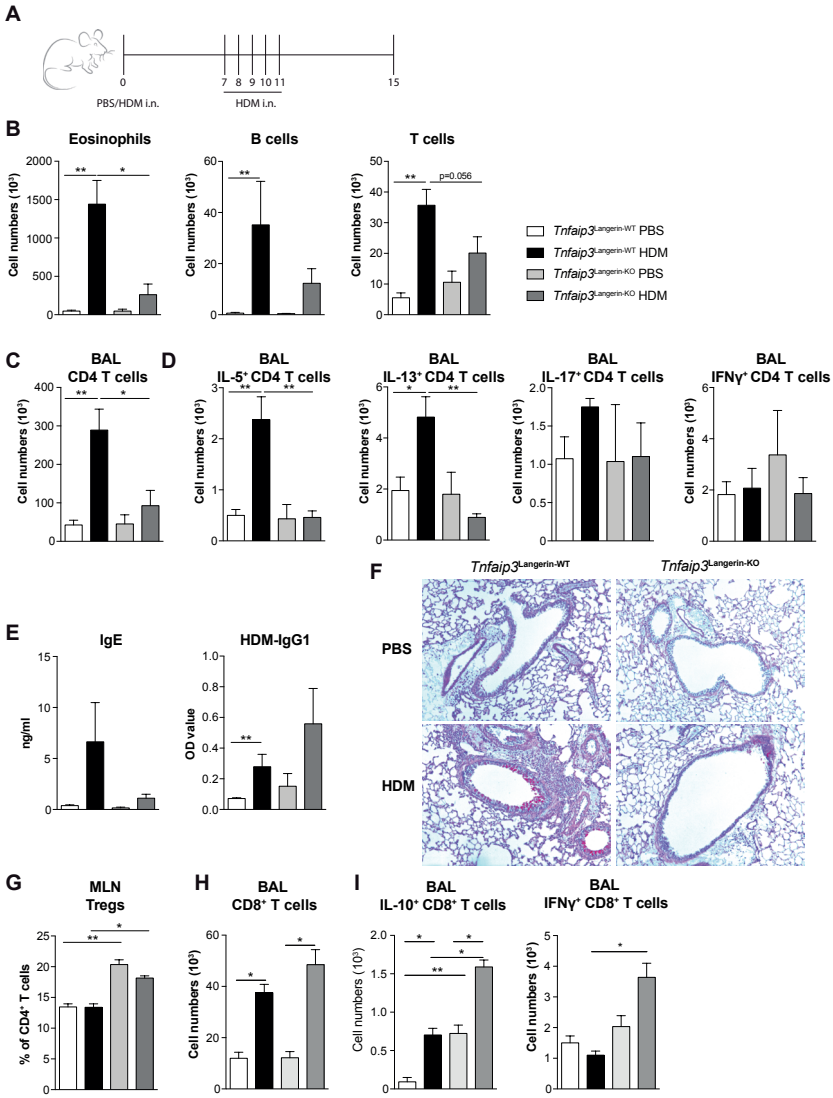

Figure 3: *Tnfaip3*$^{\text{Langerin-KO}}$ mice show reduced Th2-mediated inflammation, while Tregs, IL-10$^+$ and IFNγ$^+$ CD8$^+$ T cell numbers are increased upon HDM treatment. (A) *Tnfaip3*$^{\text{Langerin}}$ mice were sensitized i.n. with PBS or 1 µg HDM on day 0 and challenged i.n. with 10 µg HDM daily between days 7 and 11 to induce allergic asthma. Analysis was performed at day 15. (B) Numbers of eosinophils, B cells and T cells determined in BAL by flow cytometric analysis. (C) Number of CD4$^+$ T cells in BAL by flow cytometric analysis. (D) Number of IL-5$^+$, IL-13$^+$, IL-17$^+$, and IFNg$^+$ CD4 T cells in the BAL by flow cytometric analysis. (E) Serum total IgE and HDM-specific IgG1 levels. (F) Histological analysis of mucus producing goblet cells in the airways by periodic acid staining. (G) Percentages Tregs of CD4$^+$ T cells by flow cytometric analysis in MLN of *Tnfaip3*$^{\text{Langerin}}$ mice that underwent the allergic asthma protocol. (H) Number of CD8$^+$ T cells in BAL by flow cytometric analysis. (I) Quantification of IL-10$^+$ and IFNγ$^+$ CD8$^+$ T cells in the BAL by flow cytometric analysis. Results are presented as mean ± SEM of n = 2-6 mice per group and representative of two or more independent experiments. * = $p < 0.05$, ** = $p < 0.01$.

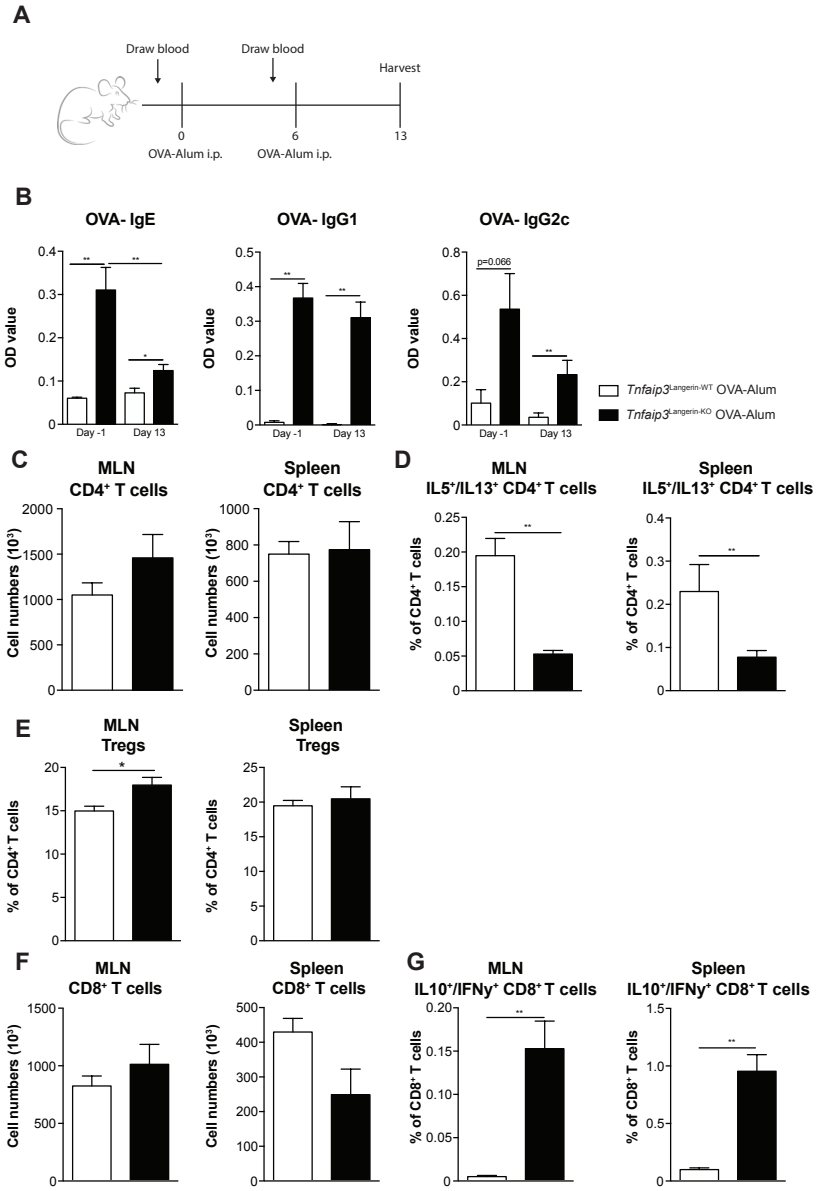

Figure 4: Systemically induced Th2 inflammation, using OVA-alum is hampered in *Tnfaip3*$^{\text{Langerin-KO}}$ mice. (A) *Tnfaip3*$^{\text{Langerin}}$ mice were treated i.p. with OVA-Alum on day 0 and 6. Blood was drawn at day -1 and 5, and analysis was performed at day 13. (B) Quantification of OVA-specific IgE, IgG1 and IgG2a determined by ELISA. (C) Quantification of CD4$^+$ T cells in the MLN by flow cytometry. (D) Flow cytometric gating and quantification of IL-5$^+$/IL-13$^+$ CD4$^+$ T cells in the MLN and spleen by flow cytometry. (E) Percentage of Tregs in MLN and spleen by flow cytometry. (F) Quantification of CD8$^+$ T cells in the MLN and spleen by flow cytometry. (G) Quantification of IL-10$^+$/IFNγ$^+$ CD8$^+$ T cells in the MLN and spleen by flow cytometry. Results are presented as mean ± SEM of n = 6 mice per group. * = $p < 0.05$, ** = $p < 0.01$.

Tnfaip3$^{\text{Langerin-WT}}$ mice, whereas cDC1s were increased in PBS-treated *Tnfaip3*$^{\text{Langerin-KO}}$ mice compared to PBS-treated *Tnfaip3*$^{\text{Langerin-WT}}$ mice. HDM exposure increased the total numbers of DCs, cDC1s, and cDC2s in both *Tnfaip3*$^{\text{Langerin-KO}}$ and *Tnfaip3*$^{\text{Langerin-WT}}$ mice. No differences were found in DC subset numbers between HDM-treated *Tnfaip3*$^{\text{Langerin-KO}}$ and *Tnfaip3*$^{\text{Langerin-WT}}$ mice (Figure 6B).

As co-inhibitory marker expression by DCs is implicated in Treg and IL-10^{+} induction (27), we examined PD-L1, PD-L2, ICOSL, and TGF-b on DC subsets after HDM exposure. No differences were observed for PD-L2, ICOSL, and TGF-b expression, as well as the immune suppressive cytokine IL-10 (data not shown). In contrast, we found an increased expression of PD-L1 not only in pulmonary cDC1s, but also in cDC2s and moDCs of naïve *Tnfaip3*$^{\text{Langerin-KO}}$ mice, which was enhanced after HDM exposure (Figure 6C). Everts *et al*, showed that IL-12 secretion by cDC1s is

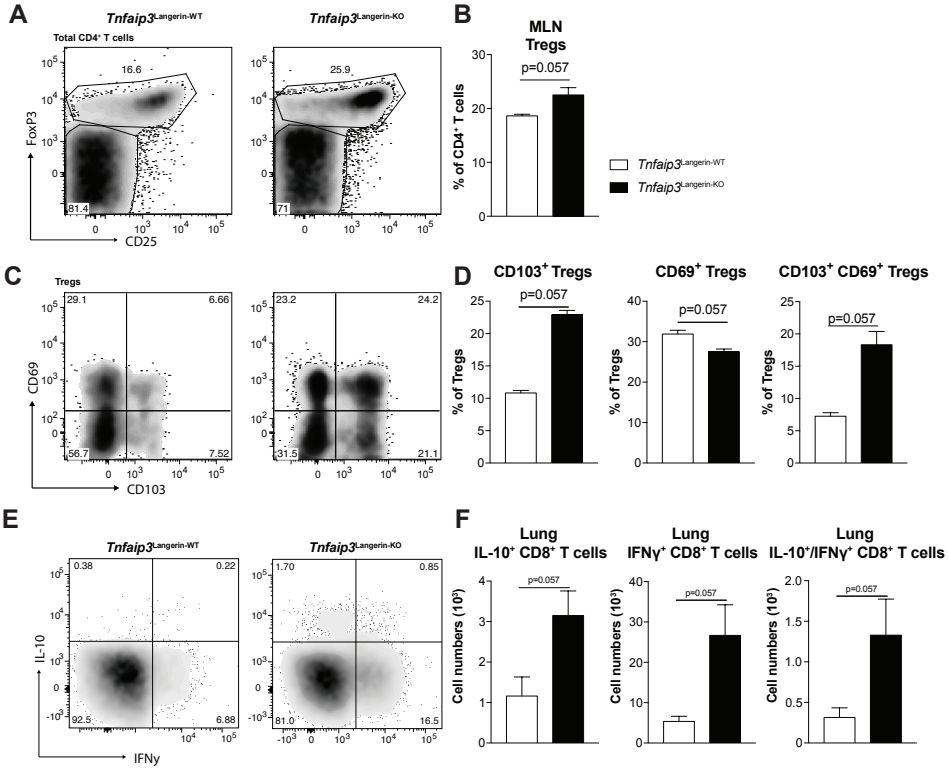

Figure 5: Tregs and CD8^{+} T cells are already increased in untreated *Tnfaip3*$^{\text{Langerin-KO}}$. (A) Flow cytometric gating of Tregs in the MLN. (B) Quantification of Tregs in the MLN by flow cytometry. (C) Flow cytometric gating of CD69 and CD103-expressing Tregs in the MLN. (D) Enumeration of CD69 and CD103-expressing Tregs in the MLN by flow cytometry. (E) Flow cytometric gating of IL-10^{+} and IFNγ^{+} CD8^{+} T cells in the lungs. (F) Quantification of IL-10, IFNγ, and IL-10/IFNγ-secreting CD8^{+} T cells in the lungs by flow cytometry. Results are presented as mean ± SEM of n = 4 mice per group.

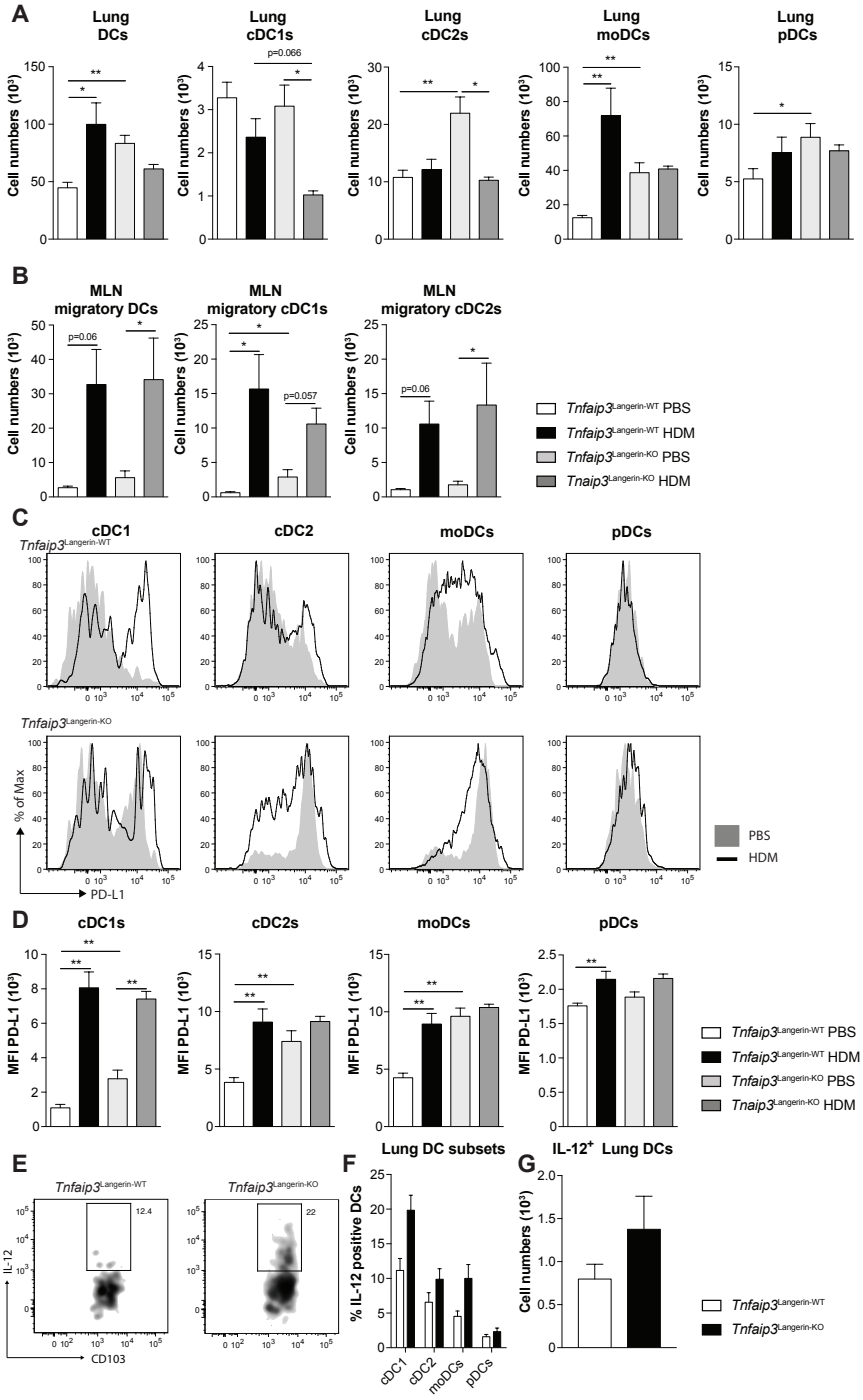
A
Lung DCs
Lung cDC1s
Lung cDC2s
Lung moDCs
Lung pDCs
Cell numbers (10³)
p=0.066
B
MLN migratory DCs
MLN migratory cDC1s
MLN migratory cDC2s
p=0.06
p=0.057
Tnfaip3Langerin-WT PBS
Tnfaip3Langerin-WT HDM
Tnfaip3Langerin-KO PBS
Tnaip3Langerin-KO HDM
C
cDC1
cDC2
moDCs
pDCs
Tnfaip3Langerin-WT
Tnfaip3Langerin-KO
% of Max
PD-L1
PBS
HDM
D
cDC1s
cDC2s
moDCs
pDCs
MFI PD-L1 (10³)
E
IL-12
CD103
12.4
22
F
Lung DC subsets
% IL-12 positive DCs
cDC1
cDC2
moDCs
pDCs
G
IL-12+ Lung DCs
Cell numbers (10³)

Figure 6: TNFAIP3 deletion in cDC1s increases PD-L1 and IL-12 expression in all pulmonary DC subsets (A) Quantification of total DCs, cDC1s, cDC2s and moDCs in lung of PBS or HDM-treated *Tnfaip3*^{Langerin} mice by flow cytometry. (B) Quantification of total migratory DCs, migratory cDC1s and migratory cDC2s in the MLN by flow cytometry. (C) Expression level of PD-L1 in different DC subsets of *Tnfaip3*^{Langerin} mice by flow cytometry. (D) Quantification of MFI of PD-L1 in cDC1s, cDC2s, moDCs, and pDCs by flow cytometry. (E) Flow cytometric gating of pulmonary IL-12-secreting cDC1s. (F) Enumeration of the percentages IL-12 secreting DCs per subsets by flow cytometry. (G) Quantification of IL-12-expressing DCs in the lung by flow cytometry. Results are presented as mean ± SEM of n = 2-6 mice per group and representative of two or more independent experiments. * = $p < 0.05$, ** = $p < 0.01$.

essential for inhibiting Th2 immune responses (12). Alongside the Th2 suppressive properties of IL-12, its expression is also essential for the production of IFNγ$^+$ by CD8$^+$ T cells (28). Strikingly, we found increased IL-12 secretion by pulmonary cDC1s of naïve *Tnfaip3*$^{Langerin\text{-}KO}$ mice as compared to *Tnfaip3*$^{Langerin\text{-}WT}$ mice. IL-12 production was also increased in cDC2s and moDCs of *Tnfaip3*$^{Langerin\text{-}KO}$ mice but to a lesser extent as cDC1s (Figure 6E-F). Besides the increase in frequency also the total number of IL-12-expressing DCs in the lungs was increased in naïve *Tnfaip3*$^{Langerin\text{-}KO}$ mice compared to *Tnfaip3*$^{Langerin\text{-}WT}$ mice.

This implicates that TNFAIP3 deletion in cDC1s increases the expression of the co-inhibitory molecule PD-L1 and Th2-suppressive cytokine IL-12, not only in *Tnfaip3*-deficient cDC1s, but unexpectedly also in all other lung DC subsets.

IL-12 and IFNγ are essential for PD-L1 expression on DC subsets in *Tnfaip3*$^{Langerin\text{-}KO}$ mice

IL-12 is essential for the induction of IFNγ secretion by CD8$^+$ T cells (28), and IFNγ can induce PD-L1 expression (29). To determine whether IL-12 and/or IFNγ are responsible for the increased PD-L1 expression in all lung DCs in *Tnfaip3*$^{Langerin\text{-}KO}$ mice, we treated mice with anti-IL-12p40 or anti-IFNγ antibodies for 10 days prior to a single HDM exposure (Figure 7A). Strikingly, blocking IL-12 completely prevents PD-L1 upregulation after HDM exposure in all pulmonary DC subsets of *Tnfaip3*$^{Langerin\text{-}KO}$ mice. Anti-IFNγ antibodies also inhibited the upregulation of PD-L1 in DCs of *Tnfaip3*$^{Langerin\text{-}KO}$ mice, although less vigorously than anti-IL-12 treatment (Figure 7B-C). CD86 expression by DC subsets in both *Tnfaip3*$^{Langerin\text{-}WT}$ and *Tnfaip3*$^{Langerin\text{-}KO}$ mice was increased after HDM exposure, but unaltered by blocking either IL-12p40 or IFNγ (Figure 7D).

We therefore conclude that, IL-12p40 or IFNγ blockage can separately reduce the increased expression of PD-L1 on DCs subsets in *Tnfaip3*$^{Langerin\text{-}KO}$ mice, indicating that IL-12 expression by pulmonary cDC1s and IFNγ secretion by CD8$^+$ T cells are essential for the increase in PD-L1 expression after HDM exposure in *Tnfaip3*$^{Langerin\text{-}KO}$ mice.

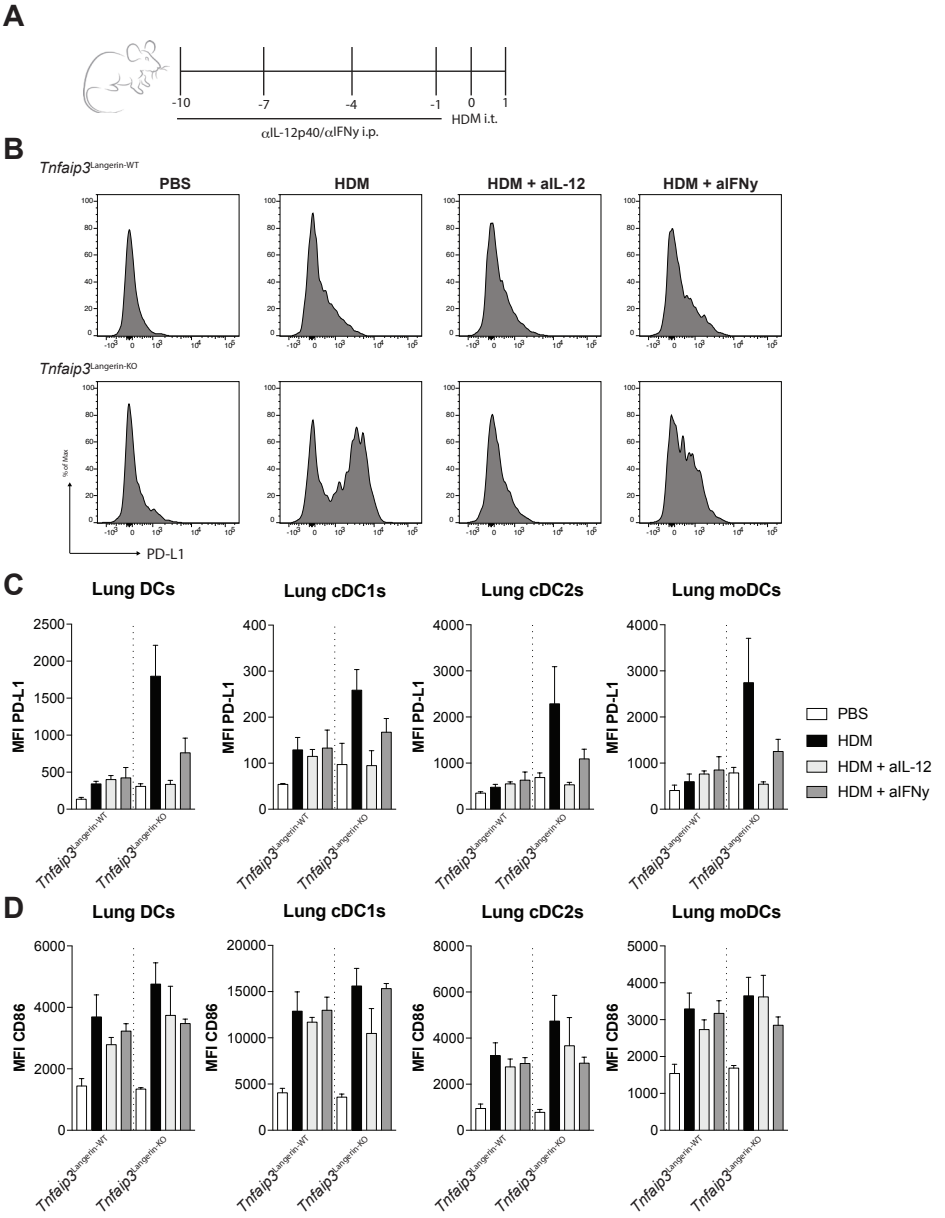

Figure 7: IL-12 and IFNγ are essential for PD-L1 expression on DC subsets in *Tnfaip3*$^{\text{Langerin-KO}}$ mice (A) IL-12p40 and IFNγ were neutralized in *Tnfaip3*$^{\text{Langerin}}$ mice by i.p. injections with anti-IL-12p40 and anti-IFNγ on day -10, -7, -4, and -1. *Tnfaip3*$^{\text{Langerin}}$ mice were treated i.t. with PBS or 100 µg HDM on day 0. Analysis was performed on day 1 (B-C) Expression of PD-L1 in DCs of *Tnfaip3*$^{\text{Langerin}}$ mice. (C) Quantification of MFI of PD-L1 and CD86 per DC subsets by flow cytometry. Results are presented as mean ± SEM of n = 3-4 mice per group.

DISCUSSION

Conventional cDC1s are specialized in $CD8^+$ T cell responses essential for antiviral and anti-tumor responses; however, there are conflicting data regarding the function of cDC1s in Th2-mediated disorders. Recently, it has been shown that cDC1s can suppress Th2-mediated inflammation in response to helminth infections via the induction of IL-12 (12). Therefore, we hypothesized that activation of cDC1s, mediated via TNFAIP3 depletion, would suppress Th2 development in response to inhaled HDM.

Our results show that activation of Langerin-expressing cDC1s is sufficient to suppress Th2-mediated inflammation in response to inhaled HDM, whereas heterozygous deletion of TNFAIP3 in cDC1s did not affect Th2-mediated inflammation. Not only was Th2-mediated inflammation attenuated upon locally induced inflammation in *Tnfaip3*$^{\text{Langerin-KO}}$ mice, but also systemically induced Th2 inflammation by the strong Th2-promoting adjuvant alum was abrogated. Both locally and systemically mediated protocols induced the development of highly activated Tregs and IL-10 and IFNγ-secreting $CD8^+$ T cells in the lungs and lung-draining lymph nodes. Surprisingly, TNFAIP3 deficiency in cDC1s promoted increased Tregs and $CD8^+$ T cell numbers in the steady state. TNFAIP3 deletion in cDC1s increased expression of the Th2-suppressive co-inhibitory molecule PD-L1 and IL-12, not only on cDC1s but also on all other pulmonary DC subsets. Blocking IL-12 and IFNγ both restored PD-L1 expression indicating that IL-12 and IFNγ drive PD-L1 expression in mice with *Tnfaip3*-deficient cDC1s.

Our observation that activated cDC1s can abolish the development of Th2-mediated inflammation agrees with other reports showing that cDC1s suppress Th2-mediated pulmonary inflammation upon helminth infections (12), ovalbumin (10), and HDM exposure (6). Furthermore, *Helicobacter pylori* infection suppresses allergic airway inflammation through cDC1s (30). In contrast, others have shown that cDC1s are essential for the induction of Th2-mediated differentiation in response to HDM (11,31), and that depleting cDC1s would even hamper Th2-mediated eosinophilic inflammation (11). This may be a consequence of the use of BXH-2 mice, which exhibit a myeloproliferative syndrome and aberrant IL-12p40 production (32) that might hamper eosinophilic inflammation. In line, specific depletion of lung cDC1s using Langerin-DTR mice did not affect eosinophilic inflammation (6).

cDC1s are a specialized DC subset involved in Treg induction through TGF-ß and retinoic acid dependent mechanisms both *in vitro* (10,33) and *in vivo* (34). Indeed, activating cDC1s increase Treg proportions, and especially the frequency of mucosal associated CD69/CD103 double expressing Tregs. These Tregs may contribute to dampen the Th2-mediated inflammation, either by inhibiting cDC2-mediated Th2 cell differentiation or affecting the effector phase of the Th2-mediated inflammation. Surprisingly, Treg depletion did not restore eosinophilic inflammation in our model, indicating that Tregs are not essential for the suppression of Th2-mediated inflammation by activated cDC1s. Furthermore, only PBS-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice dis-

played increased effector memory Tregs in the HDM-mediated allergic airway inflammation model, whereas this was not observed in HDM-sensitized *Tnfaip3*$^{\text{Langerin-KO}}$ mice. Taken together, these findings suggest that effector memory Tregs are not maintained during inflammatory responses and are therefore not capable of suppressing the Th2-mediated inflammation.

Whereas both cytokine expression by Th2 cells and IgE levels were not induced upon both HDM and OVA-alum treatment, surprisingly, we failed to detect any decrease in the Th2-associated immunoglobulin IgG1 in both protocols. IL-4 expression is essential for IgG1 class switching (35). However, follicular T helper (Tfh) cells, and especially IL-21 expression by Tfh cells are already sufficient for class switching to IgG1 (36,37). Furthermore, IL-10 expression is also known to induce IgG1 class switching (38,39). Hence, IgG1 class switching in *Tnfaip3*$^{\text{Langerin-KO}}$ mice may be triggered through other than Th2-associated cytokines.

Ablating TNFAIP3 expression in cDC1s increased the expression of PD-L1 and IL-12 in all pulmonary DCs, indicating that DCs in *Tnfaip3*$^{\text{Langerin-KO}}$ mice do not favor Th2 differentiation. However, we did not show that PD-L1 or IL-12 directly hamper the development of Th2 immune responses, there are several lines of evidence that suggest that this is plausible. It is well known that IL-12 inhibits Th2 (40,41) and promotes Th1 cell development (42), respectively. However, we did not observe an increased Th1 cell differentiation, but rather an increase in IFNγ-production by CD8^{+} T cells, which is also dependent on IL-12 (28). The specific increase of IFNγ-producing CD8^{+} T cells and not IFNγ-producing CD4^{+} T cells may be due to the key feature of cDC1s to drive superior CD8^{+} T cell activation (7-9). Moreover, IL-10-secretion by CD8^{+} T cells can be induced by IL-12, IL-4 or a combined IL-12/IL-4-rich environment (43,44). *Tnfaip3*-depletion in cDC1s increased PD-L1 expression not only in cDC1s, but strikingly in all pulmonary DC subsets. This is probably mediated by IFNγ produced by CD8^{+} T cells in *Tnfaip3*$^{\text{Langerin-KO}}$ mice, as IFNγ is known to induce PD-L1 expression (29). This may also account for the increased PD-L1 expression in all pulmonary DCs, since blocking the upstream instructive cytokine IL-12 or directly the effector cytokine IFNγ reduced PD-L1 expression. The main source of IL-12 in *Tnfaip3*$^{\text{Langerin-KO}}$ mice are most likely *Tnfaip3*-deficient cDC1s, as those DCs were superior in their IL-12 production as compared to other DC subsets in *Tnfaip3*$^{\text{Langerin-KO}}$ mice. Therefore, it is conceivable that the suppression of Th2-mediated inflammation in *Tnfaip3*$^{\text{Langerin-KO}}$ mice is caused by IFNγ-induced PD-L1 expression on pulmonary DCs in *Tnfaip3*$^{\text{Langerin-KO}}$ mice. Both PD-L1 expression on DCs as well as IL-10 and IFNγ-secreting CD8^{+} T cells are able to suppress Th2 cell-driven inflammation and maintain tolerance. In concert with the increased IL-12 production that favors Th1 differentiation, this creates a Th2 suppressive environment. The importance of IL-12 in maintaining the balance between Th1 or Th2 immune responses is further supported by the observation that atopic dermatitis exacerbates in psoriasis patients treated with ustekinumab (monoclonal antibody to the p40 subunit of IL-12 and IL-23) (45,46).

In conclusion, our data indicate that activating cDC1s increases their Th2-suppressive capacity and abolishes Th2-mediated inflammation in response to HDM. *Tnfaip3*-depletion in cDC1s

increases IL-12 and PD-L1 expression, not only on cDC1s but also on DC subsets indicating that the existence of cross-regulation between DC subsets. Activated cDC1s increases Treg and $CD8^+$ T cell numbers that secrete Th2-repressing cytokines IL-10 and IFNγ. Thus, implicating that the specific activation of cDC1s may represent a new pharmacological intervention strategy to inhibit Th2-mediated inflammation.

ACKNOWLEDGEMENTS

These studies were partly supported by NWO-VENI (916.11.067), European Framework program 7 (FP7-MC-CIG grant 304221.), and Netherlands Lung Foundation (3.2.12.087, 4.2.13.054JO, and 9.2.15.065FE). We would like to thank Martijn Schuijs (VIB, Ghent, Belgium), Boudewijn van der Wel, Lisette Krassenburg (Erasmus MC), and the Erasmus MC Animal Facility (EDC) staff for their assistance during the project.

AUTHOR CONTRIBUTIONS

HV, RWH, MK designed the experiments. HV, IB, JvH and DvU performed experiments and analysed data. HV, RWH and MK wrote the manuscript. All authors red and approved the final manuscripts.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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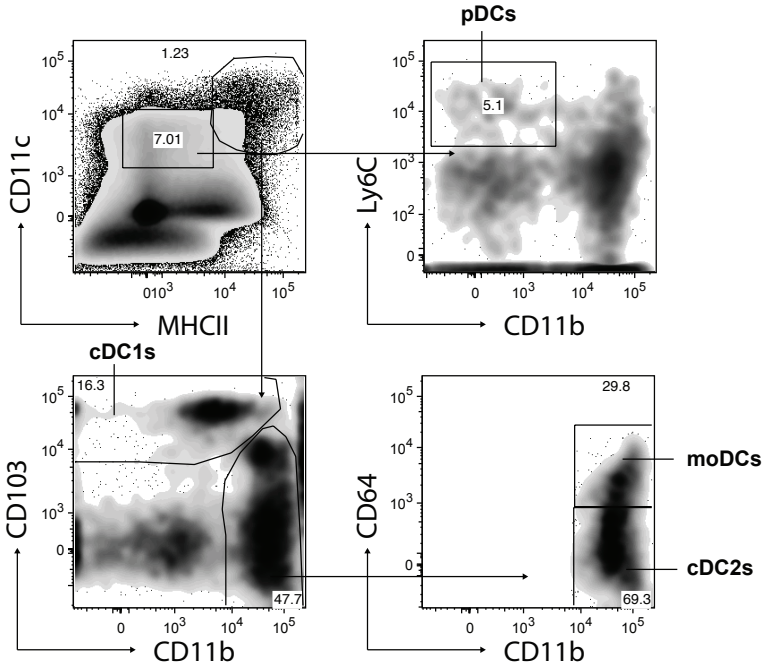
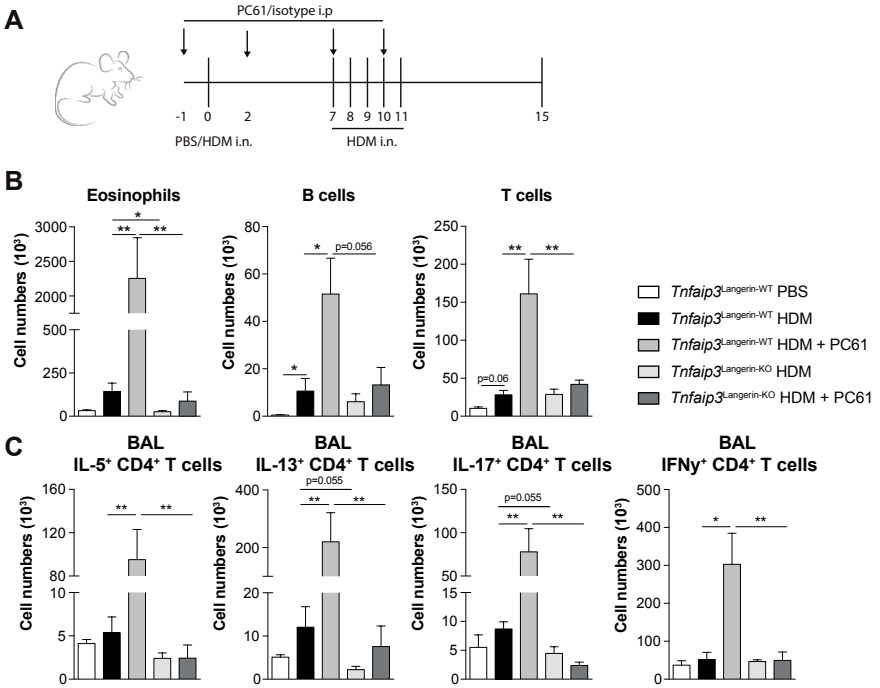


Supplementary figure 1: Gating strategy for pulmonary DC subset. Pulmonary DCs were gated according the gating strategy.



Supplementary figure 2: Tregs depletion does not restore Th2-mediated inflammation in *Tnfaip3*$^{\text{Langerin-KO}}$ mice. (A) Tregs were depleted in *Tnfaip3*$^{\text{Langerin}}$ mice with i.p. PC61 injections on day -1, 2, 7, and 10. *Tnfaip3*$^{\text{Langerin}}$ mice were sensitized i.n. with PBS or 1 µg HDM on day 0 and challenged i.n. with 10 µg HDM daily between days 7 and 11 to induce allergic asthma. Analysis was performed at day 15. (B) Quantification of eosinophils, B cells and T cells in BAL by flow cytometry. (C). Enumeration of IL-5^{+}, IL-13^{+}, IL-17^{+}, and IFNγ^{+} CD4^{+} T cells in the BAL by flow cytometry. Results are presented as mean ± SEM of n = 4-5 mice per group. * = $p < 0.05$, ** = $p < 0.01$.

Table S1: Antibodies used for flowcytometry

Antibody	Conjugate	Clone	Company
CD103	eF450	2E7	eBioscience
CD11b	PerCP-Cy5.5	M1/70	BD Biosciences
CD11c	PE-TxR	N418	eBioscience
CD19	APC-eFluor 780	ID3	eBioscience
CD207	Alexa-fluor 647	929F3.01	Dendritics
CD3	PE-CF594	145-2C11	BD Biosciences
CD3	APC-eF780	17A2	eBioscience
CD4	BV711	RM4-5	BD Biosciences
CD4	BV605	L3T4	BD Biosciences
CD64	BV711	X54-5/7.1	Biolegend
CD69	FITC	H1.2F3	eBioscience
CD8	PE-Cy7	53-6.7	eBioscience
CD86	PE-Cy7	GL1	BD Biosciences
FoxP3	PE-Cy7	FJK-16s	eBioscience
GR-1	Pe-Cy7	RB6-8C5	eBioscience
IFNy	EF450	XMG1.2	eBioscience
IL-10	PerCP-Cy5.5	JES5-16E3	eBioscience
IL-12	PE	C17.8	eBioscience
IL-13	EF660	eBio13A	eBioscience
IL-17A	AF700	TC11-18H10.1	BD Biosciences
IL-5	PE	TRFK-5	BD Biosciences
MHCII	Alexa Fluor 700	M5/114.15.2	eBioscience
PD-L1	biotin	B7-H1	eBioscience
Siglec F	PE	E50-2440	BD Biosciences

Unbiased immune profiling of asthma patients using FlowSOM

Manuscript in preparation

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ABSTRACT

Background: Asthma is a heterogeneous respiratory disease that can arise as a consequence of different underlying immunological mechanisms. In most asthma patients, the disease can be effectively treated with inhaled corticosteroids (ICS). However, a small population of asthma patients is unresponsive to this treatment. As these patients display the highest morbidity and symptom burden, it is worthwhile to investigate the underlying disease mechanism.

Aims: The aim of this study is to characterize the phenotype of immune cells in peripheral blood of asthma patients by automated analyses of flow cytometry measurements and relate these to the disease control status.

Methods: Peripheral blood of 37 asthma patients and 22 healthy controls (HC) was drawn, and surface expression of CD1c, CD3, CD4, CD8, CD11c, CD14, CD16, CD19, CD33, CD86, HLA-DR and Siglec-8 was determined using flow cytometry. Cell populations were determined using the FlowSOM algorithm, and outcomes of the analyses were confirmed by manual gating.

Results: Our results show that the immune cell composition of asthma patients is very different from HC. Asthma patients had higher proportions of blood eosinophils, B cells and monocytes, whereas $CD8^+$ T cells and neutrophil proportions were lower than those in HC. Percentages of eosinophils and neutrophils were not different between controlled asthma patients and uncontrolled asthma patients, but surprisingly, a subset of B cells expressing $CD1c^+$, and HLA-DR-expressing monocytes correlated with the Asthma Control Questionnaire (ACQ)-score.

Conclusion and Clinical Relevance: In conclusion, HLA-DR^+ classical monocytes and $CD1c^+$ B cells correlated with disease control status and could thus play a role in asthma patients that remain uncontrolled while treated with ICS. We conclude that further studies are required to explore the importance of these cell populations in human asthma.

INTRODUCTION

According to the latest Global Initiative for Asthma (GINA) guidelines, asthma is a heterogeneous disease that presents with a broad pallet of respiratory symptoms such as wheezing, shortness of breath, chest tightness and cough, in addition to variable airway obstruction (1). Clustering and defining characteristics of asthma patients have revealed that asthma is not a single disease, but rather a syndrome, that consists of various subtypes that share similar clinical characteristics (2). Asthma patients can be categorized based on clinical characteristics (phenotypes), or based on the underlying disease mechanism (so-called endotypes) (3,4). To date, most asthma patients are grouped into categories that are descriptive and provide information regarding one single dimension of the disease, such as factors that triggers the disease (e.g. exercise) (5), airway obstruction patterns (e.g. reversible and irreversible) (6), immunology (e.g. eosinophilic and non-eosinophilic) (7), or disease control based on corticosteroid responsiveness (e.g. well-controlled and uncontrolled) (8). Especially for uncontrolled asthma patients it would be beneficial to investigate the underlying disease mechanism, as these patients display the highest morbidity and symptom burden (8). Improvement of techniques to characterize immune cells and molecules enabled characterization of different immunological endotypes within asthma. However, current immunological asthma endotypes are often one dimensional and frequently based on granulocytic inflammation (eosinophilic, neutrophilic, mixed granulocytic, or paucigranulocytic) (9) or on T helper (Th) cell profile (Th2 high vs Th2 low) (10). It is generally thought that immunophenotyping of asthma patients can contribute to therapy management and pave the way for personalized medicine.

Flow cytometry is a powerful tool to identify and characterize immune cell populations, and enables high-throughput quantitative analysis of single cells. The possible number of different markers characterizing cells simultaneously using flow cytometry increases rapidly (11). Thus, flow cytometry is an ideal technology to identify immune cells in different asthma phenotypes. The majority of researchers use manual analysis of flow cytometric data. However, this has some disadvantages, as manual analysis is a very complex and time-consuming process, and as a result, not all combinations of markers are analysed on all cell types. During manual analysis, many cells are excluded from used gates, and therefore not analysed, leading to unexamined and unnoticed information (12). Furthermore, as this is a manual process, variations between researchers result in variability in flow cytometry analyses (13). Recently, advanced machine-learning algorithm techniques have paved the way for unbiased analysis of flow cytometry data (14). Techniques that ease visualisation, such as FlowSOM and SPADE can also be used to optimize and control automatic analyses of flow cytometry data (15, 16). Alterations of immunological characteristics in different asthma phenotypes (e.g. disease control based on Asthma Control Questionnaire (ACQ) are currently unknown. Therefore, here we used the machine-learning algorithm FlowSOM to analyse in an unbiased manner 15-colour flow cy-

tometry measurements of peripheral blood cells of healthy controls and asthma patients that differed in disease severity. This approach enabled us to characterize phenotypic differences between the immune profiles of healthy controls and asthma patients that are controlled, partly-controlled or uncontrolled while treated with ICS.

MATERIAL AND METHODS

Subject characteristics

Patients were diagnosed with asthma by at least one of the following parameters during the past 5 years: 1) reversibility to b2-agonists, FEV1 ³12% predicted from baseline and ³ 200 ml after 400 mg inhaled salbutamol or equivalent; 2) bronchial hyper-responsiveness to methacholine or histamine; 3) peak-flow variability of > 20% over a period of 14 days; 4) fall in FEV1 > 12% and > 200 ml when tapering treatment. Diagnosed asthma patients were included in the study at the Franciscus Gasthuis in Rotterdam, The Netherlands if they met the following inclusion criteria: 1) diagnosis of asthma, confirmed; 2) recent (<12 months) metacholine/histamine provocation test; 3) use of ICS and b2-agonist; 4) age between 18 and 50 years old; 5) signed informed consent. Asthma patients were excluded from the study if they met one of the following exclusion criteria: 1) use of systemic corticosteroid > 7.5 mg three months prior to inclusion; 2) treatment with other immunomodulatory medication; 3) concomitant pulmonary diseases (including chronic obstructive pulmonary disorders or interstitial lung diseases); 4) autoimmune diseases; 5) malignancies; 6) human immunodeficiency virus seropositivity; 7) BMI >30; 8) Age >50 years; 9) smoking > 10 pack years; 10) current pregnancy.

Healthy control (HC) subjects were recruited at the Franciscus Gasthuis in Rotterdam and the Erasmus MC in Rotterdam, the Netherlands. Healthy subjects were included in this study after giving their informed consent and were excluded if they met any of the above listed exclusion criteria, or had an FeNO > 50 ppb.

For this study, 37 asthma patients and 22 HC donated peripheral blood. Asthma control was evaluated based on two independent ACQ scores in all asthma patients. All asthma patients required minimally Global Initiative for Asthma (GINA) treatment step 3 or 4 medication (1).

The Medical Ethical Committee of the Erasmus MC Rotterdam approved this study. Further patient and HC characteristics are shown in Additional file 1: Table S1.

Flow cytometry staining

Peripheral blood was lysed to remove erythrocytes using osmotic lysis buffer (8.3% NH_4Cl, 1% $KHCO_3$ and 0.04% NA_2EDTA in Milli-Q). Lysed peripheral blood cells were stained for extracellular markers using the following antibodies: CD19-FITC (HIB19, BD biosciences), CD1c-PE (AD5-8E7, Milteyni) , CD14-PE-TxR (TuK4, Invitrogen), CD33-PE-Cy7 (VI M505, eBioscience),

CD16-PerCP-Cy5.5 (3G8, BD biosciences), Siglec-8-APC (7C9, Biolegend), CD11c-AF700 (B-Ly6, BD biosciences), CD3-APC-eFl780 (UCHT1, eBioscience), CD45-V450 (HI30, BD Biosciences), CD8-biotin (HIT8a, eBioscience), CD86-BV650 (2331FUN1, BD Biosciences), HLA-DR-BV711 (G46-6, BD biosciences), CD4-BV785 (SK3, BD Biosciences), Streptavidin-BV605 (BD Biosciences). Fixable Aqua Dead Cell Stain kit for 405 (Invitrogen, Molecular Probes) was used as live-dead marker. Cells were measured on a Flow cytometer LSRII (BD Biosciences). Manual gating analyses were performed using FlowJo V10 (Treestar). Automatic gating was performed in the R environment.

FlowSOM based unsupervised analysis and automated cell type detection

Automated analysis was done using the FlowSOM algorithm (15). First, the data were manually gated on single live $CD45^+$ cells, including all hematopoietic cells in total blood. Besides the analyses performed on all hematopoietic cells in blood, more detailed analyses were performed on $CD33^-$ lymphocytes (including B cells, T cells, NK cells and dendritic cells) and $CD33^+$ monocytes. $CD33^-$ lymphocytes and $CD33^+$ monocytes were selected from single live $CD45^+$ mononuclear cells (based on SSC and FSC expression) based on their CD33 expression. Data were compensated, transformed with an estimated logical transformation and scaled. Cells were subsequently assigned to a Self-Organizing Map with a 6x6 grid (Total blood and $CD33^-$ lymphocytes) or a 5x5 grid ($CD33^+$ monocytes). Hereby similar cells were grouped, resulting in respectively 36, or 25 clusters. Once the data were mapped onto the grid, a Minimal Spanning Tree was built to visualize similar clusters in branches. Clusters of cells with similar expression markers were clustered within metaclusters (max 10-15). The percentage of selected cells within either the cluster or metacluster of interest was computed with respect to percentage of $CD45^+$ cells, $CD33^+$ monocytes or $CD33^-$ lymphocytes.

Statistical analyses

Comparisons between groups were performed using a Mann-Whitney U test. Correlations were analysed using Spearman's rank-order correlation test. P-values were two sided, and analyses were performed using R (version 3.3.1) and GraphPad Prism (version 7). $P<0.05$ was considered statistically significant.

RESULTS

Unbiased clustering of peripheral blood cells in healthy controls and asthma patients.

To investigate a broad range of immune cells, a flow cytometric staining was designed using the markers: CD1c, CD3, CD4, CD8, CD11c, CD14, CD16, CD19, CD33, CD86, HLA-DR and Siglec-8. Combining these markers allowed identification of at least B cells, T cells, dendritic

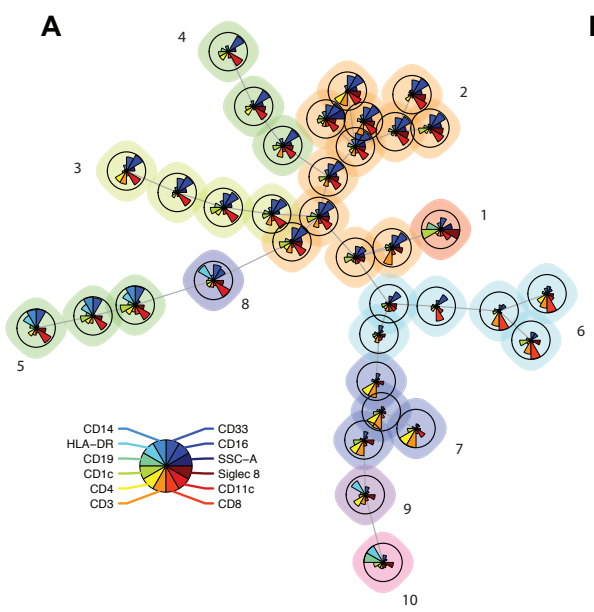
cells, monocytes, eosinophils and neutrophils in peripheral blood. A FlowSOM tree was generated from an aggregate file that included 4x10^6 live CD45$^+$ hematopoietic cells of all patients and healthy controls (HC) included in the study. In this tree 10 different metaclusters of cells with similar marker expression could be observed (Figure 1A). HCs and asthma patients were mapped to the FlowSOM tree separately. Significant increases in cell populations between cell HC and asthma patients were represented by red coloration of the nodes, whereas significant decreases were represented by blue coloration of the nodes (Figure 1B). Comparing frequencies of cells in individual metaclusters revealed significant differences between HCs and asthma patients in metaclusters 1, 4, 5 and 6 (Figure 1C). Detailed analysis of each individual included in the study showed an increase in metacluster 1, containing eosinophils in asthma patients compared to HCs. Moreover, asthma patients displayed decreased frequencies of cells in metaclusters 4, 5 and 6, which contained a subset of neutrophils, monocytes, and CD8$^+$ T cells, respectively (Figure 1D).

In conclusion, unbiased metaclustering of peripheral blood cells obtained revealed differences in various cell populations between HCs and asthma patients.

Peripheral blood of asthma patients display altered B and CD8$^+$ T cell frequencies

Significant differences were observed in the lymphocyte population, e.g. T cells in the FlowSOM Tree (Figure 1, metacluster 6, 7, 9, 10). Therefore, we next increased the sensitivity of our analysis by generating a FlowSOM Tree from an aggregate file containing CD45$^+$, SSC^{low}, CD33$^-$ lymphocytes of all study individuals. Cells were evaluated for the expression of all markers except CD33 and Siglec-8, as these markers are either not expressed on lymphocytes, or were used to select for lymphocytes. Lymphocytes comprise various cell populations, such as B cells, CD4$^+$ T cells, CD8$^+$ T cells, NK cells and DCs, that can be analysed using this analysis. From all lymphocytic cells, 15 metaclusters containing cells with similar marker expression were identified (Figure 2A). Various significant differences were observed in lymphocyte populations between HC and asthma patients (Figure 2B-C). We identified a significant increase in metacluster 3, 11 and 14, containing respectively CD1c-expressing B cells, CD86-expressing CD4$^+$/CD8$^+$ T cells and HLA-DR/CD86-expressing memory CD8$^+$ T cells in asthma patients, compared to HCs. Asthma patients displayed decreased frequencies of cells in metacluster 9, which contained HLA-DR-expressing CD8$^+$ T cells (Figure 2D). No significant differences where observed in CD4$^+$ T cells and NK cell populations between asthma patients and HCs (data not shown). DCs could not be identified in a metacluster and this was likely caused by their low numbers in pheripheral blood.

In summary, asthma patients showed different frequencies of subpopulations of CD8$^+$ T cells and CD1c$^+$ B cells compared with HC, whereas no differences in proportions of CD4$^+$ T cells or NK cells were detected.



C

Metacluster	p-value	Significant	Cell population
1	0,0000006	*	Eosinophils
2	0,22		Neutrophil subset
3	0,25		Neutrophil subset
4	0,0028	*	Neutrophil subset
5	0,014	*	Monocytes
6	0,0096	*	CD8$^+$ T cells
7	0,14		CD4$^+$ T cells
8	0,054		HLA-DR/CD11c-expressing cells
9	0,79		HLA-DR/CD4-expressing cells
10	0,73		B cells

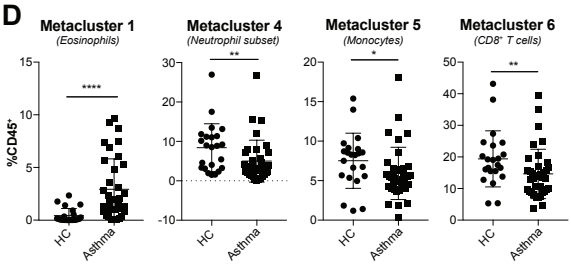
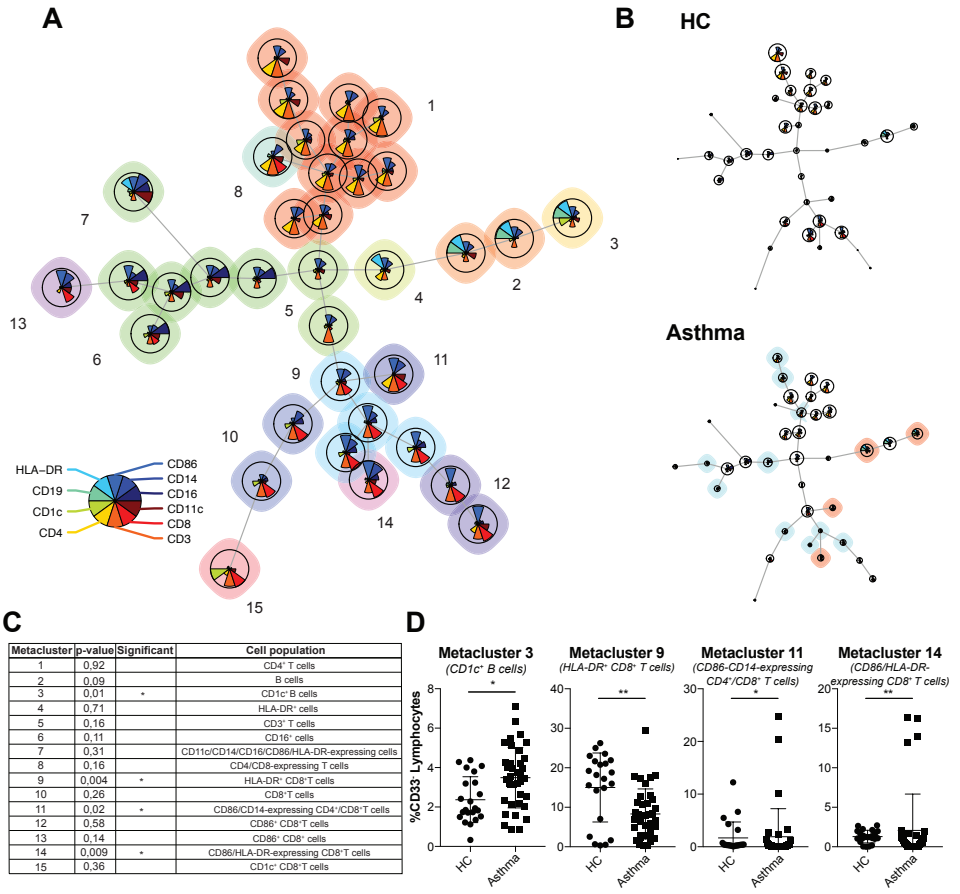


Figure 1: Unbiased clustering of peripheral blood cells in healthy controls and asthma patients. Cells from peripheral blood were stained with the extracellular panel. Single live CD45$^+$ cells for each file were exported and concatenated. This concatenated file was then analysed using FlowSOM and cells were clustered into 36 nodes. These nodes were divided over 10 metaclusters (A). Separate FlowSOM trees for healthy controls and asthma patients (B). P values for the comparison of metaclusters of healthy controls and asthma patients (C). Scatter dot plot of the frequency of the cells within the selected metacluster per individual (D). The FlowSOM algorithm was run five times to ensure reproducible results. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

Asthma patients display differences in frequencies and activation of CD14$^+$ monocytes

In the FlowSOM Tree generated from all CD45$^+$ cells, significant differences were also observed in CD33$^+$ monocytes. To improve the visibility of differences in monocytes, we generated a FlowSOM Tree from an aggregate file containing CD33$^+$ monocytes of all individuals included in the study. As CD3, CD4, CD8 and CD19 are not expressed on monocytes, only the expression of CD1c, CD11c, CD14, CD16, CD86 and HLA-DR were evaluated. The FlowSOM Tree build from all monocytes was divided into 10 metaclusters, each containing cells with similar marker

expression (Figure 3A). We found that asthma patients displayed increased frequencies of classical monocytes expressing CD1c, CD86, HLA-DR and CD11c (metacluster 8), and CD1c/HLA-DR/CD11c-expressing classical monocytes (metacluster 9), compared to HC (Figure 3B-C). In contrast, CD86 expressing classical monocytes with lower expression of HLA-DR and absent CD1c expression (metacluster 10) were lower in frequency in asthma patients, compared to HC (Figure 3D).



Metacluster	p-value	Significant	Cell population
1	0,92		CD4+ T cells
2	0,09		B cells
3	0,01	*	CD1c+ B cells
4	0,71		HLA-DR+ cells
5	0,16		CD3+ T cells
6	0,11		CD16+ cells
7	0,31		CD11c/CD14/CD16/CD86/HLA-DR-expressing cells
8	0,16		CD4/CD8-expressing T cells
9	0,004	*	HLA-DR+ CD8+T cells
10	0,26		CD8+T cells
11	0,02	*	CD86/CD14-expressing CD4+/CD8+T cells
12	0,58		CD86+ CD8+T cells
13	0,14		CD86+ CD8+ cells
14	0,009	*	CD86/HLA-DR-expressing CD8+T cells
15	0,36		CD1c+ CD8+T cells

Figure 2: Asthma patients display altered B and CD8$^+$ T cell frequencies. Single live CD45$^+$ and CD33$^-$ lymphocytes for each file were exported and concatenated. This concatenated file was then analysed using FlowSOM and cells were clustered into 36 nodes. These nodes were divided over 15 metaclusters (A). FlowSOM tree for healthy controls and asthma patients separately (B). Significant differences within between metaclusters of healthy controls and asthma patients (C). Quantification of the frequency of the cells within the selected metacluster per individual (D). The FlowSOM algorithm was run five times to ensure reproducible results. * = p<0.05, ** = p<0.01.

These data indicate that asthma patients present with different frequencies and activation states of CD14$^+$ classical monocytes, compared to HC, whereas CD16-expressing non-classical monocytes are unaltered.

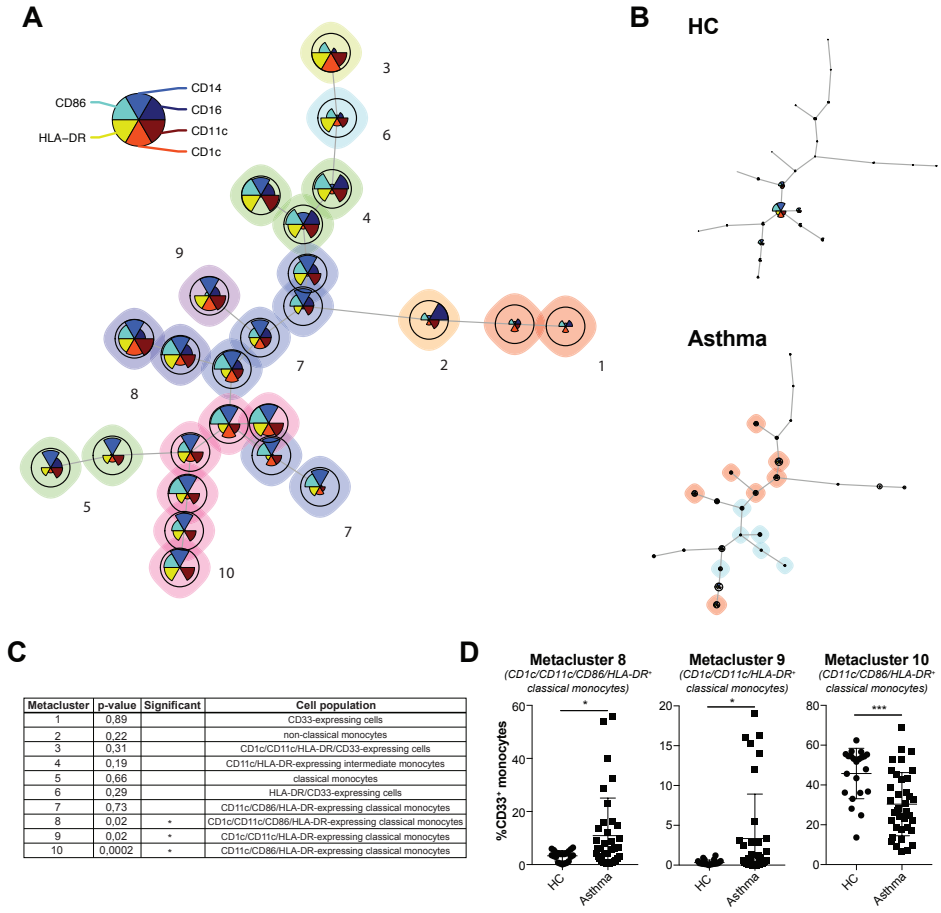
Immune profile of asthma patients depends on ACQ-score

Next, we explored whether the differences observed in the metaclusters of total blood, lymphocytes and monocytes were correlated with disease control. Metacluster 1 of total blood, consisting of eosinophils was significantly increased in all patient groups, compared to HC, whereas metacluster 4 of total blood, containing a neutrophil subset was only decreased in partly controlled and uncontrolled asthma patients compared to HC (Figure 4A). Within the lymphocyte compartment metacluster 3, containing CD1c$^+$ B cells, was specifically increased in uncontrolled asthma patients compared to HC. Metaclusters 9 and 11, containing HLA-DR-expressing CD8$^+$ T cells and CD86-expressing CD4$^+$/CD8$^+$ T cells were decreased specifically in partly controlled and uncontrolled asthma patients, whereas metacluster 14, which also contained HLA-DR and CD86-expressing CD8$^+$ T cells was specifically decreased in well-controlled asthma patients (Figure 4B). Monocyte metacluster 8, containing CD1c/CD86/HLA-DR/ CD11c- expressing classical monocytes were specifically increased in uncontrolled asthma patients, whereas metacluster 9 (containing CD1c/HLA-DR/CD11c-expressing classical monocytes) was increased in controlled and uncontrolled asthma patients. CD86-expressing classical monocytes with lower HLA-DR and absent CD1c expression (metacluster 10) were decreased in asthma patients irrespective of their disease control compared to HC (Figure 4C).

These data indicate that controlled asthma patients are characterized by increased eosinophils and CD1c/CD11c/HLA-DR$^+$ classical monocytes and decreased CD86/HLA-DR$^+$ CD8$^+$ T cells and CD11c/CD86/HLA-DR$^+$ classical monocytes. In contrast, uncontrolled asthma patients display increased eosinophils, CD1c$^+$ B cells, CD1c/CD11c/CD86/HLA-DR$^+$ classical monocytes, CD1c/CD11c/HLA-DR$^+$ classical monocytes and decreased neutrophils, HLA-DR$^+$ CD8$^+$ T cells, CD86/CD14$^+$ CD4/CD8$^+$ T cells, and CD11c/CD86/HLA-DR$^+$ classical monocytes.

CD1c$^+$ B cells and HLA-DR+ classical monocytes correlate with ACQ score

Flow cytometric analysis using the machine-learning algorithm FlowSOM revealed various differences in cell populations. However, it is important to confirm the findings by manual gating (Figure S1). In general, FlowSOM clustering resembled the manual gating. However, as expected automated clustering by FlowSOM gave more metaclusters of distinct cell populations as determined by manual gating (Figure S2). Using the FlowSOM algorithm, differences were observed in eosinophils and neutrophils in total blood. Using manual gating, similar frequencies of eosinophils were found compared to the FlowSOM analysis, however neutrophils were not different in asthma patients as compared to HC (Figure 5A). CD1c$^+$ B cells were also specifically increased in uncontrolled asthma patients. No differences were observed in CD8$^+$ T cell



Metacluster	p-value	Significant	Cell population
1	0,89		CD33-expressing cells
2	0,22		non-classical monocytes
3	0,31		CD1c/CD11c/HLA-DR/CD33-expressing cells
4	0,19		CD11c/HLA-DR-expressing intermediate monocytes
5	0,66		classical monocytes
6	0,29		HLA-DR/CD33-expressing cells
7	0,73		CD11c/CD86/HLA-DR-expressing classical monocytes
8	0,02	*	CD1c/CD11c/CD86/HLA-DR-expressing classical monocytes
9	0,02	*	CD1c/CD11c/HLA-DR-expressing classical monocytes
10	0,0002	*	CD11c/CD86/HLA-DR-expressing classical monocytes

Figure 3: Asthma patients display differences in frequencies and activation of CD14$^+$ monocytes. Single live CD45$^+$ and CD33$^+$ monocytes for each file were exported and concatenated. This concatenated file was then analysed using FlowSOM and cells were clustered into 25 nodes. These nodes were divided over 10 metaclusters (A). FlowSOM tree for healthy controls and asthma patients separately (B). Significant differences within between metaclusters of healthy controls and asthma patients (C). Quantification of the frequency of the cells within the selected metacluster per individual (D). The FlowSOM algorithm was run five times to ensure reproducible results. * = p<0.05, ** = p<0.01, *** = p<0.001

frequencies between asthma patients and HC, whereas HLA-DR+ CD8+ T cells were significantly reduced in partly controlled asthma patients compared to controlled asthma patients and HCs (Figure 5B). As FlowSOM revealed differences in the expression levels of activation markers on cell populations, manual gating was used to determine the Median Fluorescent Intensity (MFI). The MFI of CD86 was also significantly reduced in CD8$^+$ and HLA-DR$^+$ CD8$^+$ T cells of controlled, partly controlled and uncontrolled asthma patients when compared to HCs (Figure 5C). HLA-

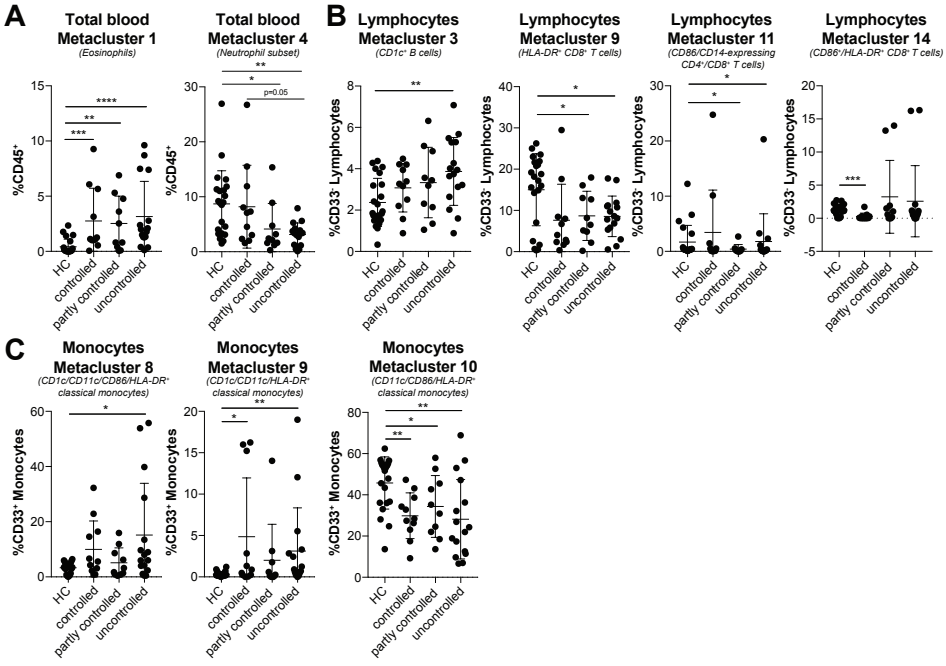


Figure 4: Immune profile of asthma patients depends on ACQ-score. Enumeration of the frequency of cells within metacluster 1 and 4 per individual of total blood (A). Quantification of cell frequencies in lymphocyte metacluster 3, 9, 11 and 14 (B). Quantification of cell frequencies in monocyte metacluster 8, 9, and 10 (C). * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

DR$^+$ classical monocytes were decreased in controlled and partly controlled asthma patients as compared to HC. MFI of CD86 was significantly decreased on classical monocytes of controlled and uncontrolled asthma patients compared to HC. MFI of CD1c in classical monocytes was only significantly decreased in controlled asthma patients compared to HC (Figure 5D). No correlation was found in the frequency of eosinophils, Th2 and Th17 cells and the ACQ-scores, as all groups of asthma patients showed increased eosinophil frequencies. In contrast, both the frequency of CD1c$^+$ B cells and HLA-DR$^+$ classical monocytes of CD45$^+$ hematopoietic cells did correlate with ACQ-scores in asthma patients (Figure 5E).

In conclusion, for various cell populations similar frequencies were determined using manual gating as compared to the FlowSOM analysis. Most interestingly, CD1c$^+$ B cells and HLA-DR$^+$ classical monocytes correlate with disease severity, and are a better predictor of disease severity as eosinophil frequency.

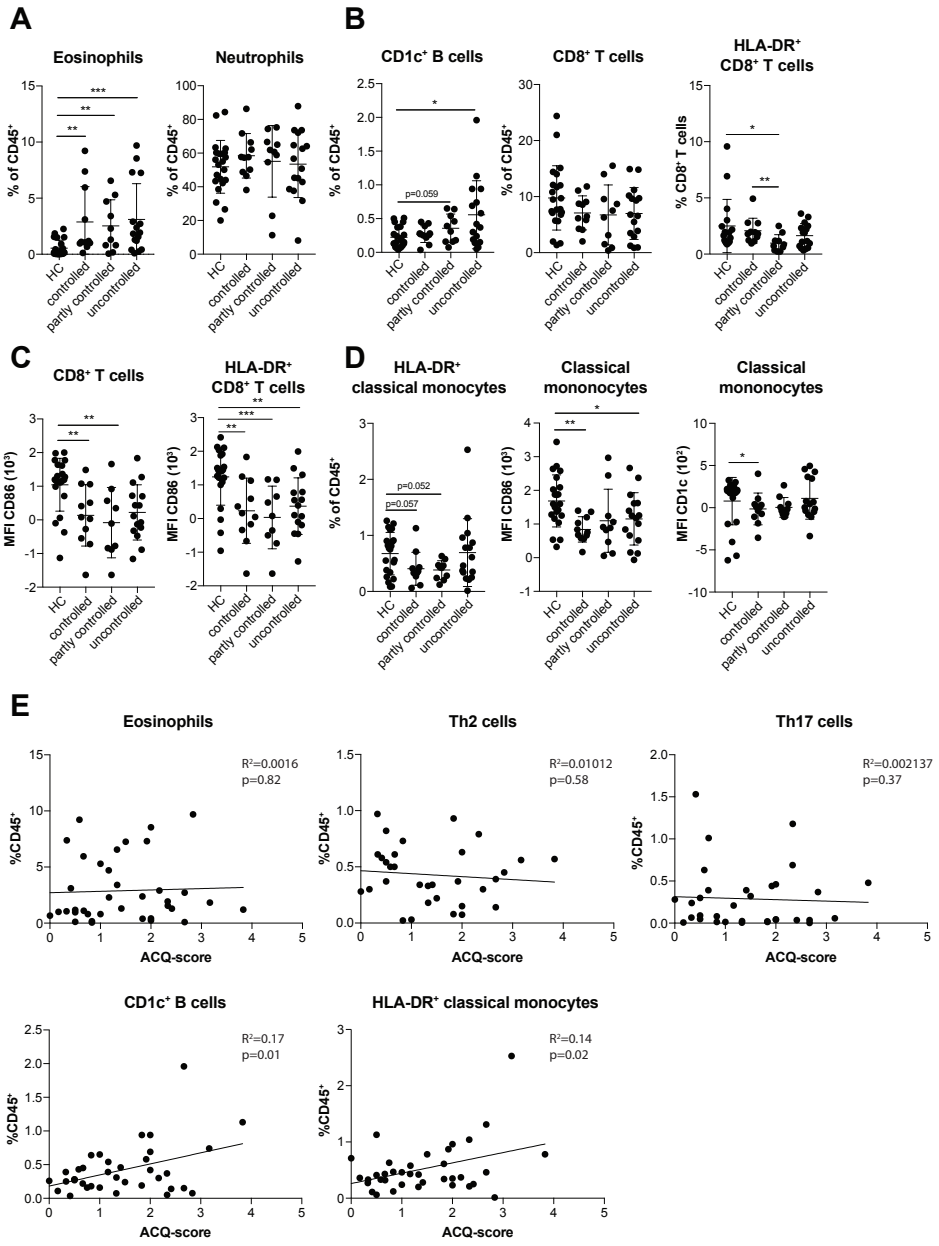


Figure 5: CD1c$^+$ B cells and HLA-DR-expressing classical monocytes correlate with ACQ. Quantification of manually gated eosinophils and neutrophils (A). Enumeration of manually gated CD1c$^+$ B cells, CD8$^+$ T cells and HLA-DR$^+$ CD8$^+$ T cells (B). Quantification of MFI of CD86 in CD8$^+$ T cells and HLA-DR$^+$ CD8$^+$ T cells (C). Quantification of HLA-DR$^+$ classical monocytes and MFI of CD86 and CD1c in classical monocytes (D). Correlation between eosinophils, Th2, Th17, CD1c$^+$ B cells and HLA-DR$^+$ classical monocytes (E). * = p<0.05, ** = p<0.01, *** = p<0.001.

DISCUSSION

Our study demonstrates that immune profiling asthma patients and HC by flow cytometry over time using FlowSOM is possible and conceives reliable data. Our results show that the immune profile of asthma patients is different from healthy controls. Asthma patients had increased percentages of blood eosinophils, $CD1c^+$ B cells, and $HLA\text{-}DR^+$ classical monocytes, whereas $HLA\text{-}DR^+$ $CD8^+$ T cells and neutrophil percentages were decreased. We observed that eosinophils and neutrophils were not different between controlled asthma patients and uncontrolled asthma patients. Surprisingly, we found that a subset of B cells, the $CD1c^+$ B cell, was specifically increased in uncontrolled asthma patients, and correlated with the ACQ-score. Also, HLA-DR-expressing monocyte proportions correlated with the ACQ-score and were only significantly decreased in controlled and partly controlled asthma patients.

Our data revealed that eosinophils are increased in asthma patients irrespectively of their disease control status. This is surprising as eosinophils are described to be sensitive to steroid treatment. As treatment adherence in asthma patients is also a known complicating factor, especially for mild and controlled asthma patients (17). Low steroid adherence could be the reason that increased eosinophil percentages are observed in controlled asthma patients.

In our study, we found that percentages of $CD1c^+$ B cells correlated with the ACQ-score. $CD1c^+$ B cells were specifically increased in uncontrolled asthma patients. CD1c ($BDCA1^+$) expression has been widely described as a marker expressed by dendritic cells (DCs) (18), but also for human marginal zone (MZ)-like B cells (19). The function of CD1c on B cells remains unknown, however CD1 isoforms are known to present lipids to clonally diverse T cells (20). Thus far, $CD1c^+$ B cells have not been described in asthma pathology, but we can speculate that this B cell subset could induce CD1c-restricted T cell activation. Surprisingly, no differences were observed in the frequencies of $CD86^+$ B cells between asthma patients and HCs, whereas a previous study reported increased expression of CD86 on B cells of allergic asthma patients. This increase in CD86 was specifically observed in $CD23^+$ B cells, but not in $CD23^-$ B cells (21). As CD23 was not included in the staining this could cause the discrepancy between the conflicting results.

We also observed that the frequency of $HLA\text{-}DR^+$ classical monocytes correlated with the ACQ-score, whereas others found no differences in HLA-DR expression on monocytes (22). Corresponding to our findings, increased expression of HLA-DR was also described in DCs cultured from monocytes of atopic asthmatics (23), indicating that the activation status of monocytes is likely implicated in asthma pathogenesis.

Our data also implies the potential for automatic analysis of flow cytometric data using machine learning algorithm FlowSOM on peripheral immune cells of patients obtained over time. Not all differences observed in the automatic analysis were confirmed by manual gating, however this can be related to fluctuations of marker intensities over time, as differences

observed in defined cell populations could most often be confirmed. This is in agreement with the FlowCAP consortium where they found lower variability in clearly defined cell subsets as compared to dim marker expressions and rare cell populations (24). Measuring peripheral blood samples of patients over time faces a lot of challenges as marker intensities can differ between patients due to biological factors but also due to technical issues that arise once samples are not processed at the same time. Therefore, it is promising to observe that the automatic analysis accurately resembled the manual gating.

There are several limitations to our study, as it remains arguable whether peripheral blood is representative for a respiratory disease. Furthermore, our data could not be corrected for medication adherence as this was not one of the parameters included in the study. Finally, this study was designed to provide a general overview of alterations in the immune system of asthmatic patients and was not designed to investigate specific differences in T cell activation. Our study suggests that the activation status of $CD8^+$ T cells in asthma patients is reduced when compared to HCs, whereas $CD4^+$ T cells were unaltered. Based on these observations it would be worthwhile to investigate the activation status of CD4 and $CD8^+$ T cells in more detail using more extensive activation markers.

Collectively, our data show that automatic analysis of flow cytometric data acquired from peripheral blood of asthma patients is feasible and closely resembles manual analysis. This unbiased analysis approach revealed that of all immune cells analysed with our study antibodies, HLA-DR^+ $CD8^+$ T cells were reduced in all asthma patients, and the percentage of CD1c-expressing B cells and HLA-DR-expressing classical monocytes showed a significant correlation with disease control. No correlation was observed between eosinophils and disease control. Therefore, $CD8^+$ T cells, HLA-DR^+ classical monocytes and $CD1c^+$ B cells could play an important role especially in asthma patients that remain uncontrolled while treated with ICS. These cell populations must be carefully characterized and their importance for the pathogenesis for asthma be further explored.

ACKNOWLEDGEMENTS

These studies were partly supported by NWO-VENI (916.11.067), European Framework program 7 (FP7-MC-CIG grant 304221.), and Netherlands Lung Foundation (3.2.12.087 and 4.2.13.054JO). HV was supported with a long-term EAACI Fellowship to visit the lab of BNL. We would like to thank Bevinda Ferreira, Simone Mulder, Bianca Timmermans and respiratory physicians (Sint Franciscus Gasthuis, Rotterdam, The Netherlands) for their assistance during the project.

AUTHOR CONTRIBUTIONS

HV, RWH, MK designed the experiments. HV, IT, SVG performed experiments and analysed data. HV, RWH and MK wrote the manuscript. All authors red and approved the final manuscripts.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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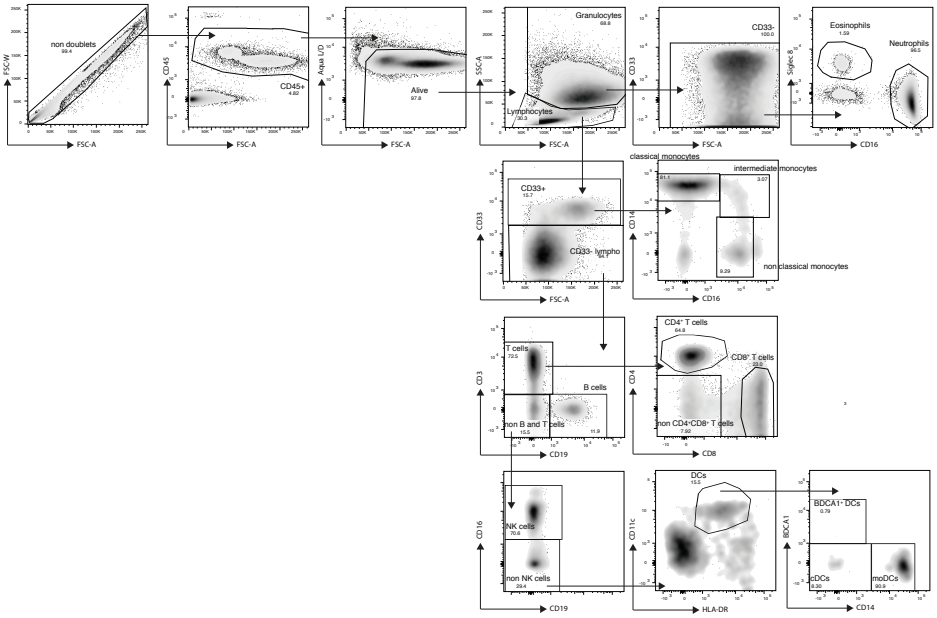


Figure S1: Manual gating on peripheral blood. Cells were gated manually according to the following gating strategy.

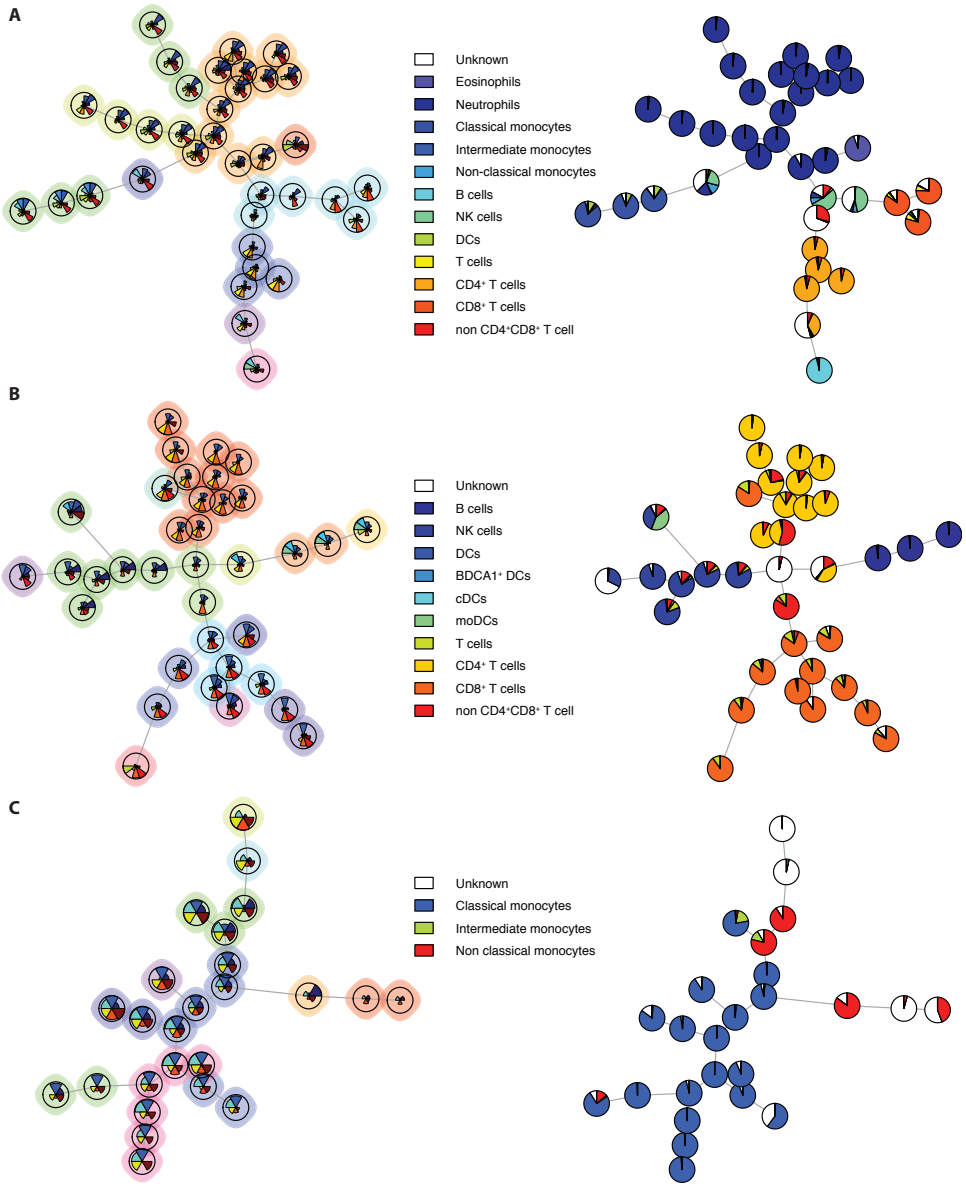


Figure S2: Comparing manual gating to automated clustering by FlowSOM. CD45$^+$ blood cells of an individual were mapped to the FlowSOM tree. The manual gating of the cells was plotted onto the node, to visualize what the origin of these cells was using the manual gating (A). CD33$^-$ lymphocytes of one individual were mapped to the FlowSOM tree and compared to the manual gating (B). CD33$^+$ monocytes of one individual were mapped to the FlowSOM tree and compared to the manual gating (C).

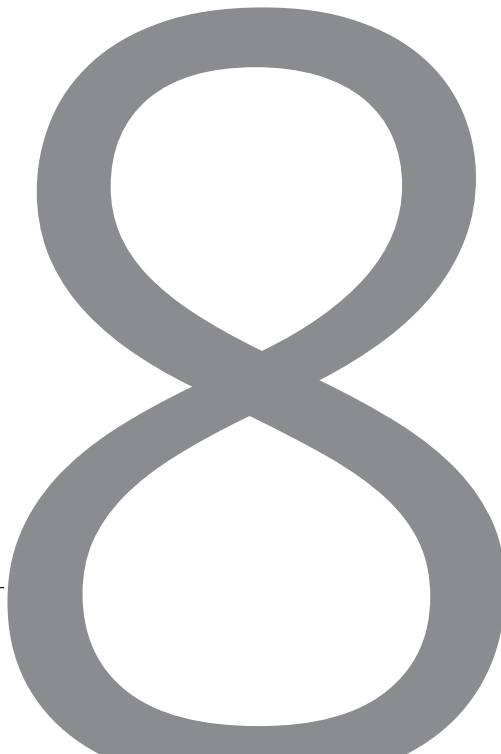
Table S1: Clinical characteristics of individuals included in the study

	HC	controlled	partly controlled	uncontrolled
n	22	11	10	16
Age	30,2 (22-47)	34,0 (19-48)	27,9 (16-43)	29,2 (18-49)
Sex (M/F)	5/17	2/9	2/8	1/15
BMI	22,85 (18,9-26,6)	24,2 (19,2-30,6)	24,14 (18,6-29,4)	26,7 (20,6-31,6)
FEV1		100,5 (69-123)	98,4 (83-119)	96,3 (74-119)
ACQ score		0,4 (0-0,67)	1,08 (0,75-1,41)	2,34 (1,5-3,83)
FeNO		20,2 (6-47)	29,4 (6-70)	33,19 (9-92)

Table S2: Extracellular markers used for flow cytometric staining

Marker	Clone	Company
CD3	UCHT1	eBioscience
CD4	SK3	BD Biosciences
CD8	HIT8a	eBioscience
CD11c	B-Ly6	BD Biosciences
CD14	TuK4	Invitrogen
CD16	3G8	BD Biosciences
CD19	HIB19	BD Biosciences
CD33	VI M505	eBioscience
CD45	HI30	BD Biosciences
CD86	2331FUN1	BD Biosciences
BDCA1	AD5-8E7	Milteyni
HLA-DR	G46-6	BD Biosciences
Siglec 8	7C9	Biolegend

General Discussion



In this thesis, we addressed different immunological mechanisms that contribute to the heterogeneity observed within asthma. We characterized the role of B cells and B-T cell interaction in the development of house dust mite (HDM)-induced Th2-mediated airway inflammation and remodelling. We demonstrated that B cells, B-T cell interactions and Tfh cells are dispensable for eosinophilic inflammation but necessary for airway remodelling in an asthma model using continuous allergen exposure **(Chapter 2)**. By depleting the negative regulator TNFAIP3 in myeloid cells and dendritic cells (DCs) we found that the activation status of pulmonary DCs controls development of eosinophilic and neutrophilic airway inflammation **(Chapter 4)**. Furthermore, we established that IL-17RA-signalling is dispensable for Th2-mediated eosinophilic inflammation. Also, neutrophilia can develop in mice with TNFAIP3-deficient myeloid cells and defective IL-17RA-signalling **(Chapter 5)**. In **Chapter 6,** we show that activation of Langerin-expressing pulmonary cDC1s, by depletion of TNFAIP3, reduces Th2-mediated eosinophilic inflammation and instead activates regulatory T cells, as well as IL-10 and IFNg-expressing $CD8^+$ T cells. Finally, we characterized various immune cells in the peripheral blood of asthma patients with differences in disease control status and demonstrated that eosinophils, Th2 cells and Th17 cells are not predictive for disease control status, whereas CD1c-expressing B cells and HLA-DR-expressing classical monocytes are **(Chapter 7)**.

In this chapter, we will discuss various different immunological mechanisms that control heterogeneity as observed in within animal models and asthma patients.

TH2-MEDIATED EOSINOPHILIC INFLAMMATION

Type-2 cytokines are secreted by T helper (Th) 2 cells (IL-4, IL-5 and IL-13), type 2 innate lymphoid cells (ILC2s) (IL-4, IL-5, and IL-13) (1), and T follicular helper (Tfh) cells (IL-4) (2). These cytokines are essential for the pathogenesis of allergic asthma, and drive eosinophilic inflammation (IL-5), IgE class switching by B cells (IL-4) and goblet cell hyperplasia (IL-13) (1, 3). Th2 differentiation is induced in the lung-draining lymph node by pulmonary DCs that encountered allergens in a Th2-instructive cytokine milieu created by the airway epithelium (**Chapter 1 and 3**) (4). Recently, the importance of Tfh cells was described for Th2 development, as naïve T cells do not directly differentiate into Th2 cells in an HDM-driven asthma model. Th2 cells first undergo a IL-4-committed Tfh stage during allergen sensitization before further differentiation into memory Th2 cells during the challenge phase (5). Others also described that Tfh cells promote the differentiation of effector Th2 cells and thereby contribute to eosinophilic inflammation (6). However, we have shown in **Chapter 2** that B cells and B-T cell interaction are dispensable for eosinophilic inflammation, but not for airway remodelling after chronic exposure to HDM. As final Tfh differentiation is dependent on B cells and interaction between B-T cell (7), our data indicates that after chronic exposure to HDM, eosinophilic inflammation

can develop in the absence of Tfh cells. Eosinophilic inflammation observed following chronic allergen exposure in the absence of B and Tfh cells may be induced or supported by direct activation of ILC2s **(Chapter 2)**. As ILC2s can secrete IL-5 in response to pro-inflammatory cytokines secreted by the airway epithelium upon allergen recognition (8). Furthermore, Th2 cytokine-secreting T cells in the broncho-alveolar lavage are not affected by the absence of B cells and Tfh cells. This suggests that moDCs can directly induce Th2 differentiation locally in the airways upon chronic allergen exposure, leading to eosinophilic inflammation (**Chapter 2**). Our data corresponds to recent work that revealed that B cells are essential for the expansion of Th2 cells in the lungs and memory T cells in the lung-draining lymph node only when the antigen dose is limiting (9). These data indicate that B cells are dispensable for eosinophilic inflammation when allergens are abundantly present. Taken together, the importance of B cells and Tfh cells in driving pulmonary inflammation largely depend on the experimental model, allergen type, and kinetics of the exposure.

Furthermore, we showed that chronic exposure to allergens provoked inducible bronchus associated lymphoid tissues (iBALT) structures **(Chapter 2)**. IBALT structures in asthma patients are larger in size and number as compared to healthy controls, however iBALT numbers do not correlate with disease severity (10). In contrary, another study found no differences in iBALT numbers between asthmatic or non-asthmatic cross-country skiers, arguing the involvement of iBALT in asthma pathogenesis (11). In other inflammatory conditions, such as influenza infection, the production of viral-specific immunoglobulins in serum is dependent on iBALT structures (12). This could indicate that in allergies and asthma, allergen-specific immunoglobulins could also depend on iBALT structures. Therefore, the exact function of iBALT structures for asthma pathogenesis remain unknown and should be investigated. To investigate this, chronic allergen-driven asthma models should be used, as more acute models consisting only a sensitization and challenge phase do not induce iBALT structures.

TH17-MEDIATED NEUTROPHILIC AIRWAY INFLAMMATION

In particular, late-onset patients that display more severe forms of asthma have a neutrophilic inflammation or a mixed eosinophilic/neutrophilic inflammation that is associated with the presence of Th1 and Th17 cytokines (13, 14). These severe asthma patients are often unresponsive to corticosteroid treatment and suffer from severe asthma exaggerations, as both Th17 cells and neutrophils are unresponsive to corticosteroid treatment (15-17). In **Chapter 4**, we found that *Tnfaip3*-deletion in all pulmonary DC subsets induced a neutrophil-associated severe asthma phenotype upon HDM treatment. This neutrophilic inflammation was associated with increased numbers of IL-17 and IFNg-producing $CD4^+$ T cells. HDM treatment in mice harboring *Tnfaip3*-deficient DCs induced Th17 differentiation and hampered Th2 differentia-

tion, whereas Th1 differentiation was unaltered **(Chapter 4)**. This suggests that DC activation controls asthma heterogeneity by directly affecting Th cell differentiation. In **Chapter 4** we furthermore demonstrated that HDM treatment increased the expression of cytokines that promote Th17 cell differentiation, IL-6, and IL-23 especially in *Tnfaip3*-deficient cDC2s and moDCs **(Figure 1)**. Simultaneously, cytokines that inhibit Th2 differentiation, such as IL-6 and IL-12 were also induced in *Tnfaip3*-deficient cultured moDCs upon HDM treatment. This suggests that cDC2s and moDCs are the most important DC subsets driving the development of either Th2-mediated eosinophilic or Th17-mediated neutrophilic inflammation, which is in line with the findings of other (4, 18-20).

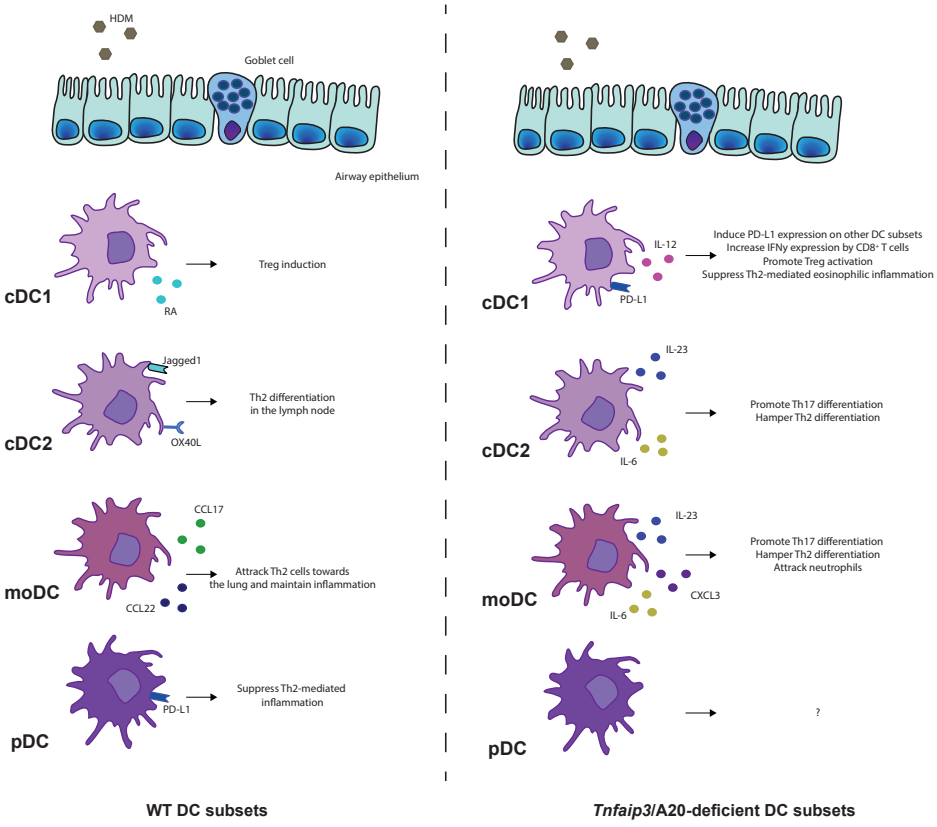


Figure 1: Alterations in HDM-activated DC function upon deletion of TNFAIP3/A20. Activation of cDC1s via *Tnfaip3*-depletion increased IL-12 and PD-L1 expression, not only in cDC1s, but also in other DC subsets, which is associated with drastically reduced Type 2 airway inflammation. In contrast, *Tnfaip3*-deficient cDC2s and moDCs express higher levels of IL-6 and IL-23 cytokines, known to drive Th17 differentiation and induce neutrophilic inflammation upon HDM treatment.

IL-17 and IFNg-producing $CD4^+$ T cells are both increased upon chronic HDM exposure, but only HDM-specific $RORgT^+$ T cells were induced by *Tnfaip3*-deficient DCs **(Chapter 4)**. Therefore, it is uncertain whether these IFNg-producing $CD4^+$ T cells are truly Th1 cells or belong to the non-classical Th17 (Th17.1) cell population (21).

Currently it is uncertain whether DC activation is also implicated in the heterogeneity as observed in asthma patients. Most DC studies were performed in predominantly Th2 high allergic asthma patients and these studies did not compare different types of asthma patients (22-25). In **Chapter 7**, we found increased frequencies of HLA-DR-expressing classical monocytes in uncontrolled asthma patients. The frequency of HLA-DR-expressing classical monocytes even correlated with disease control status. This could indicate that besides alterations in DC subsets and activation status, also the activation status of monocytes can contribute to asthma severity, not only in mice but also in human. Palikhe *et al*, showed that intermediate monocytes of severe asthma patients display increased levels of protease-activated receptor-2 (PAR-2), a G-coupled receptor activated by proteases, compared to intermediate monocytes of mild/moderate asthma patients (26). PAR-2-mediated activation induces secretion of IL-1b, IL-6 and IL-8 (27), cytokines implicated in Th17 differentiation and neutrophilic inflammation. This implies that monocytes of severe asthma patients display alterations in activation markers that could contribute to asthma heterogeneity and severity.

Furthermore, we found that in asthma patients, the percentage of neutrophils in peripheral blood positively correlated to the frequency of $RORgT^+$ Th17 cells in peripheral blood **(Figure 2A)**. Strikingly, the median fluorescent intensity (MFI) of HLA-DR, an activation marker on $CD14^+$ classical monocytes was also positively correlated to the frequency of $RORgT^+$ Th17 in the peripheral blood of asthma patients **(Figure 2B)**. In **Chapter 4** we showed that the activation status of pulmonary DCs in mice, obtained through different TNFAIP3 levels control Th2 versus Th17-mediated airway inflammation. Therefore, we wondered whether TNFAIP3 levels in human DCs would also correlate with Th17 cells. To determine this, mRNA was extracted from MACS-purified $BDCA1^+$ cDC2s of asthma patients. *Tnfaip3* mRNA levels were not different between healthy controls and patients, irrespective of their status of disease control (data not shown).

However, we found that the mRNA expression of *Tnip,* a protein that enables the function of TNFAIP3 (28, 29), was negatively correlated to the frequency of neutrophils **(Figure 2C)**, but not to the percentage of $RORgT^+$ Th17 in the peripheral blood of asthma patients **(Figure 2D)**. This suggests that also in human, the TNFAIP3 pathway controls the activation status of DCs and/or monocytes controls the type of inflammation, being either eosinophilic, or neutrophilic. Improving our understanding of factors that drive severe Th17-mediated neutrophilic asthma, will likely improve the treatment options for severe uncontrolled asthma patients.

Neutrophilic inflammation is dependent on IL-17 signalling, as IL-17A stimulates airway epithelial IL-8 production, a chemoattractant for neutrophils (30). In **Chapter 5**, we demonstrated that neutrophilic inflammation in *Tnfaip3*$^{LysM\text{-}KO}$ mice is not dependent on IL-17R signalling

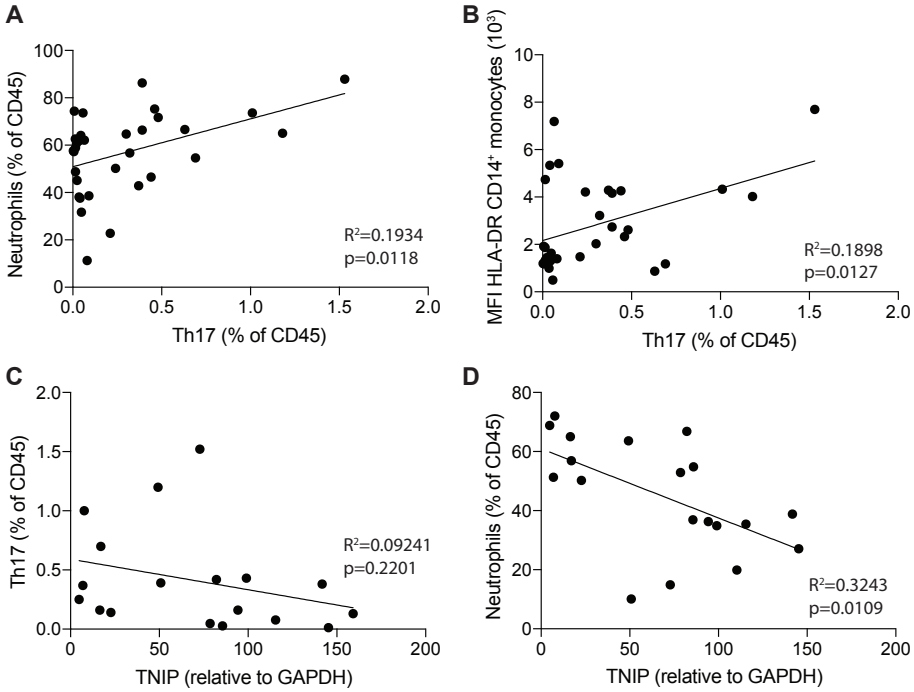


Figure 2: Increased activation of monocytes and DCs in asthmatics are associated with neutrophilia and Th17 cells. Correlation between proportion of neutrophils and RORgT$^+$ Th17 cells of asthma patients in peripheral blood by flow cytometry (A). Correlation between median fluorescent intensity of HLA-DR on CD14$^+$ classical monocytes and RORgT$^+$ Th17 cells of asthma patients in peripheral blood by flow cytometry (B). Correlation between RORgT$^+$ Th17 cells of asthma patients in peripheral blood by flow cytometry and mRNA expression of *Tnip* in MACS purified BDCA1$^+$ cDC2s of asthma patients (C). Correlation between neutrophils frequency of asthma patients in peripheral blood by flow cytometry and mRNA expression of *Tnip* in MACS purified BDCA1$^+$ cDC2s of asthma patients (D).

as neutrophilic inflammation is similar between *Tnfaip3*$^{LysM\text{-}KO}$ mice and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. Abrogating IL-17RA-signalling in combination with *Tnfaip3*-depletion in myeloid cells increased IL-23 expression, which together with defective IL-17RA-signalling, enhanced Th17 differentiation and induced spontaneous accumulation of IL-17$^+$ γδ T cells in the lungs **(Chapter 5)**. Whether these IL-17-producing γδ T cells play a detrimental or beneficial role in allergic airway inflammation and/or resolution of airway inflammation remains unknown and is likely dependent on the allergen type. Depletion of γδ-17 T cells during the resolution phase in an OVA-mediated asthma model worsened allergic inflammation (31, 32), indicating that upon OVA treatment γδ-17 T cells are beneficial during the resolution phase. However, our results indicated that the presence of high amounts of these γδ-17 T cells did not alter either eosinophilic or neutrophilic inflammation. This could also be caused by deficiency of IL-17RA-signalling which abrogates IL-17. Besides high levels of IL-17, also IL-22 and GM-CSF

were increased in lungs of mice have defective IL-17RA-signalling and harbor activated myeloid cells (**Chapter 5**), implying that upon HDM-mediated asthma models, IL-22 and GM-CSF are not beneficial nor detrimental for allergic airway inflammation.

SUPPRESSION OF ASTHMATIC AIRWAY INFLAMMATION

Development of asthmatic airway inflammation could also be caused by defective immunological suppression, or impaired airway homeostasis. Regulatory T cells (Tregs) are known for their immune regulatory functions by secretion of immunosuppressive cytokines IL-10 and expression of immunomodulatory molecules (33). Depletion of $CD4^+CD25^+$ regulatory T (Treg) cells increased airway hyper-responsiveness in mice (34). Furthermore, transfer of antigen-specific Tregs was able to prevent and suppress Th2-mediated airway inflammation in response to OVA (35, 36). These findings indicate that Tregs are essential controllers of asthmatic airway inflammation. *In vitro* studies have also shown that Tregs of non-atopic individuals can suppress cytokine secretion by $CD4^+$ T cells upon allergen stimulation, whereas Tregs of atopic individuals were unable to suppress this cytokine secretion (37).

The function of cDC1s in T helper responses in asthma remains controversial, as cDC1s have been shown to induce Treg differentiation upon allergen exposure (38, 39), but are also implicated in Th2 differentiation (40). Pulmonary cDC1s are well appreciated for their superior cross-presentation of antigens to $CD8^+$ T cells, essential for the induction of virus-specific $CD8^+$ T cells and anti-tumor immune responses (41-43).

In **Chapter 6**, we showed that increasing the activation status cDC1s by depleting TNFAIP3 in Langerin-expressing pulmonary cDC1s, inhibited eosinophilic inflammation and Th2 cell differentiation upon an HDM-mediated airway inflammation protocol. These activated cDC1s rather induced highly activated Tregs and Th2-suppressive IL-10 and IFNγ-secreting $CD8^+$ T cells. Activation of cDC1s increased IL-12 and PD-L1 expression, not only on cDC1s but also on other DC subsets, implicating interaction between different DC subsets occurs **(Figure 1)**. Increased PD-L1 expression was dependent on IL-12 and IFNγ. Depletion of Tregs in mice harboring *Tnfaip3*-deficient $Langerin^+$ cDC1s did not restore Th2-mediated eosinophilic inflammation, suggesting that IL-10 and IFNγ-secreting $CD8^+$ T cells but not Tregs suppressed the development of eosinophilic inflammation. Others have also shown that IL-10-producing cells can suppress cytokine secretion by Th2 cells (44). However, in most cases these studies focused on IL-10-producing $CD4^+$ T cells but not $CD8^+$ T cells. Our data suggests that IL-10 production by $CD8^+$ T cells could be a novel opportunity to suppress Th2-mediated airway inflammation. Furthermore, we also found decreased numbers of $CD8^+$ T cells and $HLA\text{-}DR^+$ $CD8^+$ T cells in asthma patients **(Chapter 7)**. Whether cytokine expression would also be different between $CD8^+$ T cells of healthy controls and $CD8^+$ T cells of asthma patients remains yet to be determined.

Whether the activation status of cDC1s, e.g. the expression of co-stimulatory or co-inhibitory molecules, is altered in asthmatic patients compared to healthy controls remains unknown and should be investigated.

CONCLUSION AND FUTURE DIRECTIONS

The described heterogeneity of asthma patients is mainly based on clinical characteristics, however differences within their immune system driving these clinical characteristics should be further explored. In this thesis, we have shown that the activation status of DCs can control Th2 and Th17-mediated airway inflammation and can even suppress Th2-mediated airway inflammation, dependent on the DC subsets targeted. To achieve this increased activation status of DCs, we depleted TNFAIP3 in different DC subsets (**Chapter 1**). Characterization of DC activation and subset distribution both locally and systemically in asthma patients should be performed to determine whether DC activation also controls asthmatic heterogeneity in asthmatic individuals. Our first preliminary results indicate that there are indeed differences in HLA-DR expression of monocytes and TNIP in human cDC2s, suggesting that DC and monocyte activation status could control asthma severity in human.

Next, we have shown in this thesis that the type of allergen used in experimental models and the exposure timing determine immunological processes and should therefore be chosen carefully. Animal models are often used to investigate the mechanisms driving asthmatic airway inflammation and to explore new therapeutic strategies for asthma patients. Using relevant allergens such as HDM, in physiological concentrations, and treatment schedules resembling exposure in asthma patients will improve our understanding of airway inflammation and remodelling in asthmatic subjects (**Chapter 2**). Animal models that enable investigation of heterogeneity in asthmatic airway inflammation are limited and depend on either genetic modifications, such as the activation of DCs (described in this thesis) or combined exposure with allergens and environmental triggers such as smoke, diesel exhaust particles, or viral particles (**Chapter 1**).

Investigation of the immune system both in peripheral blood and locally in lung in a longitudinal manner is essential to delineate immunological heterogeneity within the asthmatic patient population. This will enable the characterization of patients with different immunological phenotypes and should show whether the type of inflammatory response observed in these patients remains stable over time. To achieve this, collaborations are essential between clinicians and fundamental researchers, linking these immunological findings to clinical parameters using bioinformatics. This would improve our understanding of the immunological mechanisms driving heterogeneity between asthma patients and should finally result in new treatment strategies and therapeutic targets.

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Summary

English Summary

In recent years, new therapies have been established for the treatment of asthma patients. This has led to an improved quality of life and lung function for a large number of asthma patients. Despite this progress, 10% of the asthma patients suffer from symptoms while treated. These patients often present without a classically T helper (Th2)-mediated eosinophilic inflammation and rather present with a neutrophilic inflammation or pauci-granulocytic inflammation. To generate therapeutic strategies for these patients it is essential to unravel the immunological mechanism that underlies these different asthma phenotypes.

Th2 cells are critical regulators of eosinophilic asthma by their secretion of type 2 cytokines (IL-4, IL-5 and IL-13). Despite progress in many areas, it is still not completely understood which signals are needed for Th2 differentiation. Th2 cell differentiation can involve an intermediate stage through a follicular T helper (Tfh) cell stadium, as was shown in a house dust mite (HDM)-driven asthma model containing a sensitization and challenge phase. This would imply that germinal center reactions are important for Th2 differentiation. However, previous studies have shown that eosinophilic inflammation can develop in the absence of B cells, and therefore in the absence of germinal centers. In **Chapter 2** we determined whether eosinophilic inflammation was dependent on the interaction between B and T cells upon chronic HDM exposure. We found that in a chronic HDM model, eosinophilic inflammation in broncho-alveolar lavage fluid (BALf) and lung tissue can develop in the absence of B-T cell interaction and germinal centre reactions. Thus, in contrast to findings in acute HDM models characterized by sensitization and challenge phases, our data in a chronic HDM asthma model demonstrate that pulmonary eosinophilic inflammation is independent of B-T cell communication. Nevertheless, our chronic HDM model reveals that pulmonary Th2 inflammation and airway remodelling requires B cells and B-T cell interaction.

Next to patients with a Th2-mediated eosinophilic inflammation, a subset of asthmatic patients suffers from Th17-mediated neutrophilic inflammation. Currently it is unknown which mechanism controls this heterogeneity within asthma patients. As dendritic cells (DCs) drive differentiation of naïve T cells into both Th2 and Th17 cells, we wondered whether DC activation could contribute to asthma heterogeneity. To induce increased DC activation, mouse models were used in which TNFAIP3/A20, one of the negative regulators of the pro-inflammatory transcription factor NF-kB, was deleted in different DC subsets, using transgenic mice. In **Chapter 4**, we show that the activation status of DCs controls the development of either eosinophilic or neutrophilic airway inflammation. Highly activated TNFAIP3-deficient DCs induce Th17 differentiation and hamper Th2 differentiation upon HDM exposure. TNFAIP3-deficiency in specifically type 2 conventional DCs (cDC2s) and monocyte-derived DCs (moDCs) increases their potential to produce cytokines implicated in Th17-cell differentiation. Furthermore, cytokines that hamper Th2 differentiation were also increased in TNFAIP3-defecient DCs upon HDM exposure, indicating that the amount of TNFAIP3 in DCs can control the heterogeneity in granulocytic inflammation in asthma.

IL-17-signalling is implicated in both neutrophilic as eosinophilic airway inflammation, as IL-17 activates the airway epithelium to secrete chemokines mediating neutrophil chemotaxis. The influence of IL-17-signalling on eosinophilic inflammation differs depending on allergen used and time of cytokine exposure. However, whether the neutrophilic inflammation observed in chapter IV is dependent on IL-17R-signalling is investigated in **Chapter 5**. We showed that absence of IL-17RA-signalling has only limited effect on neutrophilic or eosinophilic inflammation in an HDM-driven acute inflammation model. Th2 cell differentiation is not hampered by ablation of IL-17RA-signalling, whereas mucus-producing cells are reduced. Defective IL-17RA-signalling in mice that harbour activated myeloid cells induces spontaneous accumulation of RORgt$^+$ γδ T cells and Th17 cells in the lungs. Furthermore, mice harbouring TNFAIP3-deficient myeloid cells and IL-17RA-deficiency have elevated levels pulmonary IL-23, needed for Th17 and RORgt$^+$ γδ T cell maintenance.

In the previous chapters, we showed that especially cDC2s and moDCs are essential for the development of Th2 or Th17-associated airway inflammation. However, the function of cDC1s in allergic inflammation remains controversial. In **Chapter 6** we show that activation of cDC1s by deletion of TNFAIP3 in Langerin-expressing pulmonary cDC1s is sufficient to suppress Th2-mediated inflammation. Instead of Th2 cells, allergen exposure induces development of highly activated Tregs and CD8$^+$ T cells that either express IL-10 or IFNg in the lung in mice harbouring activated cDC1s. Intriguingly, TNFAIP3 deletion in cDC1s increases expression of the Th2-suppressive co-inhibitory molecule PD-L1 and IL-12. The increased expression of these molecules was not only observed in cDC1s, but also in all other pulmonary DC subsets, indicating cross-regulation between DC subsets.

Also clinically, asthma patients comprise an heterogeneous population, including patients that are well controlled by inhaled corticosteroids, whereas other patients remain symptomatic under corticosteroid treatment. In **Chapter 7**, we investigated immunological differences in peripheral blood of controlled, partly controlled, and uncontrolled asthma patients and healthy controls by flow cytometry. Flow cytometric analysis are often performed manually, however during manual analysis cells are often excluded from used gates and not all markers are assessed on all cell types. Recently, advanced machine-learning algorithms enable unbiased analysis of flow cytometric data and ease the visualisation of these automated analysis. Thereby analysing flow cytometric data in an automated manner using these algorithms improves the interpretation of the data. Using this automated manner of analysing flow cytometric data, we show that blood eosinophils, CD1c$^+$ B cells and HLA-DR$^+$ classical monocytes are increased in asthma patients whereas HLA-DR$^+$ CD8$^+$ T cells and neutrophils are decreased as compared to healthy controls. In contrast to eosinophils, Th2, and Th17 cells, CD1c-expressing B cells and HLA-DR$^+$ classical monocytes correlate with disease severity, indicating that careful characterization of these cell populations is necessary to determine their implication for asthma pathogenesis.

In conclusion, this thesis shows that asthma is a heterogeneous lung disorder, in which both Th2 and Th17 cells can induce asthmatic symptoms. Furthermore, we have shown that the activation status of cDC2s and moDCs critically controls Th2 and Th17 differentiation, whereas activation of cDC1s abrogates development of Th2-mediated airway inflammation. Targeting the activation status of different DC subsets can contribute to asthma heterogeneity and asthma severity, or even dampen Th2-mediated immune responses. Further research should be performed to investigate whether DC activation and the secretion of cytokines contributes to the heterogeneity and severity observed in asthma patients, and whether these factors can be used as therapeutic strategy to treat uncontrolled asthma patients.

NEDERLANDSE SAMENVATTING

Astma is een chronische longziekte, die gekarakteriseerd wordt door ontstekingen, verhoogde slijmproductie en verbindweefseling in de longen. De incidentie van astma in de Westerse wereld is in de laatste tientallen jaren toegenomen. Astma wordt veroorzaakt doordat patienten allergisch zijn voor bijvoorbeeld huisstofmijt of schimmels, wat zogenaamde allergenen worden genoemd. Deze allergenen worden in de luchtwegen opgenomen door dendritische cellen (DC's) die hiermee T-cellen activeren, waardoor er een immuunreactie wordt gestart wat resulteert in de astmatische symptomen.

In de afgelopen jaren zijn er veel nieuwe therapieën ontwikkeld om astmapatiënten beter te kunnen behandelen. Dit heeft gezorgd voor een verbetering van de kwaliteit van leven en een verbeterde longfunctie voor veel astmapatiënten. Ondanks deze vooruitgang, blijft 10% van de astmapatiënten symptomen vertonen terwijl ze onder behandeling zijn met medicijnen. Deze ongecontroleerde astmapatiënten hebben vaak een ander soort ontstekingsreactie in de longen, die bestaat uit neutrofielen, in plaats van de eosinofiele ontstekingscellen die in het merendeel van de astmapatiënten verhoogd zijn. Om nieuwe therapieën te ontwikkelen voor deze patiënten is het essentieel om het immunologische mechanisme wat deze diversiteit aan ontstekingsreacties binnen astmapatiënten veroorzaakt te ontrafelen.

Astmatische ontstekingsreacties worden vaak in gang gezet door zogenaamde T-cellen. Deze T-cellen herkennen specifieke allergenen en worden geactiveerd wanneer zij deze allergenen herkennen als deze aan hun worden gepresenteerd door DC's. Er verschillende soorten T-cellen, namelijk Th1, Th2, Th17 cellen, welke verschillende soorten ontstekingsreacties in gang brengen, en regulatoire T-cellen (Tregs) die deze verschillende T-cellen afremmen. Th2 cellen zijn essentieel voor eosinofiel gedreven astma door het maken van speciale signaalstoffen, genaamd cytokines (IL-4, IL-5 en IL-13). Ondanks vooruitgang in vele onderzoeksgebieden, is het nog steeds onduidelijk welke signalen er nodig zijn voor de ontwikkeling van Th2 cellen. In kortdurende huisstofmijt-gedreven astma modellen die een sensitisatie fase en een fase van herhaalde blootstelling bevatten is gevonden dat een T-cel - na activatie door een allergeen - een Tfh-stadium doorgaat, voordat de cel uiteindelijk een Th2 cel wordt. Deze Tfh cellen bevinden zich in gebieden in de lymfeklieren waar veel interactie plaatsvindt tussen B en T-cellen, welke follikels worden genoemd. Verrassend genoeg hebben eerdere studies laten zien dat eosinofiele ontstekingsreacties ook kunnen ontstaan in de afwezigheid van B-cellen en daardoor ook in de afwezigheid van deze follikelgebieden. In **Hoofdstuk 2** hebben we onderzocht of eosinofiele ontstekingsreacties tijdens langdurig herhaalde blootstelling aan huisstofmijt ook afhankelijk zijn van de communicatie tussen B en T-cellen. In tegenstelling tot eerdere bevindingen in kortdurende huisstofmijt gedreven modellen, laten onze resultaten zien dat in de afwezigheid van B en T-cel communicatie eosinofiele ontsteking kan ontwikkelen tijdens langdurige blootstelling aan huisstofmijt. Echter, ophoping van Th2 cellen in de longen

en littekenvorming van de longen is wel afhankelijk van B-cellen en communicatie tussen B en T-cellen.

Naast patiënten die een Th2-gedreven eosinofiele ontstekingsreactie vertonen, heeft een deel van de astmapatiënten een Th17-gedreven neutrofiele ontsteking in de luchtwegen. Op dit moment is het nog onduidelijke welke signalen deze diversiteit binnen de astmapatiënten veroorzaakt. Omdat geactiveerde DC's belangrijk zijn voor de ontwikkeling van T-cellen die nog geen allergenen hebben gezien tot Th2 of Th17 cellen, vroegen we ons af of de mate van DC activatie zou kunnen bepalen welke Th cel ontwikkeling er plaatsvindt. Om dit te onderzoeken hebben we gebruik gemaakt van muismodellen waarin de activatiestatus van DC's artificieel verhoogd is doordat het *TNFAIP3/A20* gen verwijderd is waardoor er geen functioneel A20 eiwit gevormd kan worden. TNFAIP3 is een enzym dat de activiteit remt van de transcriptiefactor NF-kB, die nodig is voor cel activatie. In **Hoofdstuk 4** laten we zien dat de activatiestatus van DC's de ontwikkeling van ofwel eosinofiele of neutrofiele luchtwegontsteking controleert. Sterk geactiveerde DC's waarin het *TNFAIP3* gen geheel is verwijderd, stimuleren Th17 ontwikkeling en remmen de ontwikkeling van Th2 cellen na huisstofmijt blootstelling. Er zijn verschillende soorten DC's met elk hun eigen verschillende functie. Wij hebben gevonden dat het gebrek van TNFAIP3 specifiek in type 2 conventionele DC's (cDC2's) en DC's die ontstaan uit monocyten (moDCs) zorgt voor een verhoogde productie van cytokines die belangrijk zijn voor Th17 cel ontwikkeling. Daarnaast hebben deze geactiveerde cDC2's en moDCs ook een verhoogde hoeveelheid van cytokines die de ontwikkeling van Th2 cellen remmen na huisstofmijt behandeling.

IL-17-signalering is betrokken bij zowel neutrofiele as eosinofiele luchtwegontsteking, aangezien IL-17 het luchtwegepitheel stimuleert tot het maken van signaalstoffen die neutrofielen aantrekken naar de longen. De invloed van IL-17-signalering op eosinofiele ontstekingsreacties is afhankelijk van het allergeen dat gebruikt wordt in het astma model en het moment van blootstelling. Of de neutrofiele ontstekingsreactie in hoofdstuk 4 afhankelijk is van IL-17 receptor (IL-17R) signalering is onderzocht in **Hoofdstuk 5**. Hierin laten we zien dat de afwezigheid van IL-17RA-signalering maar een beperkt effect heeft op neutrofiele of eosinofiele ontsteking in een kortdurend huisstofmijt gedreven model dat een sensitisatie fase en een fase van herhaalde blootstelling bevat. Th2 cel ontwikkeling is niet aangetast door de afwezigheid van IL-17RA-signalering, terwijl de slijm-producerende cellen wel verlaagd zijn. IL-17RA-deficiënte muizen die geactiveerde monocyten, macrofagen en moDCs bevatten, vertonen ook spontane ophopingen van RORgt^{+} γδ T-cellen en Th17 cellen in de longen. Daarnaast is de hoeveelheid van het cytokine IL-23 in de longen van deze muizen verhoogd, wat de instandhouding van deze RORgt^{+} γδ T-cellen en Th17 cellen verzorgt.

In vorige hoofdstukken hebben we laten zien dat met name cDC2's en moDCs belangrijk zijn voor de ontwikkeling van verschillende Th cel ontstekingsreacties die gezien worden in astmatische luchtwegen. Echter, de functie van type 1 conventionele DC's (cDC1's) in allergische

ontstekingsreacties is nog steeds omstreden. In **Hoofdstuk 6** hebben we onderzocht of de selectieve activatie van cDC1's in de longen een effect heeft op allergische ontstekingsreacties, door het TNFAIP3 gen te verwijderen in cDC1's die Langerine tot expressie brengen. Hierbij hebben we gevonden dat activatie van alleen long cDC1's voldoende is om de ontwikkeling van Th2-gedreven luchtwegontstekingen te remmen. Allergeenblootstelling in muizen waarvan de cDC1's in de longen geactiveerd zijn leidt niet tot de ontwikkeling van Th2 cellen. In plaats daarvan zagen we Treg en $CD8^+$ T-cellen die IL-10 en IFNg produceren, die beide een remmende werking hebben op Th2 cel ontwikkeling en functie. *TNFAIP3* deletie in cDC1's verhoogd de expressie van PD-L1 op het celoppervlak en de productie van IL-12, welke beide Th2 cel ontwikkeling onderdrukken. Deze factoren zijn niet alleen verhoogd voor cDC1's maar ook voor andere soorten DC's in de longen, wat suggereert dat er communicatie tussen DC subsets plaatsvind.

Op basis van hun klinische karakteristieken zijn astmapatiënten zeer divers. Bij sommige patiënten is de ziekte goed behandelbaar is met inhalatiecorticosteroïden, terwijl andere patiënten ondanks de behandeling met inhalatiecorticosteroïden klachten blijven houden. In **Hoofdstuk 7** hebben we onderzocht wat de immunologische verschillen zijn in het bloed van patiënten die onder behandeling van inhalatiecorticosteroïden goed gecontroleerd, matig gecontroleerd en slecht gecontroleerd zijn. Deze 3 patiëntengroepen zijn vergeleken met gezonde controles. Om de verschillen in het immuunsysteem te bepalen is gebruik gemaakt van flow cytometrie. Hierbij werd gebruik gemaakt van geautomatiseerde analyses, waardoor het mogelijk werd om alle gemeten markers op alle aanwezige celtypes in bloed en sputum te analyseren. Met behulp van het automatische analyseprogramma FlowSOM hebben we gevonden dat eosinofielen, B-cellen die CD1c tot expressie brengen en $HLA\text{-}DR^+$ klassieke monocyten verhoogd zijn in het bloed van astmapatiënten, terwijl $HLA\text{-}DR^+$ $CD8^+$ T-cellen en neutrofielen verlaagd zijn in vergelijking met gezonde controles. In tegenstelling tot eosinofielen, Th2 cellen en Th17 cellen, correleren $CD1c^+$ B-cellen en $HLA\text{-}DR^+$ klassieke monocyten met de ernst van de ziekte. Door verder onderzoek naar deze cel populaties zou het belang van deze celpopulaties voor de ontwikkeling van astma bepaald kunnen worden.

Samenvattend, laat dit proefschrift zien dat astma een heterogene longziekte is, waarin zowel Th2 als Th17 cellen kunnen zorgen voor astmatische symptomen. Verder hebben we aangetoond dat de activatiestatus van cDC2's en moDCs een belangrijke factor is die bepaald of er Th2 of Th17 cellen worden gevormd, terwijl activatie van cDC1's de ontwikkeling van Th2 cellen onderdrukt. Verder onderzoek moet uitwijzen of de activatiestatus van DC's en de cytokines die DC's produceren bijdragen aan de diversiteit tussen astmapatiënten, de ernst van de ziekte, of het onderdrukken van Th2-gedreven astma. Daarnaast zou onderzocht kunnen worden of deze factoren kunnen dienen als nieuwe therapie voor astmapatiënten die onder behandeling van inhalatiecorticosteroïden klachten blijven houden.

Dankwoord

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In dit dankwoord wil ik graag iedereen bedanken die op enige manier bijgedragen heeft aan dit proefschrift. Zonder jullie hulp was dit nooit geworden wat het nu is.

Allereerst wil ik graag mijn promotor Prof. dr. Rudi Hendriks bedanken. Rudi, jouw enthousiasme voor wetenschap is aanstekelijk, en dit is waarschijnlijk de belangrijkste reden waarom ons lab zo'n fijne plek is om te mogen werken. Met jouw uitzonderlijke overzicht weet je altijd de zwakke plekken in een artikel aan te wijzen, maar heb je hier ook altijd direct een oplossing voor. Zonder deze suggesties was dit proefschrift daarom ook niet hetzelfde geweest. Ondanks dat ik niet direct door jou werd begeleid was de samenwerking de afgelopen jaren heel direct en stond je deur altijd open. Jouw advies is mij heel waardevol, en ik ben blij dat ik ook in de toekomst kan blijven werken op jouw afdeling.

Daarnaast wil ik natuurlijk mijn co-promotor Dr. Mirjam Kool bedanken. Ik kan me mijn sollicitatiegesprek met jou en Ingrid nog goed herinneren. We zouden een klein team binnen de afdeling Longziekten gaan vormen, met jou als begeleider en Ingrid als analist. Toen ik enkele maanden later begon, bleken de plannen iets aangepast en was de I&R (Inflammation & Remodelling) groep gevormd, en kreeg je meerdere PhD studenten en analisten onder je begeleiding. Ondanks dat de groep iets groter was geworden dan oorspronkelijk gepland, was jouw begeleiding altijd heel persoonlijk. Ik kan mij geen betere begeleidster voorstellen Mirjam! Je biedt altijd een luisterend oor, en bent heel oplossingsgericht. Ook ben ik je erg dankbaar dat je mij de vrijheid hebt gegeven om zelf keuzes te maken binnen mijn onderzoek. Dit heeft mij veel geleerd en er zeker voor gezorgd dat ik een zelfstandigere onderzoekster ben geworden. Ik hoop in de toekomst nog vaak samen te kunnen brainstormen over ons gezamenlijke favoriete cel type: de dendritische cel!

Ook wil ik graag dr. Bernt van den Blink bedanken voor zijn supervisie de eerste paar jaar van mijn promotie. Jouw achtergrond als longarts zorgde ervoor dat we nooit het klinische aspect uit het oog verloren.

Prof.dr. Henk Hoogsteden, bedankt voor de mogelijkheid om mijn onderzoek in het laboratorium van de afdeling Longziekten te mogen doen.

Prof. dr. Bart Lambrecht, bedankt voor alle input gedurende mijn promotie. Ik heb heel veel bewondering voor jouw enorme kennis van de literatuur en het vermogen om in te spelen op nieuwe technieken en onderzoeksmogelijkheden. Heel erg bedankt dat ik een gedeelte van mijn onderzoek heb mogen uitvoeren bij jouw afdeling in het VIB.

I would also like to thank Prof. dr. Hamida Hammad for all your input on the various projects of my thesis and for the supervision during the Longfonds fellowship.

Ik wil ook graag de overige leden van de kleine commissie, Dr. Erik Lubberts en Dr. Janneke Samsom bedanken voor het grondig doorlezen van mijn proefschrift. Daarnaast wil ik ook graag de overige leden van de commissie, Prof. dr. Maria Yazdanbakhsh, Prof. Dr. Bjorn Clausen, Dr. Gert-Jan Braunstahl bedanken voor het deelnemen aan de commissie.

Zonder jou, Ingrid, was dit proefschrift niet hetzelfde geweest, en de periode op het lab al helemaal niet. Vanaf dag één ben jij bij alle aspecten van dit project betrokken geweest en dit ging veel verder dan alleen praktische ondersteuning. Van het bedenken van experimenten, opzetten van nieuwe technieken, tot het uitvoeren van gigantische experimenten; een betrokkenere analiste kan ik me niet voorstellen. Daarnaast heb ik van jouw kritische kijk op experimentele procedures heel veel geleerd. Naast onze samenwerking heb ik ook genoten van alle cappuccino's en lunches. Deze gezellige sfeer heeft mijn promotietijd echt onvergetelijk gemaakt en ik ben ook heel blij dat jij me bijstaat tijdens mijn verdediging als paranimf!

Je hoort regelmatig dat promoveren af en toe een eenzaam proces kan zijn, maar als promovendus op het lab Longziekten heb ik dit nooit zo ervaren. Daarom wil dan ook graag iedereen van het lab bedanken voor alle onvergetelijke momenten, de altijd gezellige sfeer en jullie hulp. Daarnaast wil ik graag nog een paar personen in het bijzonder bedanken: Jennifer, hoe had ik ooit zonder jou al die enorme experimenten kunnen uitvoeren? En al die qPCRs die je hebt gedaan, zodat ik de laatste maanden ongestoord kon schrijven. Daarnaast heb ik ook altijd heel erg genoten van alle gezellige momenten. Menno, dank je wel voor al je hulp bij het opzetten van alle procedures voor de Iphase studie. Zonder jouw ervaringen met humaan materiaal was dit nooit zo soepel en vlot verlopen. Daarnaast bood je altijd de helpende hand bij de grote muisexperimenten als dit nodig was. Melanie, bedankt voor alle hulp met de Iphase studie en natuurlijk de luchtwegfunctiemetingen. Daarnaast wil ik de overige analisten, Marjolein, Margaretha, Koen, Myrthe en Sara bedanken voor het beantwoorden van al mijn vragen en jullie gezelligheid.

Wat is het een voorrecht om samen te kunnen werken met andere PhD-studenten waarmee je ervaringen kunt delen. Als eerste wil ik graag de medepromovendi van de I&R-groep: Caroline, Peter, Tridib, Thomas en Denise (ofwel de 'roomies') bedanken. Het kunnen delen van ervaringen en jullie feedback hebben het promotietraject heel erg versoepeld. Daarnaast wil ik ook Irma en Bobby bedanken voor jullie steun. Als de drie PhD's die aan astma werkten hebben we veel samengewerkt en zelfs samen een klinische studie opgezet. Het gezellige congres in Roemenië zal ik nooit vergeten. Veel succes nog met het afronden van jullie proefschriften! Ook

wil ik graag Sanne, Sabine, Floris, Pauline, Simar en Jasper bedanken voor jullie hulp en steun. Pauline in het bijzonder, bedankt voor het wegwijs maken in “R”! Naast de PhD studenten wil ik uiteraard ook Alex, Odilia, Ralph en Saravanan bedanken. Saravanan, bedankt voor alle hulp met de mRNA-sequencing data. Zonder jou was de analyse niet zo soepel gegaan.

Tijdens mijn promotietraject heb ik drie studenten mogen begeleiden: Lisette, Boudewijn en Denise. Heel erg bedankt voor jullie hulp bij alle experimenten die hebben bij gedraagt aan mijn proefschrift. Lisette, mijn eerste student en jouw eerste onderzoeksstage. Bedankt voor al je gezelligheid tijdens alle experimenten, en ik ben benieuwd hoe het met je opleiding gaat! Boudewijn, zoals jij zonder moeite omschakelde vanuit je geneeskunde opleiding naar een basaal onderzoeksproject heb ik altijd heel erg bewonderd. Niet voor niks heb je hiervoor de Gerrit-Jan Mulder prijs gekregen. Ik hoop ook dat je na je coschappen besluit wetenschappelijk onderzoek te blijven combineren met het arts zijn. Denise, een meer betrokkene en daarnaast ook gezellige studente dan jij, heb ik me niet kunnen wensen. Zoals jij alle projecten (en het waren er veel!) naar je toetrok en je eigen maakte was heel knap. De hoeveelheid werk die je verzet hebt in je stageperiode is dan ook gigantisch, en hebben mij de gelegenheid gegeven om wat meer op het schrijven te focussen. Ik ben dan ook super blij dat je afgelopen september bent kunnen starten als PhD-student bij Mirjam.

Ook wil ik graag Prof. dr. Yvan Saeys en Sofie van Gassen bedanken voor alle hulp en ondersteuning tijdens de periode bij het VIB. Ik heb enorm veel geleerd over bioinformatica en in het bijzonder over FlowSOM en de enorme mogelijkheden die hiermee gepaard gaan. Ook wil ik graag Martijn Schuijs bedanken voor alle hulp bij de experimenten die we hebben uitgevoerd in het VIB. Ondanks dat je zelf altijd een enorme hoeveelheid experimenten lopende had, wist je altijd tijd vrij te maken om mij te helpen. Justine en Kim, ook heel erg bedankt voor jullie hulp.

Een klinische studie opzetten en draaiende houden is een enorme teamprestatie, en ik wil dan ook graag de researchverpleegkundigen van het Erasmus MC en het SFG, Annemarie, Marjolein, Bevinda, Simone en Bianca bedanken voor jullie hulp, advies en het includeren van alle patiënten. Daarnaast wil ook graag alle betrokken artsen en arts-assistenten bedanken in het SFG, en in het bijzonder Dr. Gert-Jan Braunstahl. Zonder patiënten die willen deelnemen aan klinische studies zouden klinische studies niet bestaan, dus ik zou ook graag alle astmapatiënten willen bedanken voor het deelnemen aan de studie.

Sandra en Jan Piet, mijn eerste kennismaking binnen het Erasmus MC was als jullie student bij de afdeling Reumatologie. Bedankt dat jullie al zo snel tijdens mijn stage mee wilden denken

over een baan als PhD-student na mijn afstuderen en mij hebben aanbevolen bij Mirjam en Rudi. Zonder jullie was ik nooit op deze promotieplaats terechtgekomen.

Lieve vrienden, bedankt voor alle gezellige feestjes, etentjes en borrels de afgelopen jaren.

Lieve familie, bedankt voor jullie onvoorwaardelijke steun en gezelligheid. Het hebben van een hechte familie zo dichtbij is mij heel veel waard. Lieve pap, mam en Moniek, bedankt dat jullie altijd voor mij klaar staan, jullie steun betekent heel veel voor mij! Ingrid, bedankt dat jij me tijdens de promotie wil bijstaan als paranimf. Ik prijs me gelukkig dat ik een zusje heb waarmee ik al die tenniswedstrijden, volleybalwedstrijden, winkelmomenten en city-trips heb kunnen beleven.

Lieve Ruud, ik ben jou heel dankbaar voor al je steun. Jij helpt me altijd weer herinneren welke dingen er het belangrijkst zijn in het leven. Het leven is mooi samen met jou!

Heleen

PhD Portfolio

Summary of PhD training and teaching

Name PhD student:	H. (Heleen) Vroman
Erasmus MC Department:	Pulmonary Medicine
Research School:	Molecular Medicine
PhD period:	2012-2017
Promotor:	Prof.dr. R.W. Hendriks
Supervisor:	Dr.ing. M.Kool

PhD training

General courses, seminars and workshops

2012

– Laboratory Animal Experimentation (4,3 ects)

2013

– Advanced Immunology course (3 ects)
– NVVI Lunteren symposium 2013: 'The immune system on fire: The intracellular logistics of inflammation' (0,5 ects)
– Workshop on Adobe Photoshop and Illustrator (0,3 ects)
– NRS animal model day (0,3 ects)
– Course on R (2,0 ects)
– Mucosal Immunology (0,5 ects)

2014

– NVVI Lunteren symposium 2014: 'Mucosal Immunology: crossing borders' (0,5 ects)
– BROK-course (2,0 ects)
– NRS animal model day (0,3 ects)

2015

– NVVI Lunteren symposium 2015: 'Immunity and Science Fiction: the next 50 years in Immunology' (0,5 ects)
– Biomedical English writing course (2 ects)

(Inter)national scientific presentations and conferences

17th Molecular Medicine day, Rotterdam (poster)	2013	0,3
NRS Young Investigator, Amsterdam (poster)	2013	0,3
Abcam meeting, Brugge (poster)	2013	0,5
NVVI Winter School, Noordwijkerhout (oral)	2013	0,5
EMDS, Erlangen (oral)	2013	1,0
NRS animal model day (oral)	2014	0,3
Longdagen, Utrecht (poster)	2014	0,5

18th Molecular Medicine day, Rotterdam (poster)	2014	0,3
13th International Meeting on Dendritic Cells, Tours (poster)	2014	1,0
NVVI Winter School, Noordwijkerhout (poster)	2014	0,5
EAACCI Winter School, Brasov (oral)	2014	1,0
ERS Annual Conference, Estoril (oral)	2014	1,0
ERS Annual Conference, Munchen (oral)	2014	1,0
Longdagen, Utrecht (oral)	2015	0,3
ICMI 2015, Berlin (poster)	2015	1,0
NVVI Winter School, Noordwijkerhout (poster)	2015	0,5
NVVI Winter School, Noordwijkerhout (oral)	2015	0,5
19th Molecular Medicine day, Rotterdam (poster)	2015	0,3
Longdagen, Ermelo (poster)	2016	0,5
20th Molecular Medicine day, Rotterdam (poster)	2016	0,3
International Conference of Immunology (oral)	2016	1,5
Keystone Asthma symposium (oral)	2017	1,5

Teaching

- Winter course, Research Master Infection & Immunity, Erasmus University, Rotterdam.
  Lecture: 'The immunology of asthma' (0,5 ects)
- Advanced Immunology Course, Postgraduate School "Molecular Medicine", Erasmus University, Rotterdam.
  Lecture: 'Dendritic cell subsets and antigen processing' (0,5 ects)

Supervising students

- Supervising Master internship 6 months L. Krassenburg, Erasmus University, Rotterdam (10 ects)
- Supervising Master internship 6 months B. van der Wel, Erasmus University, Rotterdam (10 ects)
- Supervising Master internship 9 months D. van Uden, Erasmus University, Rotterdam (15 ects)

Awards

- NRS Young Investigator Travel Grant for EMDS, Erlangen
- NRS Young Investigator Travel Grant for International Conference of Immunology, Melbourne
- ERS Travel Grant for ERS, Estoril
- Long-term research fellowship from the Dutch Lung Foundation (Longfonds) 2016
- Long-term research fellowship from the European Academy of Allergy and Clinical Immunology (EAACI) 2016

Curriculum vitae

Heleen Vroman was born in Goes on October 17th in 1989. She attended the Buys Ballot College in Goes and graduated in 2007 after which she started her studies in Biomedical Sciences at Utrecht University. After obtaining her Bachelor of Science degree, Heleen continued with a Master in Biology of Disease at Utrecht University and obtained her degree in 2012. During this period, she performed a 9 month internship at the department of Pathology at the UMC Utrecht in Utrecht and a 6 month internship at the department of Rheumatology at the Erasmus MC in Rotterdam. In 2012, she started her PhD project at the department of Pulmonary Medicine at the Erasmus MC in Rotterdam. Her PhD project focusses on the effect of dendritic cell activation on the development of asthmatic immune responses under the supervision of Prof. dr. Rudi W. Hendriks and Dr. Mirjam Kool. During her PhD-project, Heleen obtained two long-term research fellowships from the Longfonds and EAACI. These fellowships enabled her to visit the VIB in Ghent, Belgium to work with transgene mice harbouring T cells reactive to house dust mite and analyse flow cytometry data using the FlowSOM algorithm, under the supervision of Prof. dr. Bart N. Lambrecht and Prof. dr. Hamida Hammad. The results of her PhD project are described in this thesis and will be defended in June 2017 in Rotterdam. After obtaining her PhD, Heleen will work as a Post-Doc at the Tumor Immunology group at the department of Pulmonary Medicine of the Erasmus MC.