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IL-17 and TNF-α are essential mediators of *M. catarrhalis* triggered exacerbation of HDM allergic airway inflammation

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CONTENT

ABBREVIATIONS

°C	degree Celsius
µl	micro liter (10^{-9}1)
µM	micro molar
%	percent
Ag	antigen
Ab	antibody
APC	antigen presenting cell
Aqua dest	destilled water
ATP	adenosine tri-phosphate
BAL	bronchoalveolar lavage
Bp	base pairs
BCR	B cell receptor
BSA	serum albumin bovine
DMSO	dimethylsulfoxide
DNA	desoxribonucleic acid
DNase	desoxyribonuclease
EAE	experimental autoimmune ecephalomyelitis
e.g.	example given
ELISA	enzyme-linked immunosorbent assay
M.cat	*Moraxella.catarrhalis*
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescin isothiocyanate
g	gramm
h	hour
IFNγ	interferon gamma

Ig	immunoglobulin
IL	interleukin
IL-17 AF $^{-/-}$	deficient in interleukin 17 AF genes
KO	knockout
l	liter
LN	lymph node
mAB	monoclonal antibody
M.catarrhalis	Moraxella catarrhalis
ME	mercaptoethanol
mg	milligramm
MHC	major histocompability complex
min	minutes
ml	milliliter
mM	millimolar
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
Pg	picogramm
R	receptor
rm	recombinant murine
RNA	ribonucleic acid
RNase	ribonuclease
RORγt	RAR related orphan receptor γt
RT	room temperature
TBE	Tris-Borat EDTA
T-bet	T-box transcription factor, T-box21
TCR	T cell receptor
TNF	Tumor necrosis factor
γδTCR	gamma delta T cell receptor

T_H	T helper
U	Unit
V	Volts
Vs	versus
WT	wildtype
w/v	weight per volume

1. INTRODUCTION

1. ASTHMA

1.1 EPIDEMIOLOGY

Asthma is a disease of global importance as demonstrated by statistics relating to morbidity, mortality and associated healthcare expenditures (Brusselle et al. 1994). Approximately 300 million people worldwide suffer from asthma, with increasing prevalence among people in most European countries. This trend is also evident in less developed countries and has been attributed to implementation of westernised lifestyles. Estimations predict that if this rising trend persists, an additional 100 million people will be afflicted with asthma by 2025 (Masoli, M.,et al. 2005). In the UK, approximately 5.4 million people are currently receiving treatment for asthma, representing one of the highest prevalence in the world. The incidence is higher in children and affects approximately 1 in 10, thus making this the most common chronic childhood disease in the UK, while mortality statistics indicated 1100 deaths attributed directly to asthma in 2009 (UK, A. Facts for journalists). Economic costs including hospital admissions, physician visits and pharmacological expenditures associated with the condition rank as one of the highest among chronic diseases (Bahadori, K., et al.2006)

1.2 DEFINITION AND DIAGNOSIS

Asthma is a disease with heterogeneous symptoms that can broadly be defined as a disorder of the conducting airways resulting in variable degrees of airflow obstruction that is reversible or non-reversible.

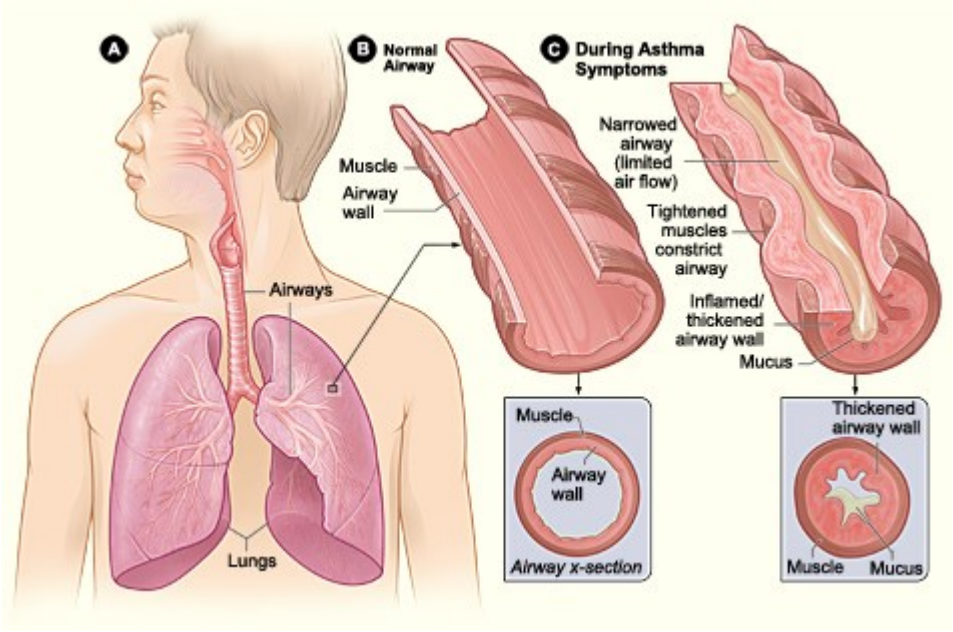


Figure 1.1 Right: healthy bronchus with clear airway; Left: inflamed, contracted smooth muscle (https://upload.wikimedia.org/wikipedia/commons/4/4a/Asthma_attack-illustration_NIH.jpg)

The pathology of asthma is characterized by airway inflammation, alterations in airway smooth muscle growth and structural changes throughout the conducting airways which contribute to airway hyper responsiveness (AHR), mucosal inflammation and airway remodeling (Patterson et al. 2014).

Asthma attacks are characterized by wheezing, cough, shortness of breath and chest tightness or any combination these and are reported in 90% of patients with asthma.

However, these symptoms alone are not sufficient for diagnosis and more information about chest symptoms during different time points of the day, cough or shortness of breath during stress or after exercise is required for a differential diagnosis.

A family history of asthma and/or other allergic disease is also a useful diagnostic marker as asthma clusters in families, as well as worsening of symptoms in response to known triggers such as pets, house-dust mites, tobacco smoke or cold/humid air (Barnes et al. 2008).

1.3 CLASSIFICATION

Asthma has been grouped under various phenotypes and broadly divided into two forms, namely allergic and non-allergic asthma (Hassan et al. 2012). Allergic asthma is the most

common form of disease and is characterised by the presence of allergen-specific IgE usually indicated by the positive skin-prick test (SPT) to the panel of common environmental allergens. In contrast, non-allergic asthma is not driven by allergen although patients can present symptoms of allergic disease such as eosinophilic infiltration in the lung. Alternatively, a number of asthmatic patients have robust neutrophilic responses in the airways which is indicative of a more severe, steroid-resistant asthma phenotype or viral-induced asthma exacerbation. In the accordance with these subsets, *Woodruff et al.* classified two unique molecular phenotypes based on the extent of Th2 inflammation; groups were defined as 'Th2-high' and 'Th2 low' with the latter known as poor responders to current asthma therapies (Woodruff et al. 2016).

1.4 DISEASE MODELS OF ALLERGEN –DRIVEN ASTHMA

Asthma pathophysiology is associated with airway inflammation; however multiple immune pathways are implicated in the activation of inflammatory components that bring about disease symptoms. Most of the research on cellular components and mechanisms of asthma has been focused on allergic asthma induced by environmental allergens. These findings are largely derived from studies of allergen-challenged atopic patients and analyses of biopsies, bronchial alveolar lavage (BAL), sputum and serum and *ex vivo* studies of clinical samples.
In addition, murine models of allergic airway inflammation were used to investigate the role of cellular and molecular components in vivo: genetically modified mouse strains, mono-clonal antibodies that block a singular component of the immune system or local gene overexpression are tools in asthma research. The most common mouse model of acute allergic airway inflammation is based on systemic sensitisation with Ovalbumin (OVA) as surrogate allergen together with an adjuvant aluminium hydroxide (alum). This is followed by multiple allergen challenges of the airways over 1-2 weeks, although variation of this treatment regime has been described including the route of delivery of the sensitising allergen and the number of challenges administered (Conrad et al. 2009). Other allergens that are commonly used include complex aeroallergens, e.g. cockroach extract, ragweed or house dust mite (HDM) which is utilised in this study. House dust mite is a common cause of allergic symptoms and asthma worldwide. The mites gut contains potent digestive enzymes (proteases) that persist in their feces and exoskeleton which are major inducers of allergic reactions such as wheezing and allergic reactions (Subramaniam et al. 2016) .

The house dust mite (Dermatophagoides pteronyssinus) are microscopic organisms that thrive in warm and humid houses with lots of human and animal skin. The protein substances that are excreted of the mites are inhaled or touch the skin upon which the body reacts with IgE antibody production. On following exposures, IgE antibodies can cause the release of histamine that leads to swelling and irritation of the upper respiratory passages.

2 ASTHMA EXACERBATION

2.1 EPIDEMIOLOGY AND CLINICAL CONSEQUENCES OF EXACERBATION

Asthma exacerbations are more common in female than in male patients, and the higher prevalence of asthma in adult women contrasts with the higher prevalence of asthma in male children (Bjornson und Mitchell et al. 2000).

Loss of control of symptoms can result in an exaggerated lower airway response to environmental triggers which leads to acute exacerbation of the disease (Johnston et al. 2007). Allergens, environmental pollutants, medication, cold/humid air, exercise and occupational irritants have been identified as triggers, however virus infections account for the large majority of severe episodes. Respiratory viruses (RVs) such as respiratory syncytial virus (RSV) are the most frequently detected viruses during exacerbations but also other viruses such as coronavirus, adenovirus, bocavirus, influenza and parainfluenza. It has been also shown that bacterial pathogens, such as *H. influenza*, *S. pneumoniae, P. aeruginosa and M. catarrhalis* increase the secretion of mucus, which is found in infected asthma or COPD patients by characteristic goblet cell hyperplasia and often result in exacerbation of the disease (Bisgaard et al. 2002). Further, the group of Bisgaard has shown that colonization of the hypopharyngeal region of neonates with *S.pneumoniae, H.influenzae*, or *M.catarhalis* or a combination of these organisms leads to increased risk for recurrent wheeze and asthma early in life.

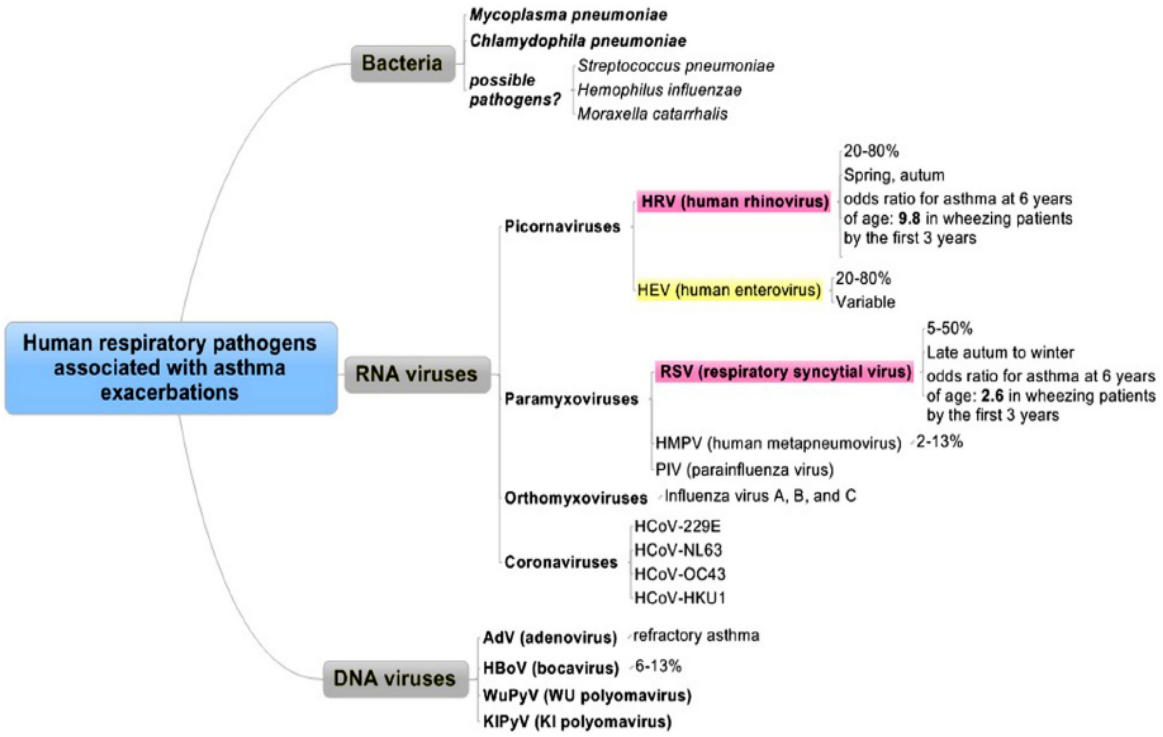


Figure 1.3 ***Human respiratory pathogens associated with asthma exacerbations.***

Exacerbations are often events in the clinical course of COPD, associated with significant mortality. Exacerbation of COPD increased the percentage of mortality in patients from 8% to 23% after 1 year of follow up. Exacerbations are correlated with accelerated loss of lung function and quality of life and increased healthcare costs (Seemungal und Wedzicha et al. 2015).

2.2 INFECTION AND ALLERGIC ASTHMA

A fundamental question which *Green el. et* has asked is why infections that usually manifest as a self-limiting mild disease of upper airways in healthy individuals cause severe lower respiratory tract infections and initiate asthma exacerbations in individuals (Green et al. 2002)

Studies have highlighted a strong association between atopy, allergen exposure and respiratory bacterial infections with an increased risk of exacerbations of asthma in children and adults (Bisgaard et al. 2007a). In addition, research indicates that atopic sensitisation and respiratory viral infections in early life may interact to increase the risk of asthma inception and development. Notably, Bisgaard et al. evaluated hypopharyngeal samples of a

community-based cohort of 321 neonates at 1 month of age of. 21% of the infants were colonized with *M.catarrhalis, S.pneumoniae, H.influenzae,* or a combination of these organisms, and bacterial infection was significantly associated with persistent wheeze, or acute severe exacerbation of wheeze. Blood eosinophils counts and total IgE at the age of 4 years were significantly increased in children neonatally colonized with a single bacterium or several microbes at the same time. Interestingly, specific IgE was not significantly affected in these children. Asthma was significantly increased and the reversibility of airway resistance after $ß_2$-agonist administration at 5 years and was decreased in colonized children as compared to non-colonized children (Bisgaard et al. 2007a).

3. *M. CATARRHALIS* INFECTION

M. catarrhalis is a gram-negative, aerobic, oxidase-positive diplococcus bacterium that was first described in 1896 (Frosch et al. 1896). The organism is also known as *Micrococcus catarrhalis, Neisseria catarrhalis,* and *Branhamella catarrhalis* (Berk et al. 1990). Currently, it is considered to belong to the subgenus *Branhamella* of the genus *Moraxella*. Previously, *M.catarrhalis* was associated with no significant pathogenic consequences and were considered as a saprophyte of the upper respiratory tract (Gordon J.E et al. 1921).

Until recently, the complex mechanisms of colonization and pathogenesis of *M.catarrhalis* were unknown due to the limited research activities. However, research efforts have increased over the past decade and various bacterial components have been isolated, characterized and identified as possible vaccine antigens.

After years of confusion and misidentification, *M.catarrhalis* is receiving attention as a pathogen that causes morbidity and mortality. The bacterium often causes infections in young children, is frequently associated with respiratory tract infections in adults causing lung disease and in rare cases can even cause systemic infections. Furthermore, the resistance to antibiotics suggests that the incidence of these infections may continue to rise (Karalus et al. 2000).

Most studies of the immune response against human infection with *M.catarrhalis* focused primarily on systemic immune responses. However, the bacterium is primarily responsible for local infections of the mucosal surfaces. Therefore, future studies that evaluate human mucosal immunity may identify responses that are critical for prevention of colonization or the resolution of infections caused by *M.catarrhalis* (Karalus et al.2000).

3.1 *M. CATARRHALS* IN RESPIRATORY EPITHELIAL CELLS

M. catarrhalis is a mucosal pathogen that may cause childhood otitis media, exacerbate asthma and trigger chronic obstructive lung disease (COPD). *M.catarrhalis* can invade bronchial epithelial cells, type II pneumocytes and primary small airway epithelial cells. *M.catarrhalis i*nvasion is dependent on cellular microfilaments and bacterial viability. Invasion of lung epithelial cells results in colonization and infection of the respiratory tract despite recognition by cell surface Toll-like receptor 2 (TLR2) and the intracellular surveillance molecule NOD1 (Seybold et al. 2007). It has been shown that *M.catarrhalis* adheres to epithelial cells of different mucosal tissues such as the nasopharynx and lungs and consequently, strong adhesion to epithelial cells is considered an important virulence trait of *M.catarrhalis*. Upon epithelial cell adhesion, bacteria express various pro-inflammatory genes, including those encoding for tumor necrosis factor-α (TNF-α), Interleukin Il-1ß, IL-6 and IL-17 and chemokines CXCL1, CXCL2 and CCL20. These genes are known to be induced during acute infection by many bacterial pathogens and serve as a general alarm signal to initiate a strong immune response by the host. (Stefan P. 2010 ; Brusselle et al. 1994).

4. THE IMMUNOLOGY OF ASTHMA

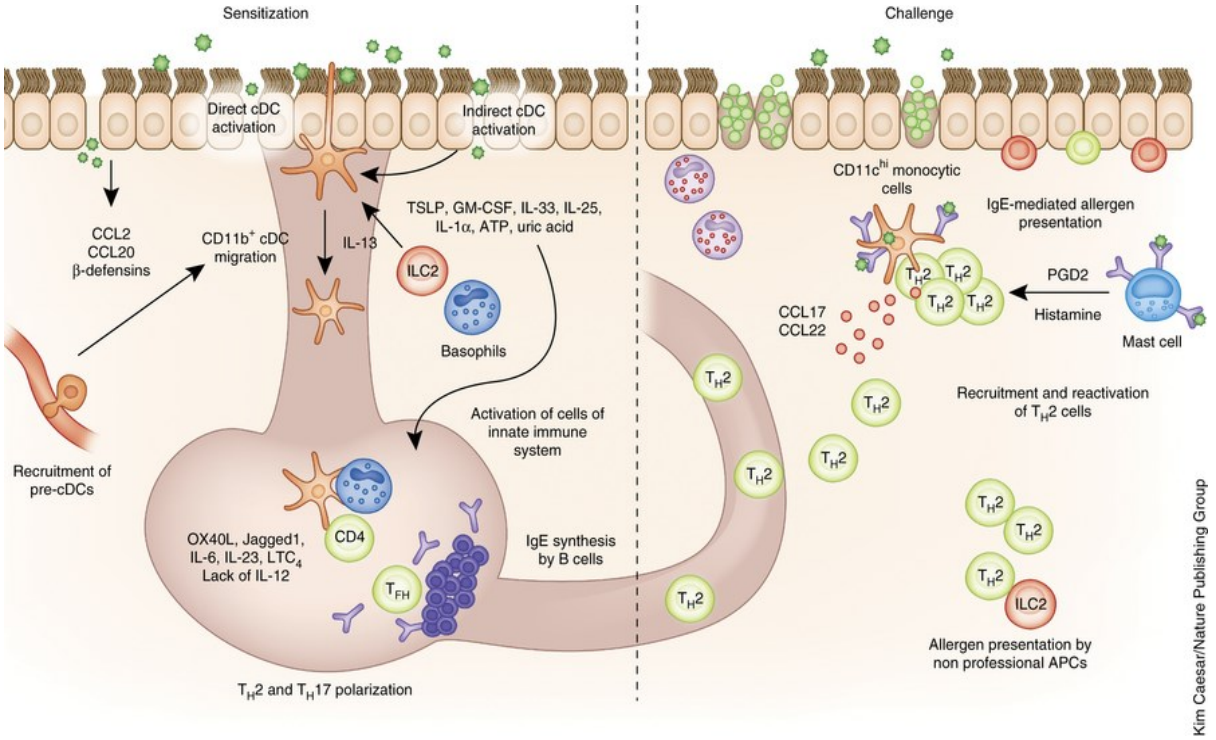


Figure 1.2 ***Left: sensitization phase; Right: challenge phase of asthma***

Direct and indirect cDCs and epithelial cells express pattern-recognition receptors and can be activated directly by allergens. In response to allergens, lung epithelial cells produce chemokines such as CCL2 and CCL20 that attract immature pre-cDCs. Activated epithelial cells produce cytokines (IL-1α, GM-CSF, IL-25, IL-33 and TSLP) and danger signals (ATP and uric acid) that favor the maturation of CD11b+ cDCs Activated lung CD11b+ cDCs then migrate to the draining mediastinal lymph nodes, where they induce TH2 and TH17 responses. Migration is stimulated by IL-13 produced from ILC2 cells. Some helper T cells start producing IL-21 and adopt a follicular helper T cells to induce IgE in B lymphocytes. In the lymph nodes, DCs receive help from basophils to activate TH2 responses. ***Taken from/modified :*** *Bart N Lambrecht, nature immunology , 2014*

Asthma is a very common disease that affects many people, men and women, young and old, worldwide. The major hallmark for asthma was T-helper type 2 (Th2) cell for a very long time. However, recent discoveries show that it is not always the case; also neutrophils which are IL-17 dependent contribute to the inflammation. They are usually controlled by T-helper type 17 (Th17) cells or by a newly discovered cell subset conferring the innate lymphoid cells type 3 (ILC-3) which also secrete IL-17 (Lambrecht and Hammad et al.2014).

One of the early steps in allergic sensitisation is the generation of antigen-specific T cell responses, which are initiated by antigen-presenting dendritic cells (DC) (Goke et al. 2001).

Dendritic cells survey the lungs for invading pathogens and inhaled antigens in a DC-network located under the airway epithelium. Epithelial cells provide adjuvant signals in response to antigens that direct the migration and maturation state of DCs and T cells (Thornton, E.E. et al. 2012). DCs then migrate to the draining lymph node to present antigen and costimulatory signals to the T cells, which then in turn migrate to the airways to secrete cytokines and other mediators. These mediators direct the asthmatic response in the lung, for example IL-5 which promotes airway eosinophilia whereas IL-17 recruits neutrophils to the lung. The magnitude of both acute and chronic asthma attacks correlates with the number of eosinophils and neutrophils present in the lung and with the increase in bronchial hyper- responsiveness. From biopsy studies, it is known that infiltrating eosinophils degranulate at the subepithelial site, but also deeper in the interstitium (Dooper et al. 1993). Presumably, toxic eosinophil derived products such as eosinophilic cationic protein and major basic protein are able to induce shedding of epithelial cells, as seen in adults with asthma, where sloughing of the epithelium has occurred. IL-13 seems to be involved in goblet cell metaplasia and mucus production (Barrett and Austen 2009). In addition, allergen-specific, long-lived memory T cells are generated and react immediately and strong upon repetitive contact with an immunological known antigen.

4.1 T HELPER CELLS AND ASTHMA

Over the last years T helper 2 (TH2) cells, a subpopulation of $CD4^+$ T cells emerged to play an important role in asthma pathogenesis. By releasing a number of typical cytokines, these cells direct a number of inflammatory events that lead to the formation of the disease. The major cytokines induced in asthmatic patients are IL-4, IL-5 and IL-13 (Luger et al. 2009).
IL-4 upregulates the fate-determining transcription factor GATA-3 in naïve T helper cells, which in turn is essential for the initial differentiation and expansion of allergen-specific Th2 cells.
Furthermore, IL-4 can also play a significant role in triggering the basis for IgE-mediated allergic reactions. Beside its effects on differentiating B cells, IL-5 can work on eosinophils by effecting their recruitment, activation, differentiation and survival in the periphery suggesting that this cytokine plays a central factor in regulating airway eosinophilia in asthma (Rosenberg et al. 2007). While, IL-4 and IL-5 mainly acts on immune cells, IL-13 can act on airway epithelial cells and smooth muscle cells, where is mediates mucus production,

subepithelial fibrosis, and the development of AHR (Wills-Karp 2004). By secreting these three cytokines, Th2 cells exert on asthma pathogenesis on the level of T cells, B cells, and structural cells. It has been further studied in mouse models of experimental asthma the importance of these factors and demonstrated that allergic airway inflammation or AHR could not be induced in mice deficient for IL-4 , IL-13 nor animals lacking the IL-5 receptor (Brusselle et al. 1994). Although these results were clear and promising, the clinical approaches towards asthma therapy by neutralizing IL-4, IL-5 and IL-13 did not progress to clinical trials (Wenzel et al. 2007).

Additionally, over the last few years Th9 and Th17 T helper subtypes were identified, which are also involved in asthma pathogenesis. The participation of Th17 cells in asthma has been first shown in 2001 revealing high amounts of IL-17 in plasma samples and increased IL-17 mRNA levels in the pulmonary tissue of asthmatic patients (Wong et al. 2001). In 2008, this new T helper subset was identified as disease-mediating cells. It has been demonstrated that patients with severe asthma have highly activated Th17 cells in biopsies. In fact, these cells represented up to 20% of all infiltrating lymphocytes (Pene et al. 2008). These findings suggest that Th17 cells play a crucial role in the development of a severe asthma phenotype. Further studies in murine asthma model supported that these cells are glucocorticoid resistant (McKinley et al. 2008). Taken together, Th17 cells are key players of inducing allergic immune responses and are highly resistant against common treatment strategies with steroids.

Although Th1 cells have shown to act as opponents to Th2 cells which counteract ongoing allergic immune responses, few studies also demonstrate a participation of interferon γ (IFNγ) in the development of AHR (Sel et al. 2007 and Cohn 1998).

5. INNATE IMMUNITY

Innate immunity is the first line of defence against infections. In many instances, innate immunity can eliminate microbes before the adaptive immune response is able to respond. Cells of the innate immune system comprise granulocytes, macrophages, natural killer cells, γδ cells and innate lymphoid cells (ILCs).

In contrast, the evolutionary younger adaptive immune system allows a highly specific and efficient response to selected pathogenic structures. This intervention needs training and a tight regulation to prevent unnecessary damage (Janeway CA 2002). In case of reinfection, the immunological memory of the adaptive immune system enables a much faster and more

efficient response. The function of the adaptive immune system is mainly based on the activity of T and B cells and their interaction. Both are able to specifically recognise different pathogens via highly selective T or B cell receptors on their surfaces, which are generated by somatic rearrangement of the corresponding genes (Medzhitov R, and CA Janeway 2000).
The innate immune system consists of cellular and soluble components, that recognize microbes and initiates responses that lead to their elimination. The principal effector cells of innate immunity are neutrophils, mononuclear phagocytes and natural killer (NK) cells (Biron et al. 1999) . These cells attack microbes that have reached epithelial barriers in order to invade the host tissue. Each of these cell types play a distinct role in the response to microbes. Notably, macrophages and NK cells secret cytokines that activate phagocytes and stimulate a sequelae of soluble mediators and cellular reactions, leading to inflammation. Inflammation consists of recruitment and activation of leukocytes and several plasma proteins into the site of infection and elimination of infectious agents (Mayer-Scholl et al. 2004).
Phagocytes, including neutrophils and macrophages are cells whose primary function is to identify, ingest and destroy microbes. Neutrophils, also called polymorphnuclear leukocytes, are the most abundant population of circulating white blood cells and mediate the earliest phases of inflammatory responses. The nucleus of a neutrophil is segmented into three to five connected lobules. Neutrophils are produced in the bone marrow and arise from a common lineage with mononuclear phagocytes (Mayer-Scholl et al. 2004).
Macrophages and their circulating precursors, called monocytes, play a central role in innate and adaptive immunity and are important effector cells for the elimination of microbes. Bacteria taken up by macrophages reach the phagosome which will then fuse with the lysosome resulting in acidification of phagolysosome which usually kills the bacteria (Underhill und Ozinsky et al. 2002).

5.1 RECRUITMENT OF LEUKOCYTES TO SITES OF INFECTION

The early local reaction of innate immunity is the inflammatory response, in which leukocytes are recruited to the site of antigen and activated to eradicate the respiratory infection. The function of inflammation is to combat invading microbes or eliminate antigen by bringing activated effector cells into contact with foreign antigens in tissues. Inflammation is initiated by cytokines, especially IL-1 and TNF, chemokines that act on endothelial cells to recruit and activate various types of immune cells.

The composition of inflammatory leukocytes within tissues changes with time from neutrophil rich to mononuclear cell rich, which is a reflection of both, change in the recruitment of specific cell types and short life span of neutrophils. Macrophages that are recruited to sites of infection are activated by microbial products and by NK cell-derived IFNγ to phagocytose and to kill microbes. During respiratory inflammation, a variety of systemic cytokines are produced that enhance the ability of the innate immune system to clear the foreign antigen and which in rare cases may induce systemic tissue injury or death. These changes are mediated by the endocrine actions of cytokines and are collectively described as the acute phase response or the systemic inflammatory response syndrome (Annual Review of Immunology 2002).

5.2 TUMOR NECROSIS FACTOR (TNF)

5.2.1 ROLE OF TNF-α IN INFECTIONS AND IN ASTHMA

TNF-α is the most widely studied pleiotropic cytokine of the TNF superfamily. TNF- α is an important cytokine of the innate immune response, which plays a key role in the immediate host defence against invading microorganisms before activation of the adaptive immune system (Locksley RM et al. 2001). It is principally produced by macrophages in response to activation of membrane-bound pattern-recognition molecules, such as Toll like receptors, which detect common bacterial cell-surface products, such as LPSs, lipoproteins or flagellin. TNF-α is produced by several cells, including monocytes, dendritic cells, B cells, $CD4^+$ T cells, neutrophils, mast cells and eosinophils, and stromal cells, ie, fibroblasts, epithelial cells, and smooth muscle cells. TNF-α is initially produced as a biologically active, 26-kd, membrane-anchored precursor protein (membrane TNF-α), which is subsequently cleaved by TNF-α converting enzyme to release a 17-kd protein. This protein forms biologically active homodimers that act on the ubiquitously expressed TNF-α receptors 1 and 2. This receptor-ligand interaction causes intracellular signalling without internalization of the complex, leading to phosphorylation of IκBα and thus activation of the nuclear factor kB (p50-p65) heterodimer, which then interacts with the DNA chromatin structure to increase transcription of proinflammatory genes, such as *IL1ß, IL6, IL8* and *TNFα* itself. The response to TNF-

α activation is balanced by shedding of the extracellular domain of the TNF-α receptors (Brightling et al. 2008). The primary role of TNF is the regulation of immune cells. It has been discovered that TNF is identified as a mediator of endotoxin poisoning, and plays a role in septic shock. In these acute cases, monoclonal anti-TNF antibodies are of high therapeutic value (Thavarajah et al. 2009a).

TNF plays a key role in response to infection. In the response to bacteria, TNFR1 mediates the activation, recruitment, and functional efficacy of immune cells and the formation of granuloma. Both TNFR1 and TNFR2 play a role not only in the control of viral and protozoal infections via apoptosis, but also for the pathology associated with these infections. TNF mediates numerous biological effects, including fever, shock, tissue inflammation and increase in acute phase proteins. TNF targeted therapy provides disease-modifying treatment and relief to patients suffering from rheumatoid arthritis, inflammatory bowel disease and other autoimmune condition. A growing number of applications are being explored, including treatment of refractory asthma. Treatment of this severe form of asthma however has been accompanied by a growing number of reported side effects, with reactivation of tuberculosis which belongs to one of the most severe unwanted side effects.

Thus, the beneficial effects may be counteracted by pulmonary side effects including infections, pulmonary nodules, chronic fibrosis and exacerbation of lung disease. It is still questionable if discontinuing TNF-target therapy via the side effects would be a future target. (Thavarajah et al. 2009b).

The contribution of TNF-α to inflammatory responses in asthmatic airways was supported by observations that TNF-α mRNA and protein levels were increased in the airways of patients suffering from asthma (Ying et al. 1991b). Importantly, inhalation of recombinant TNF-α to normal individuals led to the development of AHR and airway neutrophilia (Thomas et al. 1995a). The mechanisms driving TNF-α induced AHR have not been fully understood. AHR could be caused by a direct effect of TNF-α on airway smooth muscle (ASM), or indirectly by the release of the cysteinyl leukotrienes C4 and D4 (Huber et al. 1988). In addition to its effects on AHR, TNF-α is a chemoattractant for neutrophils and eosinophils, increases the cytotoxic effect of eosinophils on endothelial cells, is involved in activation of T cells, and increases epithelial expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM 1) and vascular cell adhesion molecule (VCAM).

In general, TNF-α has been related to severe refractory asthma via several properties, including recruitment of neutrophils, induction of glucocorticoid resistance, myocyte

proliferation, and stimulation of fibroblast growth and maturation into myofibroblasts by promoting TGF-ß expression (Desmouliere et al. 1993; Sullivan et al. 2005).

In addition to promoting airway inflammation and AHR, TNF-α might play a central role in airway remodelling in severe refractory asthma

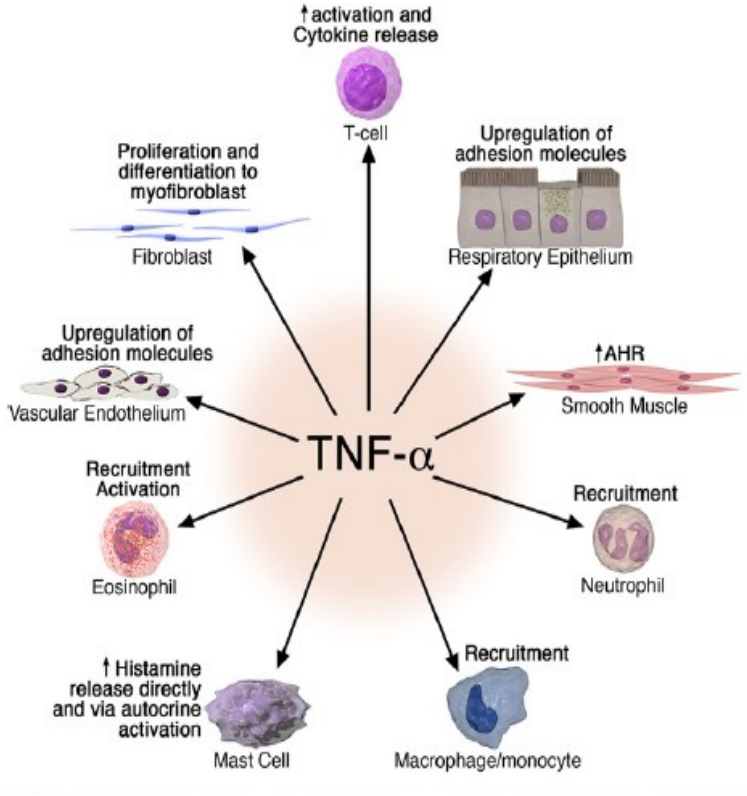


Figure 1.4 Role of TNF-α in the pathogenesis of asthma

TNF-α has many effects on the respiratory system. It can cause the recruitment of proinflammatory cells and affects the airway remodelling and induces typical asthma symptoms. Inhaled recombinant human TNF-a has been demonstrated to cause a decrease in forced expiratory volume and increased neutrophil and eosinophil recruitment. (Christophor Brightling, mike Berry, J allergy Clin Immunol 2008)

5.3 INTERLEUKIN 6 IN RESPIRATORY INFECTION AND ASTHMA

Interleukin 6 (IL-6) has a broad effect on many cells including those of the immune system and often displays hormone-like characteristics that affect homeostatic processes. IL-6 is produced by innate immune cells such as macrophages, dendritic cells, mast cells, neutrophils and by some CD4 effector T cells which stimulate immune response during infection or tissue damage leading to inflammation. It could be also secreted by non- leukocytes such as

endothelial cells, epithelial cells and fibroblasts. IL-6 is responsible for stimulating acute phase protein synthesis and supports the growth of B cells and is antagonistic to regulatory cells. Together with colony-stimulating factors, IL-6 also stimulates the production of neutrophils from bone marrow progenitors. In adaptive immunity, IL-6 can stimulate the growth of B lymphocytes that are differentiated into antibody producers. IL-6 has context-dependent pro- and anti-inflammatory properties and is regarded as a prominent target for clinical intervention. IL-6 is often induces together with the pro-inflammatory cytokines TNF-α and IL-1 in many inflammatory states and have been recognized as targets of therapeutic intervention. Circulating IL-6 plays an important role in the induction of acute phase reactions. In acute inflammatory responses *Xing el. et.* has revealed that endogenous IL-6 plays a crucial anti-inflammatory role in both local and systemic acute inflammatory responses by controlling the level of proinflammatory, but not anti-inflammatory cytokines and that these anti-inflammatory activities by IL-6 cannot be compensated for by IL-10 or other IL-6 family members (Xing et al. 1997). Moreover, it is evident that IL-6 is not only a proinflammatory marker, but also an active factor that contributes to the pathogenesis of different inflammatory diseases such as rheumatoid arthritis. High levels of IL-6 in serum have been found in asthmatic patients (Yokoyama A et al. 1995). An important question to be addressed is whether the presence of IL-6 in the lung is associated with altered lung function. In obese asthmatic patients, the increased levels of IL-6 in serum also correlated with impaired lung function (Dixon AE et al. 2006). Together, it is indicated that the presence of IL-6 in the lung airways correlates with an impaired lung function in different subsets of asthmatic patients, and suggests that IL-6 can be directly involved in the pathogenesis of asthma.

6. ADAPTIVE IMMUNITY

When naïve T cells are activated in the presence of IL-4, it induces T cell differentiation into the Th2 subset, which then generates large amounts of IL5, IL-4 and IL-13. These cytokines will activate eosinophils, mast cells and basophils which plays an important role in allergic reactions. Additionally, Th2 cytokines promote B cells to produce IgA and IgE isoforms of antigen specific antibody. IL-4 and transforming growth factor (TGF)-ß results in the generation of Th9 which will produce IL-4 and IL-13 similar to Th2 cells as well as IL-9. Both Th2 and Th9 subsets are upregulated by the activation of GATA-3. In response to

extracellular bacteria, immune cells will generate large amounts of both TGF-ß and IL-6 an additional stimulation of IL-21 and IL-23 will differentiate the naïve T cells into Th17 cells under the differential control of a transcription factor called RORγT. This T cell subset will then produce large amounts of IL-17 which can activate neutrophils to kill many bacteria and fungi. Not all T cell differentiation is pathogenic specific. Some T cell differentiation occurs to support immune functions to all infection responses. In response to pathogens IL-21 and IL-27 are generated, naïve T cells via the induction of transcription factor Bcl-6 home to B cell follicles in SLOs. Once there Tfh cells express cytokines and costimulatory molecules to assist in the germinal centre reaction of B cells.

The danger of immunopathology is characterized by persistent immune responses which produces a regulatory subset of T cells (Tregs) that dampens the inflammatory processes. Tregs can be produced directly from thymic selection (natural Tregs) as well as differentiated (induced Tregs) unlike other T cell subtypes under the influence of environmental factors such as TGF-ß and retinoic acid. The suppressive activity of Tregs is mediated by the active expression of the transcription factor FoxP3.

Finally it is important to note the immunological diseases that occurs when any of these processes occur in an unyielding or in the absence of a traditional immunological/pathogenic trigger. The role of Th1 and Th17 has been elucidated in multiple autoimmune diseases such as type 1 diabetes and multiple sclerosis (Komiyama et al. 2006). Additionally the robust of Th2/Th9 responses has been linked to allergy and asthma. Tissue fibrosis and loss of functional organ architecture is resulted via unresolved Th2 and Th17inflammation (Puel et al. 2010). Collectively, the appropriate immune homeostasis for the host depends on the regulation of activation and immune suppression.

6.1 INTERLEUKIN 17A

Interleukin 17 is a pro-inflammatory cytokine induced by IL-23 and produced by T-helper cells. The function of IL-17 is to bind to a type I cell surface receptor called IL-17R. IL-17 mediates the delay type reactions by increasing chemokine production in various tissues. Signaling from IL-17 recruits neutrophils and monocytes to the site of inflammation in response to invasion by pathogens. The most important role of IL-17 is the involvement in inducing and mediating proinflammatory cytokines. IL-17 has been described to act synergistically with TNF and IL-1 in promoting inflammation and the production of other

cytokines such as IL-6, G-CSF, TGF-ß which has many functions, such as airway remodeling. IL-17 is able also to induce the production of chemokines (including IL-8, GRO-α and MCP-1) which attracts other cells including neutrophils but not eosinophils. IL-17 function is also essential to a subset of CD4+ T-Cells called T helper cells (T_h17) cells.

Interleukin 17 has six members in the (IL-17) cytokine family, including IL-17A, IL-17B, IL17C, IL17D, IL17E and IL-17F. IL-17A and IL-17F are well understood in the biological function and regulation and have a similar protein structure. They play protective roles in host defense against certain pathogens at epithelial and mucosal barriers. Aujela SJ el.et. has showed that IL-17A and IL-17F are important for the clearance of the extracellular bacteria *Kelebsiella pneumoniae*, which infects the lung (Aujla et al. 2008). Ishigame et al. has also demonstrated that when these both cytokines are defective the animals become more sensitive to the S.aureus which demonstrates the largely redundant function of these cytokines (Ishigame et al. 2009). As already mentioned IL-17F has been shown to have a proinflammatory role in asthma which has been well characterized both in vitro and in vivo. IL-17F is clearly expressed in the airways of asthmatics and its expression level correlates with disease severity. Moreover, a coding region variant (H161R) of the IL-17F gene is inversely associated with asthma and encodes an antagonist for the wild-type IL-17F. IL-I7F activates the MAP kinase and utilizes IL-17RA and IL-17RC as its receptor. IL-17F is produced from several cell types such as Th17 cells, basophils and mast cells and shows a wide tissue expression pattern including the lung (Kawaguchi et al. 2009) . has showed that overexpression of IL-17F gene in the airway of mice is associated with airway neutrophilia, induction of many cytokines, an increase in airway hyperactivity and mucus hypersecretion (Kawaguchi et al. 2009). Collectively, IL-17F plays an important role in allergic airway inflammation and have important therapeutic implications in asthma.

Although asthma is classically associated with eosinophilia and Th2 cytokines, some patients show a neutrophil-predominant disease with an absence of Th2 cytokines. In particular, patients with more severe forms of asthma seem to have neutrophilic inflammation with less reversible airway obstruction and a mixed Th1 and Th17 cytokine milieu (McKinley et al. 2008). The role of IL-17 and Th17 cells in allergic asthma has been fully elucidated, as this cytokine has been shown to be sometimes involved in protection and in other cases (most cases) in the development of inflammation or immune pathology . Thus, IL-17 can be pro or anti-inflammatory cytokine depending on the time point of neutralization or administration of IL-17 for example, IL-17A plays a protective role in asthma only during the challenge phase

(Schnyder-Candrian et al. 2006). Another study showed that high exposure to diesel exhaust particles is associated with both exacerbated asthma symptoms and increased serum concentrations of IL-17A in children with atopic asthma (Brandt et al. 2013). Severe forms of asthma are characterized by increased airway remodelling. IL-17A contributes to remodelling by promoting fibroblast proliferation and by counteracting the anti-inflammatory role of Tregs in some experimental asthma models driven by HDM or ozone. (Zhou et al. 2015). In mice and humans, IL-17 can also cause direct contraction of bronchial smooth muscle cells and thus cause bronchial hyperreactivity (BHR) in the absence of neutrophilic inflammation. It remains unclear where in asthmatic airways, IL-17A and IL-17F are produced. There is also a complex interaction between Th17 cell driven asthma and tumor-necrosis factor (TNF). Pulmonary and systemic amounts of TNF are increased in patients with severe steroid-resistant asthma, although some studies have not confirmed this (70-79 the immunology of asthma). In an adoptive transfer model of Th17 OVA-specific T cells, neutralization of TNF led to reduced neutrophilic influx in the lung tissue and airspaces which was associated with improved lung function parameters such as lung compliance but not airway hyper-resistance. In clinical trials, the results of TNF blockade have also been variable (Berry et al. 2006a). Thus, it is still unclear whether TNF blockade in IL-17-rich neutrophilic asthma improves also steroid responsiveness.

6.2 TH1 IN INFECTIONS AND ASTHMA

Th1 cells, typically mediate antiviral, antibacterial and in some cases also antifungal immune responses, by activating macrophages through the production of IFNγ, whereas cytokines secreted by Th2 cells are responsible for immunity against worms and the production of IgG and IgE, the recruitment of eosinophils and clearance of extracellular parasites.

It has been well accepted that the decrease in microbiological burden which started in the beginning of the 20th century caused an increase in atopic diseases and particularly asthma. This observation was later stated as the hygiene hypothesis (Strieter et al. 2002). Decreased exposure to pathogens during the early postnatal development skews the immune system towards Th2 response. Recently, this hypothesis was complemented by the discovery of different regulatory T cells (Tregs) which play a crucial role in dampening Th2 driven inflammation (Rautava et al. 2004). Commensal bacteria of the airways and the gut influence

the Th1/Th2 balance in the lung via the induction of Tregs (Atarashi und Honda 2011), also via secretion of short chain fatty acid (Wong 2006).

The role of Th1 cells in asthma remains still controversial. Moreover, studies could not show airway hyperresponsiveness after adoptive transfer of OVA specific Th1 cells, other groups were able to induce AHR (Shaw et al. 2005). However, Th1 and Th2 responses may even act in combination to augment each other's activity to induce airway inflammation and AHR (Ford et al. 2001). It has been demonstrated in a mouse model of allergic asthma that Th1 and Th2 cells were recruited together to the airway after sensitization with the model antigen OVA. However, Th1 cells were not only predominantly found early after the challenge their presence seems moreover to be a prerequisite for the recruitment of Th2 cells to the airways (Randolph et al. 1999).

Observations were different concerning the function of Th1 cells in mucus hypersecretion, as one feature of bronchial asthma. Cohn et al showed that the adoptive transfer of antigen-specific Th1 cells induced much less mucus than Th2 cells, mucus hypersecretion was totally absent in OVA challenged mice (Cohn et al. 1998).

At least the typical Th1 cytokine IFN-γ has already been shown to play a role in the development of AHR and in aggravating inflammatory events in asthma. Nevertheless, more studies are required to fully elucidate the role of Th1 cells and especially of IFN-γ in asthma pathology (Vock et al. 2010a).

2. AIMS OF THE STUDY

Bacterial infections such as *M.catarrhalis*, *Haemophilus influenza* and *streptococcus pneumonia* are main triggers of asthma exacerbations. While Th17-mediated inflammation is clearly involved in the asthmatic responses, it is unknown whether *M.catarrhalis* infection triggers pulmonary Th17 immune responses and thus exacerbates pathogenesis of this disease. Besides IL-17, we also wondered whether epithelial-derived TNF-α which has been identified as an initiator and regulator of Th17 immunity also plays a role in *M. catarrhalis* induced asthma excacerbations.

Given that bronchial epithelial cells are the primary site of *M.catarrhalis* infection, it may be hypothesised that *M.catarrhalis* infection of the airway epithelium induces TNF-α and thus provided a link between infection and Th17 driven allergic inflammation.

In this study, our aims are to:

- To study the induction of TNF-α to *M.catarrhalis* airway infection.
- To study in a mouse model of *M.catarrhalis* infection and HDM-induced allergic airway disease the effects on TNF-α and Th17 mediated immune responses.
- Define a mechanistic role for *M.catarrhalis*-induced TNF-α in Th-17 induced exacerbation of allergic airway disease.
- To determine the time point of *M.catarrhalis* infection on the augmentation of allergic airways disease (AAD).
- To study the interaction between TNF-α and Th17 in the exacerbation of allergic airway inflammation.

3. MATERIAL

3.1. ANTIBODIES

3.1.1. UNCONJUGATED

α-IL-6 PE capture beads	BD Biosciences,Sane Jose,CA
α-TNF PE capture beads	BD Biosciences
α-IL-1ß PE capture beads	BD Biosciences
α-IL-17 PE capture beads	BD Biosciences
α-IL-5 PE capture beads	BD Biosciences
α-IL-13 PE capture beads	BD Biosciences
α-IFNγ PE capture beads	BD Biosciences
α- mouse IL-6 (clone MP5-20F3)	Berlin Max Planck Institute
α-mouse TNF-α (clone MP6-XT22)	Berlin Max Planck Institute

3.1.2 CONJUGATED

α-CD4-V450(RM4-5)	BD
α-CD8a-V500 (53-6.7)	BD
α-CD16/CD32 (Fc block)	BD
α-CD25-PE-Cy7 (PC61.5)	eBioscience, San Diego, USA
α-CD11b-Phycoerythrin (PE)	eBioscience
α-CD45-APC	eBiolegend
α-CD3-FITC	BD,
α-Ly6C-V450	eBioscience
α-Ly6G-V500	Biolegend San Diego, USA
α-IL-5-PE	eBioscience
α-IL13-AlexaFluor 647	eBioscience
α-IFNγ-PerCP-Cy5.5	Biolegend
α-FoxP3-AlexaFlour 700	eBioscience
α-IL17-AlexaFlour 700	eBioscience
α-γδTCR-FITC	eBioscience
α-IL-6 PE	BD Biosciences
α-TNF PE	BD Biosciences
α-IL-1ß PE	BD Biosciences
α-IL-17 PE	BD Biosciences

α-IL-5 PE	BD Biosciences
α-IL-13 PE	BD Biosciences Sane Jose, CA
α-IFNγ PE	BD Biosciences
α-mouse IgE (biotinylated)	BD Pharmingen NJ, USA
α-mouse IgG1 (biotinylated)	BD Pharmingen NJ, USA

3.2 CHEMICALS

99,8%	Sigma-Aldrich,Steinheim
Aqua dest	Braun AG, Melsungen
Agarose	Sigma- Aldrich, Steinheim
Albumin bovine (BSA)	Sigma- Aldrich, Steinheim
Ampicillin	Sigma- Aldrich, Steinheim
ß-Mercaptoethanol (ME)	Biomol, Hamburg
BSA Albumin Fraction V	Serva Elektroph, Heidelberg
BM blue, POD Substrate	Roche, Mannheim
Brefeldin A	Sigma-Aldrich Steinheim
Chloroform 99%	J.T Baker
Clicks RPMI	Biochrom, Berlin
Collagenase D	Roche, Switzerland
Complete Protease-Inhibitor Tablets	Roche, Switzerland
DiffQuick®	Dade-Behring, Marburg
DNase I	Roche, Switzerland
DNA loading day 6×	Fermentas, Switzerland
DNA away	M&P
Dulbecco's Phosphate Buffered Saline	Biochrom , Berlin
Eosin G	Merck, Darmstadt
Erythrocyte lysis buffer	Sigma-Aldrich, Steinheim
Ethanol,absolute	Merck, Darmstadt
Ethidiumbromid 1% (1mg/ml)	Roth, Karlsruhe
Fetal Calf Serum	Greiner, Frickenhausen
Hämatoxilin II, (Gill)	Biochrom, Berlin
Ionomycin	Sigma-Aldrich, Steinheim
Isofluran	Baxter, Erlangen
Isopropanol	Roth, Karlsruhe
Ketamin 50 mg/ml	Inresa GmbH,
Non-essential amino acids	PAA, Austria
Penicillin G/Streptomycin 100× solution	Thermo Fischer Sc.
Phosphate Buffered Saline (PBS)	Biochrom, Berlin
Phorbol 12-myristate 13-actetate (PMA)	Sigma, Steinheim
Phosphate buffered saline (PBS)	Biochrom, Berlin

2-Propanol	Sigma, Steinheim
RNase away®	M&P
RNase A	Biozym Scientific GmbH, Oldendorf
Rompun 2%	Bayer, Leverkusen
Protease Inhibitor Cocktail Tablets Complete	Roche, Switzerland
Streptavidin/phosphate	Sigma, Steinheim
Sodium Phosphate (Na_2HPO_4)	Merk, Darmstadt
Sodium carbonate ($NaHCO_3$)	Merk, Darmstadt
Sulferic acid (H_2SO_4)	Merk, Darmstadt
Saponin	Sigma-Aldrich, Steinheim
Schiff's Reagent	Roth, Karlsruhe
Tween 20	Roth, Karlsruhe
TRIS_HCl	Invitrogen
Trypan blue	Gibco

3.3 KITS

IFNγ ELISA: Due Set ELISA mouse IFNg	
FoxP3-Fixation Kit	eBioscience
Rotor-Gene SYBR® Green PCR Kit	Qiagen, Hilden
RNeasy® Mini Kit(50)	Qiagen, Hilden
RNase-Free DNase Set	Qiagen, Hilden
RevertAid RT Reverse Transcription Kit	Thermo Fisher Scientific, Jdstein
Cytometric Bead Array (CBA) Mouse/Rat soluble	
Protein master Buffer Kit	BD

3.4 LABORATORY EQUIPMENT AND CONSUMABLES

AriaIII Cell Sorter	Becton Dickinson, Heidelberg
Braun Vasofix®Safety Braunüle G18 grün	Roth
Biosphere®Plus safe Seal Micro Tubes	Sarstedt, Nümbrecht
Casy® Cell Counter	Schärfe Systems
Cell culture dish (150×20 mm, 100×20 mm,)	Becton Dickinson, Heidelberg
Cell culture plate (96-, 48-, 24-well flat bottom, 96- well round bottom)	Greiner/Coster, Frickenhausen
Colombia Agar with 5% Blood	BD
CryoTube™ Vials	Thermo Fisher Scientific
Cuvettes, Gene Pulser,	

0.4cm (Nr. 165-2088)	BioRad
ELISA plates	Thermo Fisher Scientific
ELISA reader	Thermo Fisher Scientific
Filtertippes (10µl, 100µl, 1000,µl)	Starlab, Hamburg
GasPak^{TM} EZ Campy Counter system red	BD
Incubator	Heraeus, Hanau
Hamilton Pipette	Hamilton
House dust mite (HDM)	Greer, USA
Heating block TCR 200	Roth, Karlsruhe
Hypodermic needle 25G, 26G and 27G	BD Microlance
Megafuge 1.0 R/ Biofuge Pic (Rotors '2704/'3328),1700 rpm equals 470g	Heraeus, Hanau
Maxi-Sorp plates	Thermo Fisher Scientific
Microscope BX51	Olympus
Microwave	Sharp
Nanodrop ND- 1000 Spectrophotometer	PeqLab
Nylon cell stainer	BD Falcon,,NJ, USA
Nylon mesh	GE Health care
Object slide over slip	Menzel
Object slides	Menzel
PAA	Pasching
Pasteur pipette	Brand
PCR cycler	Biometra
Reaction cups, 1.5 and 2 ml	Eppendorf
Reaction tubes, 15 and 50ml	Greine
Rotor-Gene Strip Tubes & Caps	Star Lab, Hamburg
Rotor Gene 3000®	LTF
Sterile Filter, 100 mm	BD
Sterile filter, 24 mm	BD
Sephadex coloms	GE Healthcare
Sintilation counter	Life technologies
Whatman paper	
Schleicher+Schüll	

3.5 MEDIA; BUFFERS AND SOLUTIONS

Cell lysis buffer per 5×10^7 cells or 1 tail tip:

500µl TNE (see below), 7.5 µl proteinase K (10mg/ml), 25 µl pronase E (10 mg/ml) and 50 µl SDS solution (10%), freshly prepared.

Digestion medium:

RPMI 1640, 10% FCS, 1×non-essential amino acid, Pen/Strep (100 U/ml final)
, 1mg/ml Collagenase D (stock 50mg/ml, 1:50), 20 µg/ml DNase I (stock 10 mg/ml, 1:500)

ELISA blocking solution

1× PBS with 1% BSA

Coating buffer:

0.1 M $NaHCO_3$, pH 8.2

EM cell freeze medium:

80 % feeder cell medium, 10 % DMSO

FCS:

Serum was inactivated by heating at 56 °C for 40 min, cooled to room temperature (RT) and filtered sterile

HDM solution for sensitization of mice:

100µgHDM in 50µl PBS per animal

Anaesthetics (5×):

10 ml ketamine (50 mg/ml)/3.125 ml Rompun in PBS.

Restimulation medium:

3 ml of RPMI per well with 50ng/mL PMA (stock 1 mg/mL , 1:20000 dilution), 750 ng/mL ionomycin (stock 1 mg/mL, 1:1333 dilution), 5µg/ml Brefeldin A /stock 10 mg/mL, 1:2000)

RPMI culture medium:

500ml medium supplemented with 10 % FCS, 5 ml non-essential amino acids, with L-glutamine (2mM final), penicillin/streptomycin solution (100 U/ml final)

Saponin buffer:

0.2 % saponin (w/v) dissolved in PBS and 2 % FCS (v/v) added.

ELISA washing buffer:

PBS with 0.05% tween (g/v)

TNE solution:

10ml 1M Tris (pH 8.0), 20 ml 5 M NaCl, 2ml 0.5 M EDTA diluted ad 1000 ml aqua dest.. and autoclaved.

3.6 MICE AND CELLS

Mice were used for asthma and infection experiments or for organ extraction. Animals were kept under specific pathogen free conditions (SPF) in individual ventilated cages (IVC) at 20 °C and artificial light dark cycles. Food and water were provided *ad libitum.* The following inbred mouse stains and bacteria were used.

Mice

C57BL/6J mice, wildtype	Jackson laboratories
IL-17AF$^{-/-}$ mice, C57BL/6	kprovided by Prof. Prinz, Hannover

Bacteria

Moraxella catarrhalis	human isolates, provided by Prof. Mutters, Marburg

3.7 ENZYMES

DNaseI	Invitrogen
Hot start DNA polymerase	Novagen
Proteinase K	Qiagen
RNase	Biozym Scientific GmbH

3.8 OLIGONUCELOTIDES

Primers for quantitive real-time PCR:

L-32:

L-32 forward	5’ GCA AGT TCC TGG TCC ACA AT 3’
L-32 reverse	5’ GGG ATT GGT GAC TCT GAT GG 3’

mIL-17:

mIL-17 forward	5’ AAG GCA GCA GCA ATC ATC CC 3’
mIL-17 reverse	5’ GGG TCT TCA TTG CGG TGG AG 3’

IFNγ:

IFNγ forward	5’ GCT TTG CAG CTC TTC CTC AT 3’
IFNγ reverse	5’ GCA GGA TTT TCA TGT CAC CA 3’

IL-6:

IL-6 forward	5’ CCG GAG AGG AGA CTT CAC AG 3’
IL-6 reverse	5’ CAG AAT TGC CAT TGC ACA AC 3’

3.9 SOFTWARE

Adope illustrator CS3	Adobe
Cell Quest Pro	Becton Dickinson
FlowJo	Tree Star
Cell^{F}- Imaging Software	Olympus
FCAP Array^{TM} software	BD
Relevation^{TM}	Dynex
Rotor Gene Software	Qiagen

3.10 STATISTICAL ANALYSIS

Statistical calculation for asthma experiment was performed with 1 way ANOVA Analysis including the Bonferroni multiple Comparison Test. Associated data points were analysed by Grubb’s test to exclude significant outliers.
(http://www.graphpad.com/quickcalcs/Grubbs1.cfm)

3.11 DATABASES AND ONLINE TOOLS

Blast:	http://blast .ncbi.nlm.nih.gov/blast.cgi
Ensemble mouse:	http://www.ensembl.org
Primer design and analysis:	http://frodo.wi.mit.edu/primer3/

4. METHODS

4.1 CULTURE OF *MORAXELLA CATARRHALIS*

For mouse infection experiments, 25 µl of a *M. catarrhalis* frozen aliquot were plated on Columbia agar plates and incubated at 37 °C overnight. The next day, the plates were washed off and suspended in sterile PBS.
To determine the effect of infection, mice were inoculated i.n with 200 * 10^6 CFU per mouse in a final volume of 50µl of *Moraxella catarrhalis*

4.2 INFECTION OF MICE WITH *M. CATARRHALIS*

C57BL/6 mice and IL-17AF $^{-/-}$ mice were anesthetised with Ketamine/Rompun solution.
Their neck slightly overstretched and 50µl of bacteria-in-PBS-solution was applied intranasally. Mice were killed after 2hs and on days 1, 4, 7 and 12 after infection, Blood, BAL and Lung were removed prepared and analysed as described.

4.3. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is a biochemical method of detecting and quantifying antibodies or antigens in a liquid sample which bind to a surface-bound capture antibody. In a second step an antigen-specific detection antibody is bound onto the antigen that receives either another amplifying antibody which carries and enzyme such as horseradish peroxidise (HRP) or alkaline phosphatase (AKP) or is linked to the enzyme itself.
The substrate for the enzyme causes a colour change which is detectible by photometric analysis.
Systemic levels of HDM-specific IgG1 were determined by Elisa. MaxiSorp 96-well plates were coated with 50µg/ml HDM extract overnight at 4°C. samples were added the next day and incubated overnight at 4°C with the corresponding biotin-labeled anti-Ig antibody. Plates were washed and incubated with streptavidin-peroxidase for 30 min at RT.After development with substrate the reaction was sropped using H2SO4 and ODs.

4.4 SURFACE STAINING

Lungs were cut into 2–5 mm pieces and incubated for 30 min at 37°C in the incubation medium (IM) (RPMI-1640 with L Glutamine and NaHCO3, 10% FCS, 16 non-essential amino acids , 100 mg/mL streptomycin,120 mg/mL penicillin supplemented with1 mg/mL Collagenase D and 20 mg/mL DNase I. The pre-digested lungs were minced through 100 mm nylon cell strainer diluted with IM and centrifuged at 1700 rpm for 5 min. Cell pellets were resuspended in erythrocytes lysis buffer (8 g/L NH4Cl; 1 g/L KHCO3; 37,2 mg/L EDTA) and incubated at room temperature supplemented with 50 ng/mL PMA,750 ng/mL ionomycin and 10 mg/mL brefeldin A for 4 h. T-cells extracellular staining was performed in PBS with 1%FCS and presence of BD Fc Block anti-CD16/CD32 (93) and fluorophore-labelled antibodies. The following antibodies for extracellular T-cell staining was performed with anti-CD4-V450 anti-CD8a-V500 and anti-CD25-PE-Cy7; nature for 3–5 mins. Then, cells were washed with IM, centrifuged and resuspended in IM.

4.5 INTRACELLULAR CYTOKINE STAINING (ICS)

After extracellular staining, T-cells were fixed with FoxP3-Fixation Kit and permeabilized with 0,3% Saponin, 1% FCS in PBS followed by an intracellular staining in presence of BD Fc Block anti-CD16/CD32 with the following fluorophore-labelled antibodies: IFNɣ-PerCP-Cy5.5 ; IL-17-AlexaFluor 700 and FoxP3-AlexaFluor 700 ; IL-5-PE ; IL-13-AlexaFluor 647 . Every staining included respective negative and isotype controls. Fluorescence signals were acquired by flow cytometry and analyzed using FACSDivaTM software.

4.6 CYTOMETRIC BEAD ARRAY (CBA)

Cytometric Bead array (CBA) is a flow cytometry application that allows users to quantify multiple proteins simultaneously. The BD CBA system uses the broad dynamic range of fluorescence intensity so that beads can be mixed and run simultaneously in a single tube. This method significantly reduces sample requirements and time to results in comparison with traditional ELISA and Western blot techniques. CBA Flex sets make it easy to build a

multiplex by following five simple steps. The finished assay can be acquired on a variety of dual-laser flow cytometers and analysed using FCAP^{TM} Array software.

Broncho alveolar lavage (BAL) fluid and lung homogenate supernatants were tested for secreted cytokines such as IL-6, IL-1ß, TNF, IL-17 and IFNg by CBA flex assay.

96-well plates U-bottom were coated with first antibody mixture (each well 1µl capture bead antibody pro cytokine). Next 50µl of samples or standard will be added to each well and incubated for 1 hour in dark. Second antibody mix will be then added and shacked for 5 min, incubated for 2h in dark. After 2 hours centrifuge at 1200 rpm for 5 min. discard the supernatant, add 180µl wash buffer per well and measure using FACS Array Bioanalyzer.

4.7 MOUSE MODEL FOR ADJUVENT- FREE ACUTE ALLERGIC ASTHMA

The induction of allergic asthma is based on inflammatory reactions against an otherwise harmless allergen. For this model C57BL/6J wildtype and IL-17AF$^{-/-}$ mice were exposed intranasally (i.n) to house dust mite extract (HDM, 100mg in 50 µL PBS) by weekly (days 0,7,14, and 21) intranasal challenges of slightly anesthetized mice (i.p) with Ketamine/ Rompun. Two days after the last HDM challenges, mice were sacrificed and blood, bronchoalveolar lavage fluids (BAL) and lungs were collected for further analysis.

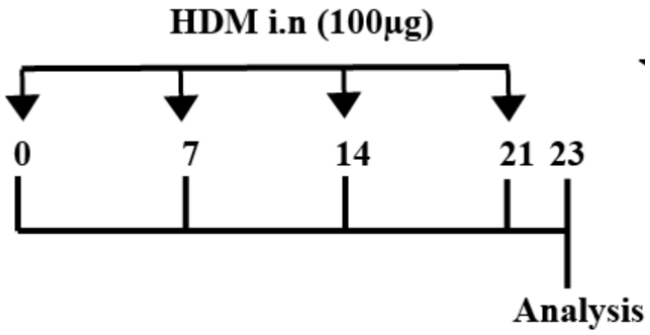


Figure 2: Challenge protocol for acute murine experimental asthma induced by house dust mite (HDM) . *Numbers indicate days of treatment and analysis.*

4.8 PREPERATION OF SERUM

Mice were killed with Isoflurane. Blood was drawn from axillary blood vessels for serum antibody analysis. After clotting (45 mins at RT), serum was separated by centrifugation at 4000 rpm for 20 minutes and adjacent quantification of cytokines by ELISA as described above. Meanwhile, serum samples were stored at -80 °C.

4.9 BRONCHOALVEOLAR LAVAGE (BAL)

After 48 hours after the last HDM/PBS application, BAL weas taken using a syringe filled with 1 mL of protease inhibitor diluted in PBS. The BAL was diluted 1:1000 for the cytospin analysis and the rest was frozen and used for the cytokine analysis.

4.10 CYTOSPIN ANALYSIS

10^5 cells were washed in cold 2% FCS-PBS twice and diluted in 100 µl of cold (PBS1% BSA). All samples were kept on ice. Normal microscope slides were used and put together with a special filter for the cytospins, all were placed into appropriate slots in the cytospin with the cardboard filters facing the center of the cytospin. In case there were few cells available, about 100 µl of cold PBS with 1% BSA aliquots were added into each well and spun for 1-2 minutes. This will serve to wet the filter and allow more cells to reach the slide. The lid of the cytospin was carefully placed over the samples and spinned at maximum speed for 1-3 minutes. The filters were removed from their slides without contacting the smears on the slides. Each slide was examined under the microscope to be sure that the cells have annealed properly. The cells should appear to have normal morphology and should be lying flat on the slide. For staining purposes, the cells should also be in a flat layer on the slide.
The slides were left to dry overnight and afterwards stained with Diff – Quick staining.

4.11 LUNG EMBEDDING

Proceeding the BAL, the left lung was disconnected from the rest of the organ close to the bifurcation of the lungs by using a thin thread. This part of the lung was frozen in liquid nitrogen and stored for RNA analysis.
The right lung was then filled and thereby fixed with 6% paraformaldehyde (PFA).
Fixation was completed by storing the lung in 6% PFA for 24 hrs. Subsequently, the lungs were embedded in 2 % agarose and cut into 6 mm thick pieces and embedded in paraffin wax. The embedding of the lungs was conducted in Marburg in the Institute of Pathology of the University Hospital Gieβen and Marburg.

4.12 PREPERATION OF LUNG SLIDES

The paraffin-embedded lungs were cut into thin slices of 3 µm thickness by using a microtome and placed onto object slides.

4.13 HAEMATOXYLIN-EOSIN- (HE-) STAINING

For examination of pathophysiological changes and lung damage induced by allergic asthma, lung slices were stained with haematoxylin and eosin. First, the samples were de-paraffinated in xylol (2* 10 mins) before rehydration in descending alcoholic concentrations (each step 2*5 mins in 100 %- , 5 mins 96 %,- and 5 mins 70 %- ethanol).
After rinsing with water, the slides were stained in haematoxylin for 3 mins. A resting step in water for 5 mins secured persistence of the blue staining in the tissue. Next, the samples were stained in eosin for 2 mins and rinsed again with water.
Afterwards the tissue was dehydrated in different alcoholic solutions (1 min in 96 %-, 2-3 mins in 100 % ethanol followed by 2x10 mins in xylol). Histomount® was used to cover the samples with cover slides.

4.14 PERIODIC-ACID SCHIFF (PAS) STAINING

Mucus production by goblet cells can be taken as a characteristic of the asthmatic lung as goblet cells are only rarely found in healthy tissue. The periodic-acid Schiff staining (PAS) makes these cells visible. Deparaffinated and rehydrated tissue slices (see section for HE staining) were incubated in 0, 5 % periodic acid for 10 mins and washed in water for another 3 mins. The samples were transferred into Schiff's reagent for 15 mins followed by 1 min incubation in haematoxylin. After a final rinsing step, the samples were dehydrated in alcoholic solutions (1 min each 70 %, 96 % and 100 % ethanol).
After final 15 mins incubation in xylol, the object slides were covered.

4.15 IMMUNOGLOBULINS

For detection of HDM-specific IgG1 and IgG2c, Maxi-Sorp plates were coated with 50µg/ml HDM extract in bicarbonate buffer (pH 9.6) overnight at 4 °C. Subsequently, coated wells were blocked with 1% w/V BSA in PBS for 2h at RT. After washing, serum samples (1:100) were incubated overnight at 4 °C, washed and incubated with the corresponding biotin-labelled anti-immunoglobulin antibody overnight at 4 °C. Plates were washed and incubated with streptavidin-peroxidase for 30 min at RT. After development with substrate, the reaction was stopped using H2SO4 and ODs were read at 450 nm.

4.16 PREPERATION OF WHOLE LUNG HOMOGENATE

M. catarrhalis infected mice were killed by a high dose of isoflurane. Lungs were perfused with PBS through the heart puncture, before lungs were removed. The organs were cut into small pieces and digested with 1 mg/ml collagenase D and 20 mg/ml DNase I in incubation medium for 30 mins at 37 °C. Tissue pieces were sieved through nylon filters and centrifuged at 1700 rpm for 5 mins. Cell pellets were resuspended in erythrocytes lysis buffer and incubated at room temperature for 3 mins. Then cells were washed with IM, centrifuged and resuspended in IM. The resulting cells was used for restimulation experiment.

4.17 LYMPHOCYTE RESTIMULATION

For ex vivo analysis, cells were restimulated for 4 hrs with 50 ng/ml PMA and 750 ng/ml ionomycin. If intracellular cytokine staining was performed, 10 µg/ml of Brefeldin A which is a Golgi blocker, to enrich the amount of intracellular cytokine and prevent secretion.

4.18 CYTOKINE DEPLETION BY ANTIBODIES

For depletion experiments, WT mice were injected intraperitoneally (i.p) with 100µg anti-IL-6 or anti TNFα or an isotype control antibody,4 hours before *M.catarrhalis* infection.

4.19 mRNA MEASURMENT BY QUANTITATIVE REALTIME PCR (QRT-PCR)

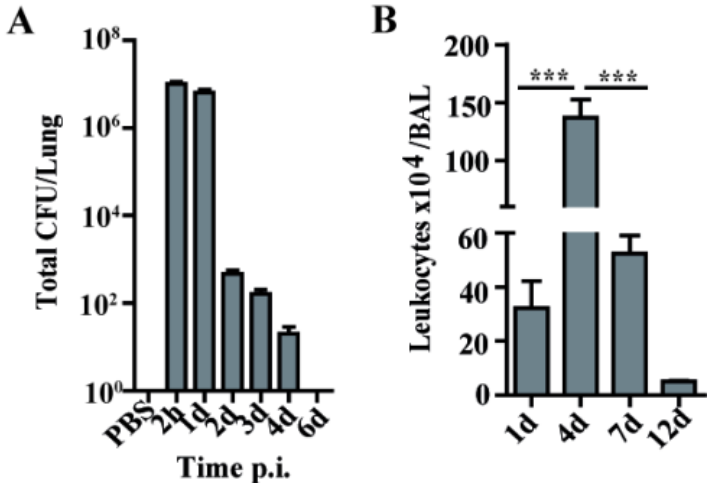
This PCR technique is used to amplify and simultaneously quantify a specific target DNA by including a fluorescent probe or dye in the reaction.
For analysis of the cytokine production of *M.catarrhalis* infected lung tissues, a Taqman® -PCR for IL-6, IFNγ and IL-17A was performed on an Rotor-gene Real-Time Analysis software 6.1 (Build 93). For RNA sample extraction, lung tissues were snap frozen and homogenization of the tissue was done by mortar and pestle (cooled to temp in liquid nitrogen bath). At the same time, transfer 600µl RLT buffer + 1% ß-Mercaptoethanol pro 25mg tissue to be homogenized .Transfer the tissue to the pestle and grind until a layer of very fine dust is all that is left, Add 70% ethanol to the homogenized tissue, followed by RNA isolation via the „RNeasy Micro Kit®" according to the manufacturers instructions. The resulting RNA content was measured by photometric analysis; cDNA synthesis was performed using the "cDNA Maxima First Strand Synthesis Kit for RT-qPCR®"with oligo-dT- Primers.
The following PCR conditions were used for the detection of IL-6, IFNγ and IL-17 expression:

The temperature profile was the following: 95°C 5 mins, 94 °C 20 secs, 45 repeats 60 °C 20 secs, 72°C 20 secs, acquiring to Cycling A(FAM). Results were normalised to L32 as a housekeeping gene by the ΔΔct method as specified by Applied Biosystems.

5. RESULTS

5.1 AIRWAY INFECTION WITH *M.CATARRHALIS* TRIGGERS LUNG INNATE AND ADAPTIVE IMMUNITY

To investigate the association between *M. catarrhalis* lung infection and allergic airway response, we first established a murine infection model and assessed the course of infection and the type of immune response triggered by *M. catarrhalis*. Two hours after intranasal inoculation, bacterial infection was fully established and bacterial titers of the lung dropped massively at day 2 p.i. and were below the detection limit at day 6, indicating that *M. catarrhalis* is rapidly cleared in WT mice (Fig.1 A). Next, we performed a kinetic of the immune response after intranasal *M. catarrhalis* infection on the absence of allergic airway disease. Total and differential cell counts of the Broncho Alveolar Lavage (BAL) demonstrated that infection induced an early and strong immigration of granulocytes (neutrophils and eosinophils) at day 4 which was followed by an influx of lymphocytes at day7 after infection (Fig.1 B, C). Accordingly, high amounts of CXCL1, CXCL10 in the BAL coincides with the prompt appearance of neutrophils and may act as a feedback loop to further attract neutrophils, NK and T cells (Fig.1 D)



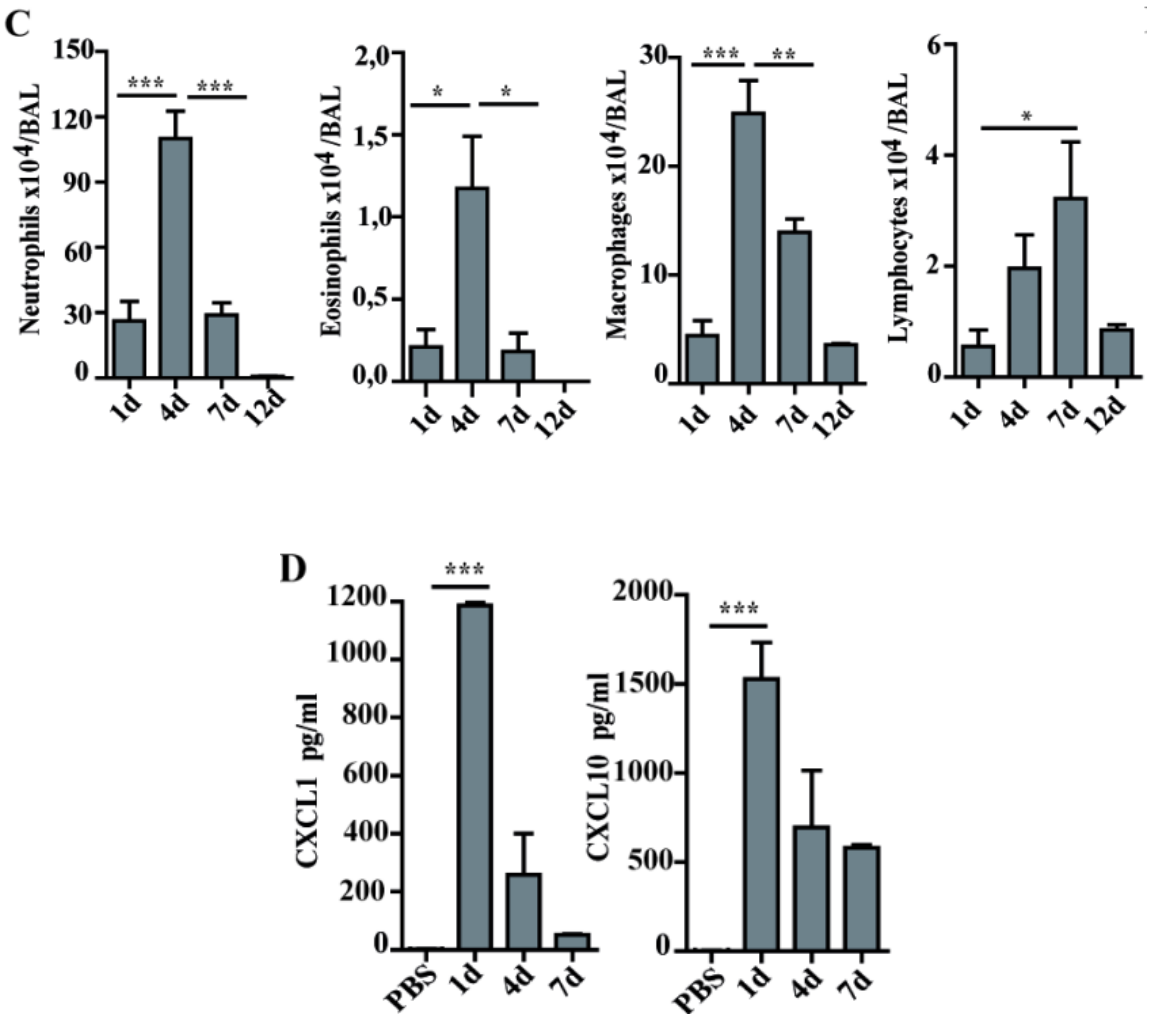
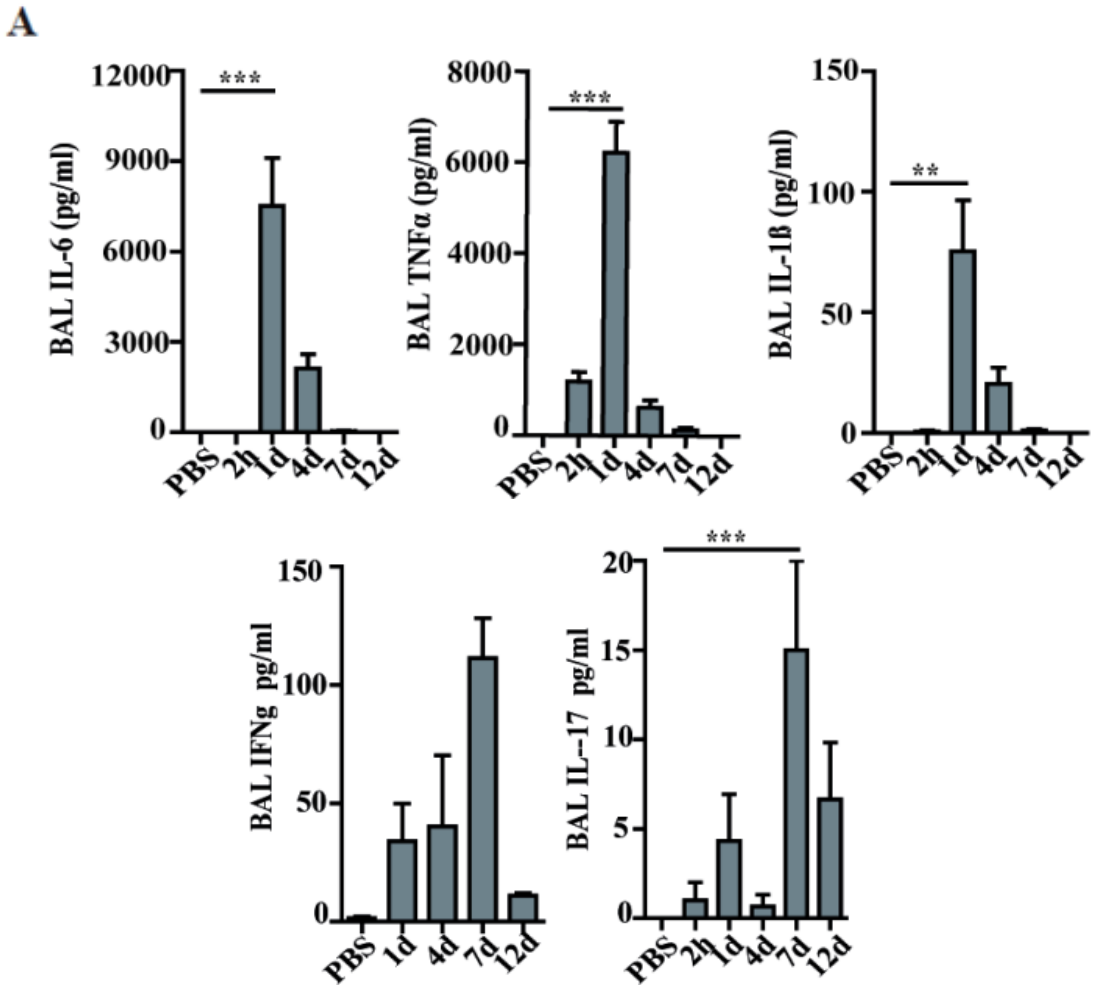


Figure 1 Characterization of M.catarrhalis induced immune responses. *Immune analysis of infection was assessed in mice that only received M.catarrhalis (i.e not HDM). (A) Time course of bacterial recovery from lung homogenates. (B) Total BALF cell counts. (C) Differential cell counts. (D) Secretion of CXCL1 and CXCL10. Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

As pulmonary colonization with *M. catarrhalis* has been shown to cause airway inflammation via the epidermal growth factor receptor (Slevogt H 2014), we performed a kinetic study of inflammatory cytokines in the BAL. While IL-6, TNF-α and IL-1ß peaked at day 1 p.i and rapidly decreased to low levels by day 4, IFN-γ and IL17 amounts were highest at day 7 p.i. (Fig.2 A).



We next determined the effect of *M. catarrhalis* infection on the survival of these mice. We found that 50% of wt mice died between day1 and day 2 post infection (Fig.2 B)

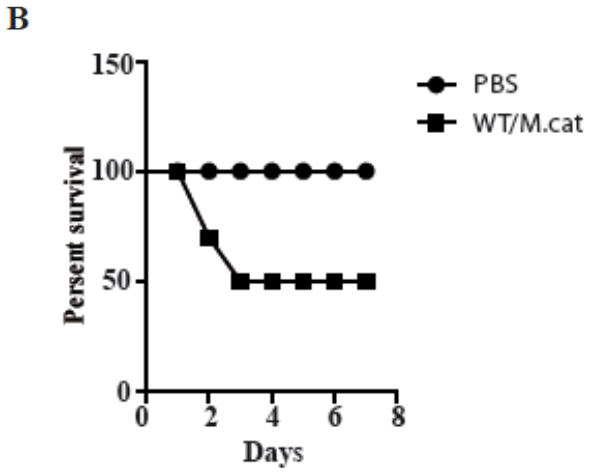


Figure 2 Characterization of M. catarrhalis induced immune responses. *The profile of infection was assessed in mice that only received M.cat (i.e not HDM). (A) IL-6, TNF, IL-1ß, IL-17, IFNγ cytokines in the BALF of wt mice was measured at day 1,4,7 and 12 after intranasal administration of 10^8 CFU of M. catarrhalis. (B) Survival curve. Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

Since IL-17 has been described as a key cytokine involved in the pathogenesis of infection triggered asthma, the frequency and the origin of IL-17 production was investigated. Due to the late occurrence of IL-17 in the BAL, we continued to analyse the mRNA expression of this cytokine in lung homogenates. Strong IL-17 expression was found at day 7 and correlated with the rise of IL-17^{+} CD4^{+} T cells at this time point (Fig.3 A, B). The majority of IL-17 secreting cells in the lung were conventional CD4^{+} T cells (70%) which increased between day 7 to 15 p.i. and only a minor fraction of TCR $\gamma^{+}\delta^{+}$ cells (14%) was positive for IL-17, (Fig. 3 C)

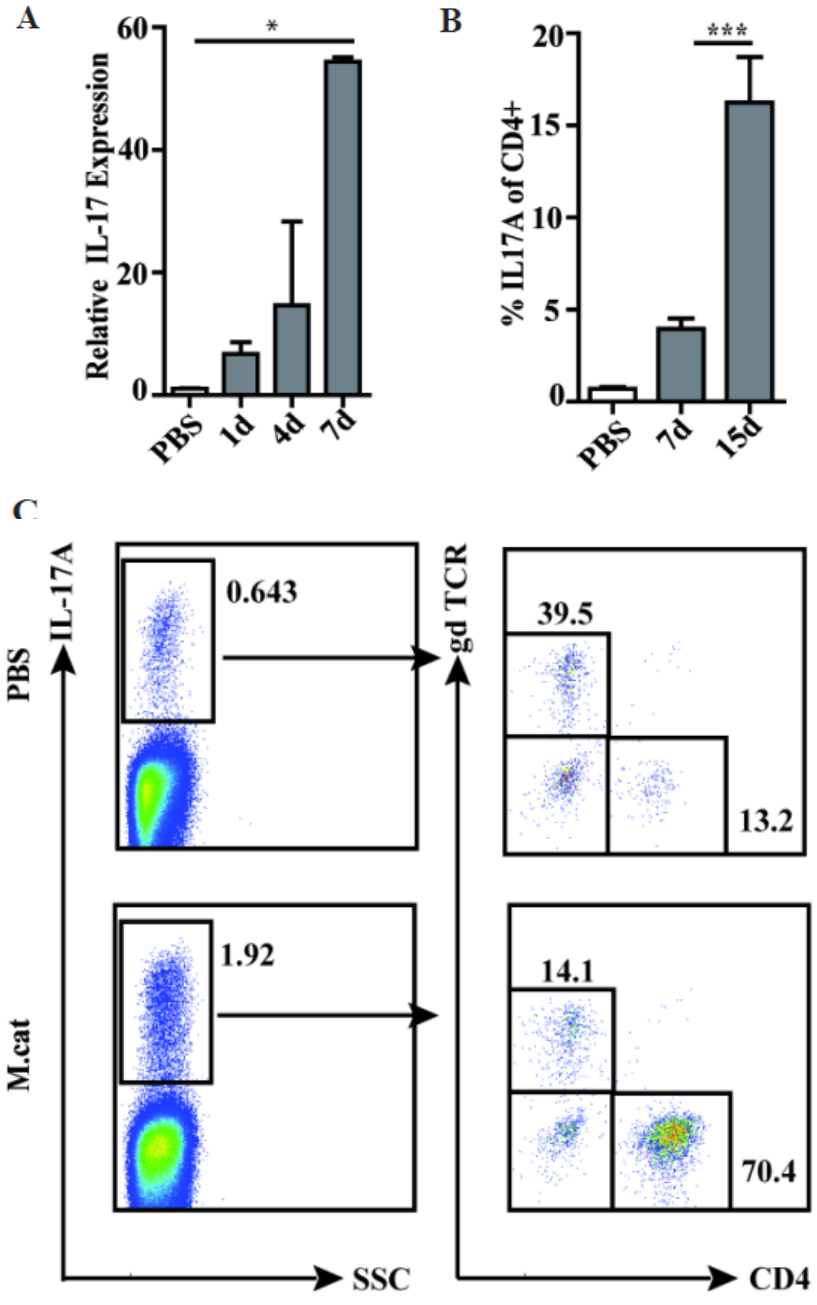
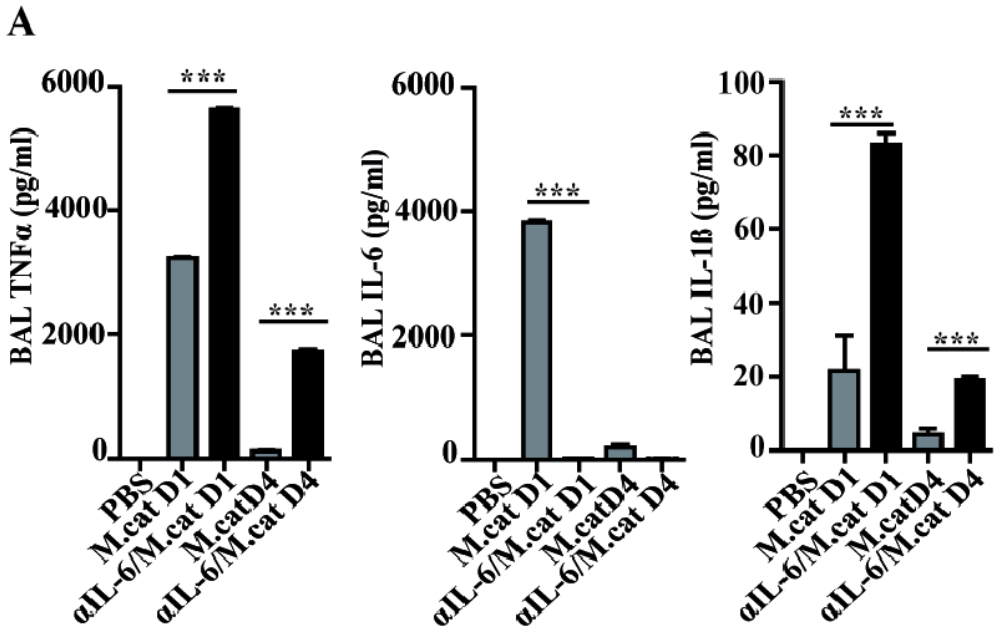


Figure 3 Analysis of IL-17 frequency and origin after M.catarrhalis infection

*(A) mRNA relative IL-17expression in lung tissue. (B) Lung lymphocytes from M.catarrhalis infected mice were analysed for the frequency of IL-17A at days 7 and 15. (C) IL-17A T cells at day 7 after infection. Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

5.2 ANTI INFLAMMATORY ROLE OF IL-6 DURING *M.CATARRHALIS* INFECTION

IL-6 is induced together with the proinflammatory cytokines TNF-α and IL-1ß after *M.catarrhalis* infection. IL-6 is known to play an important role in the induction of acute phase reactions. However, whether it plays pro- or anti-inflammatory roles in local or systemic responses after *M.catarrhalis* infection remains unclear. In this experiment, the role of IL-6 in acute inflammatory responses was investigated in animal model by using monoclonal antibody against IL-6. We found that Ab-mediated neutralization of IL-6 increased local and systemic TNF-α as well as early IL-1ß and IL-17 in the BAL (Fig.4 A, B). Importantly, anti-IL-6 treatment resulted in enhanced mortality, demonstrating that IL-6 exerts an anti-inflammatory function during pulmonary *M.catarrhalis* infection (Fig.4 C). This reveals that IL-6 has an anti-inflammatory role as shown by the reduction of local and systemic inflammatory cytokine responses.



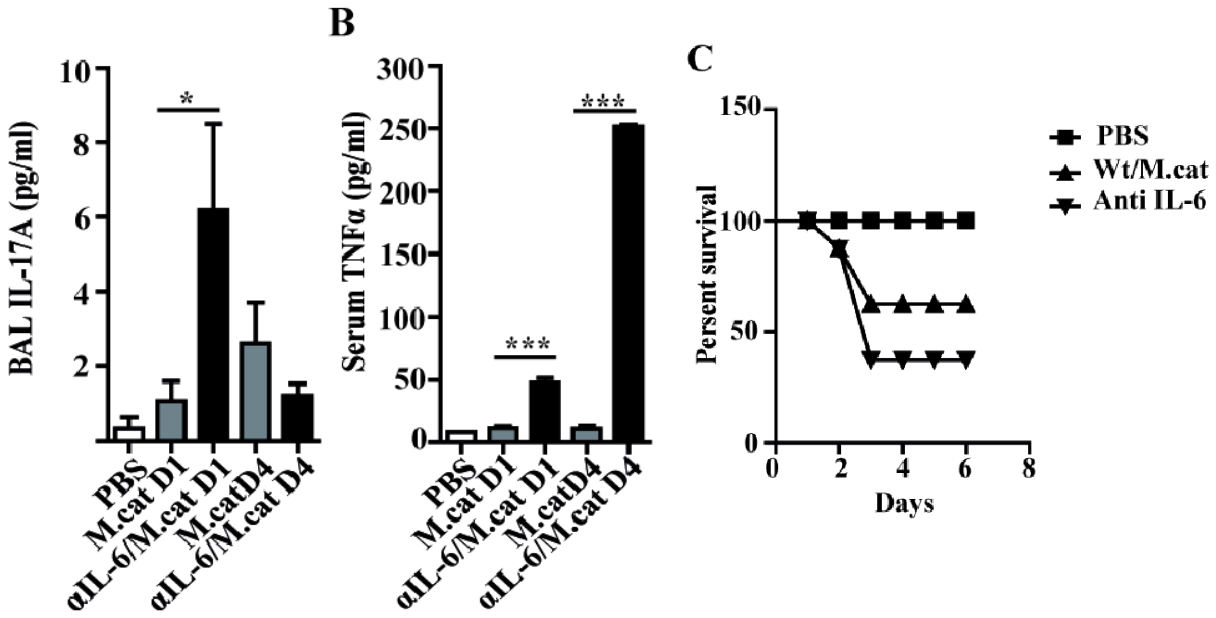


Figure 4 Anti-inflammatory role of IL-6 response to M.catarrhalis infection

*Cytokine measurement was assessed in mice that only received M.catarrhalis. (A) Mice were administered a rat anti-murine IL-6 (100µg;20F3) or an IgG1 control antibody i.p intraperitoneal before 4 hours of M.catarrhalis infection. BALF proinflammatory cytokines TNF-α, IL-1ß, IL-6 and IL-17. (B) Serum Proinflammatory TNF-a. (C) Survival. Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

5.3 PRO- INFLAMMATORY ROLE OF TNF ON *M.CATRRHALIS* INFECTION

TNF-α is a multifunctional cytokine of the immune system, involved in local and systemic inflammation. It has been discovered as a mediator of endotoxin poisoning, and plays a role in septic shock. So we wondered whether TNF-α is the mediator of inflammation and mortality. It has been demonstrated previously by *Stefan P.W.dem Vries* that TNF-α is upregulated after *M. catarrhalis* infection by human epithelial cells, to determine the source of TNF-α after *M.catarrhalis* infection in our animals. Wt mice were infected with *M.catarrhalis,* after 4 and 24 hours of infection and supernatants of epithelial cells were tested for the secretion of TNF-α by Cytometric Bead assay. As shown in Fig 4 high levels of TNF-α were secreted by epithelial cells. To determine the role of TNF-α pro or anti-inflammatory cytokine after *M.catarrhalis* infection. We administered in vivo a TNF-α blocking monoclonal antibody (aTNF-α) and we found that the absence of TNF-α decreased the recruitment of neutrophils at day 4 compared to WT infected mice.

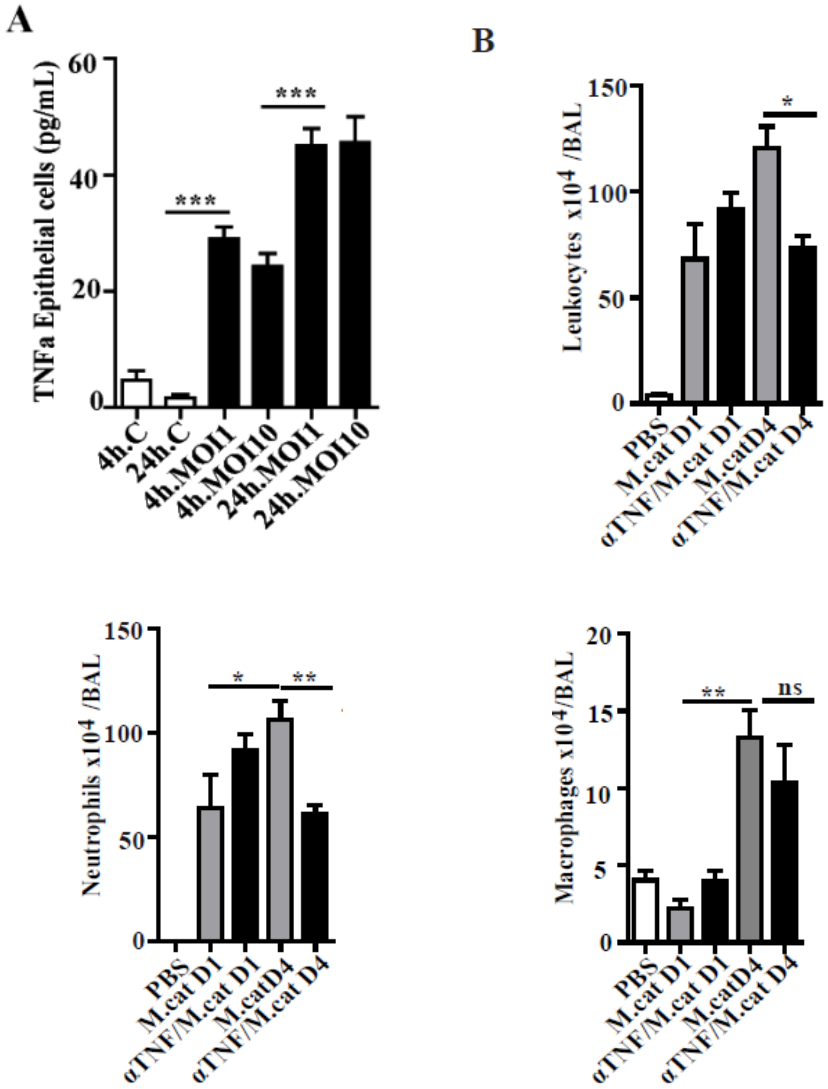


Figure 5. The effect of anti TNF-α on influx of cells after M.catarrhalis infection

Infection was assessed in mice that only received M.catarrhalis. Mice were administered a rat anti-murine TNF (100µg; MP6-XT22) or an IgG1 control antibody i.p intraperitoneal before 4 hours of M.cat infection. (A) TNF-α expressed by Tracheal epithelial cell administration with M.catarrhalis. (B) Total BAL cell counts and differential cells (neutrophils and macrophages).

Moreover, in contrast to IL-6, neutralization of TNF-α resulted in local which (BAL) and systemic (serum) reduction of IL-6, IL-1ß and IL-17 and increased IgG1with subsequent protection against *M.catarrhalis* induced death of animals (Fig 6 A, B, C and D). Collectively, these results indicate that TNF-α plays a proinflammatory role in inducing neutrophils and IL-6, IL-1ß and IL-17 cytokines leading to a pathogenic response to *M.catarrhalis* infection.

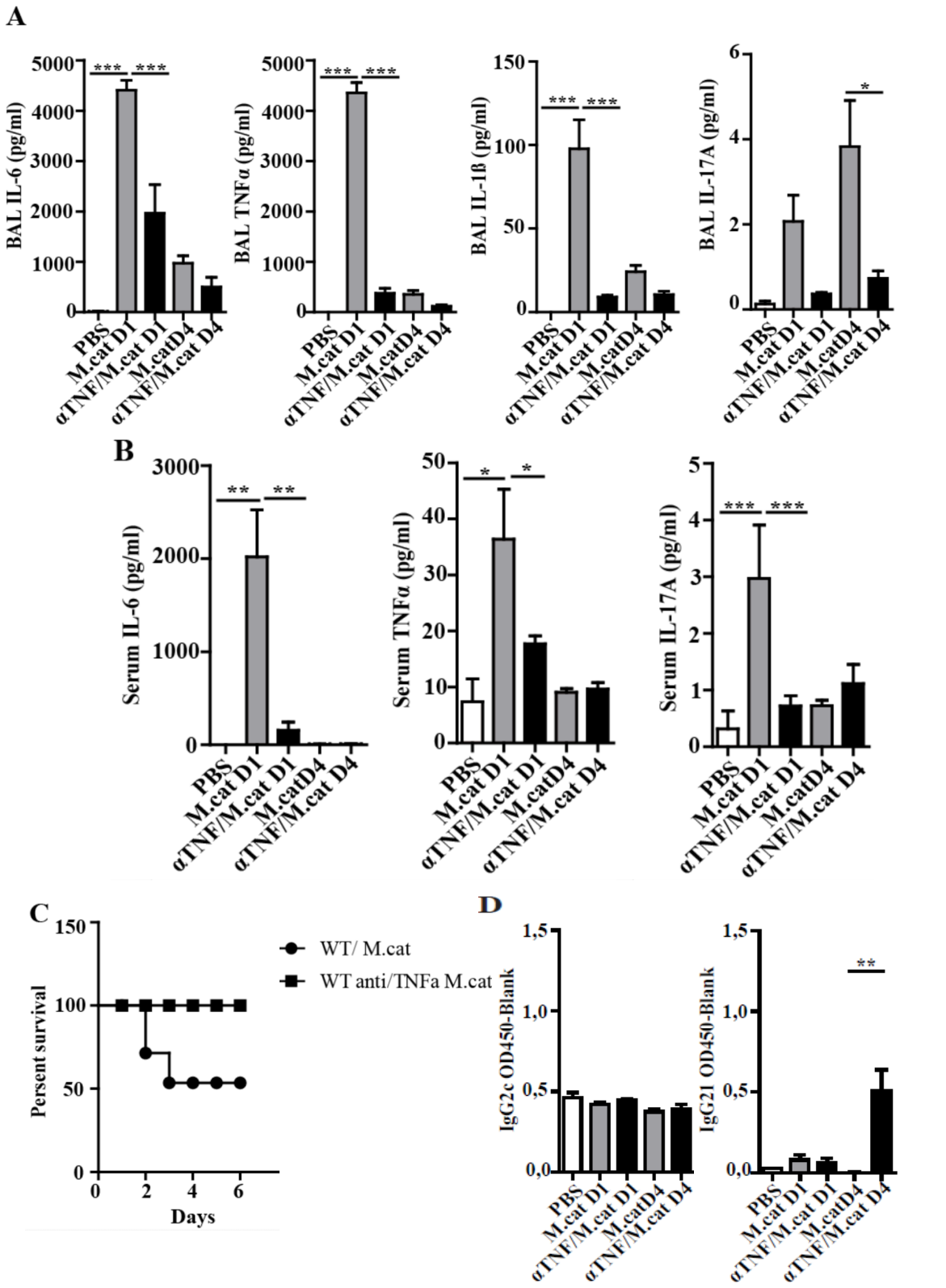


Figure 6 The effect of anti TNF-α on cytokine secretion after M.catarrhalis infection

*The protein levels of IL-6, TNF-α and IL-17 was measured after M.catarrhalis infection as indicated in different time points (A,B) BALF and Serum pro-inflammatory cytokines IL-6, TNF, IL-1ß and IL-17. (C) Survival. (D) Levels of HDM specific IgG1 and IgG2c antibody titers were measured by the Elisa technique. . Data were from two independent experiments (n=6 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

5.4 PULMONARY INFECTION WITH *M.CATARRHALIS* AUGMENTS HDM INDUCED AIRWAY RESPONSES

To determine the mechanisms underlying *Moraxella catarrhalis* induced exacerbation of asthma, we used a HDM asthma model which is clinically more relevant to humans. HDM is a common allergen associated with asthma, which is used to induce allergic airway phenotype.

The established model of HDM induced in C57BL6/J mice were infected with *M. catarrhalis* intranasally after second exposure with house dust mite extract (HDM) on day 8 through intranasal administration and monitored the ensuing allergic airway inflammation on day 23 (Fig.7 A). As expected, HDM exposure induced more robust BALF inflammation than did PBS treatment. Inflammatory cell infiltration was increased in the airways of mice infected during HDM exposure (Fig.7 B). HDM-triggered inflammation was dominated by enhanced neutrophilic, eosinophilic and lymphocytic infiltrates (Fig.7 C)

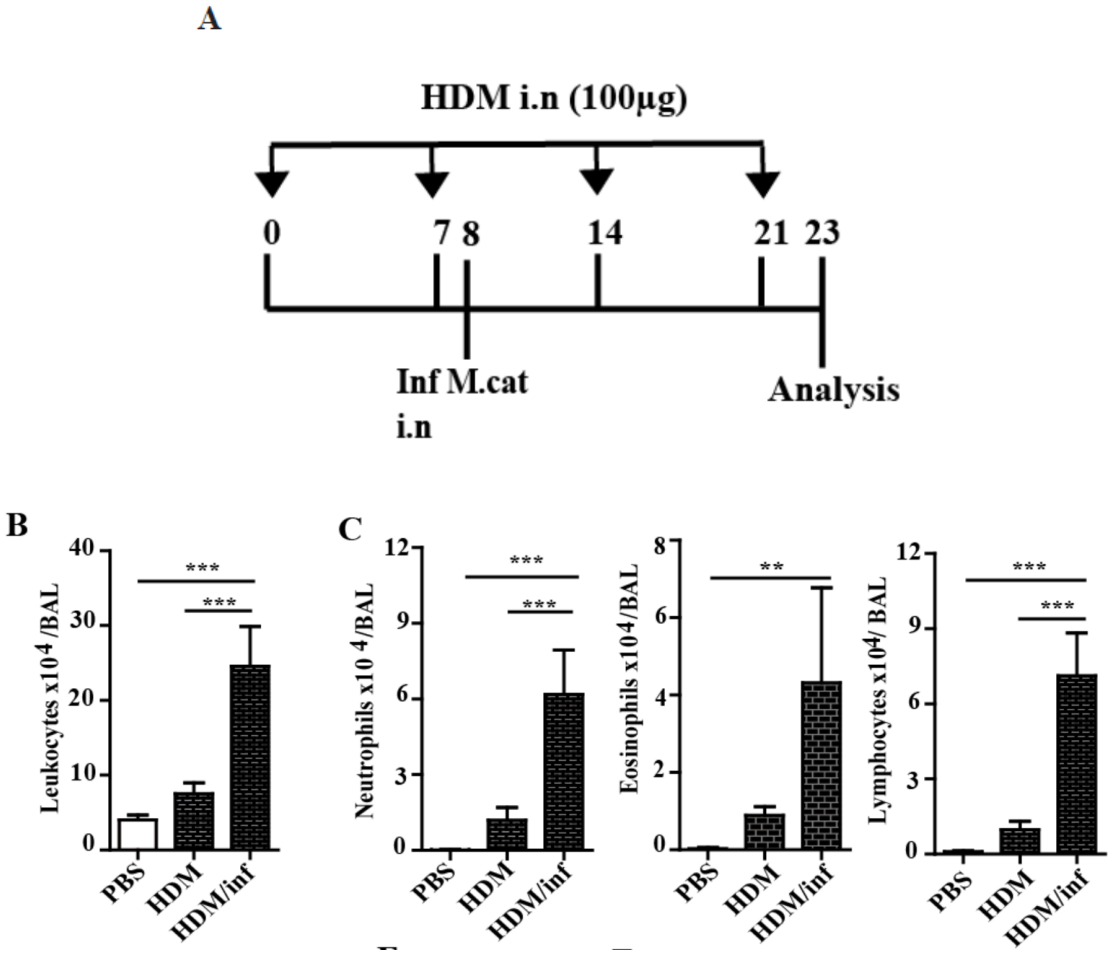


Figure 7 M.catarrhalis during HDM exposure increases neutrophilic and eosinophilic infiltrates to the airways *(A) HDM asthma induction Protocol in, Intranasal. (B)Total BALF cell counts (C) Differential cell counts, Neutrophils, Eosinophils, Lymphocytes.*

Furthermore, the predominant sign of allergic asthma is lung inflammation and mucus production. Therefore, Lung slices were stained for mucus production by PAS staining and goblet cells. As visualized by PAS staining, PBS control mice had healthy lungs whereas the HDM allergic mice showed mucus-producing goblet cells within the lung epithelium However, M.catarrhalis infection during HDM exposure showed significantly increased airway inflammation, goblet cell hyperplasia and mucus production from the lung compared to HDM allergic group (Fig.8 A, B, and C).

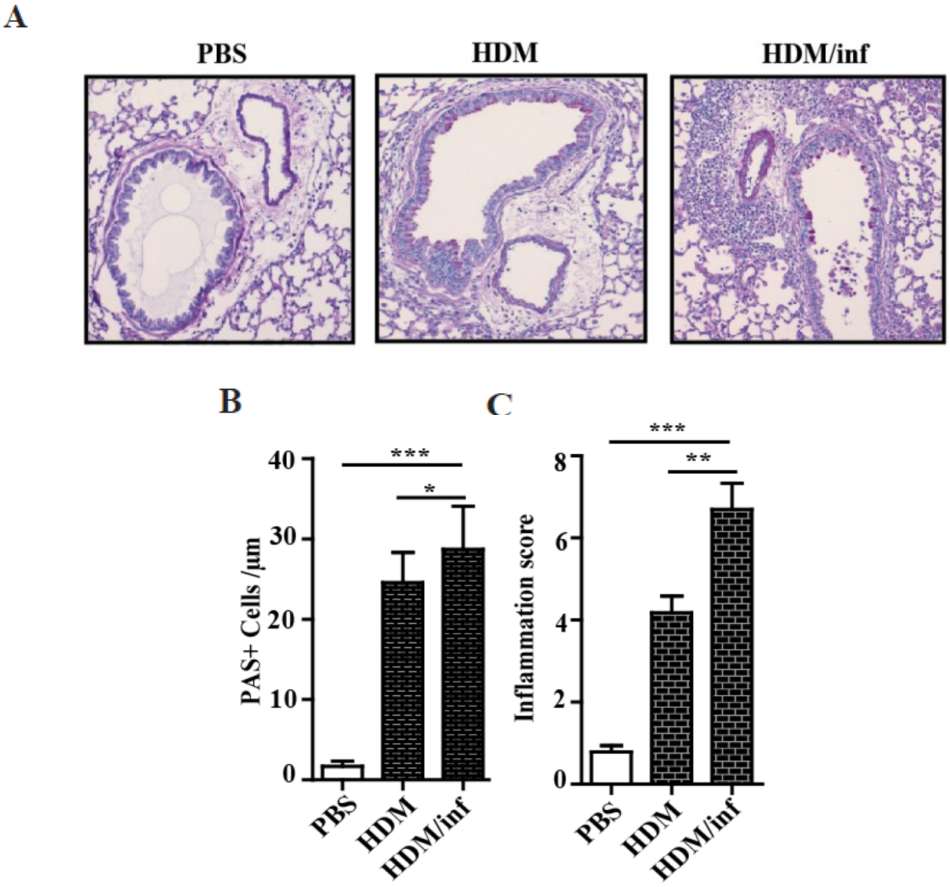


Figure 8 M.catarrhalis infection increases mucus production and airway inflammation during HDM exposure *(A). Representitive PAS stained lung tissue sections from mice either PBS, HDM or HDM and M.catarrhalis infection. Scale bars, 200 µm.. (B) Goblet cell counts from lung tissue. (C) Inflammation score. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

5.5 T CELL PROFILE AND CYTOKINE PRODUCTION FROM MICE INFECTED WITH *M.CATARRHALIS* DURING HDM CHALLENGE

M.catarrhalis is the bacterium most commonly isolated during COPD exacerbations. We wondered whether *M.catarrhalis* may promote neutrophilic asthma by suppressing Th2 mediated responses, and by inducing potent neutrophilic inflammation that is driven by Th17 responses. We compared HDM allergic mice with those that additionally infected with *M.catarrhalis*. In line with the increased lymphocytic infiltration in BAL, the concentration of IFNγ, IL-17 and the frequency of Th1, Th17 and Th2 were enhanced in lung tissue from mice infected during HDM challenge (Fig.9 A).

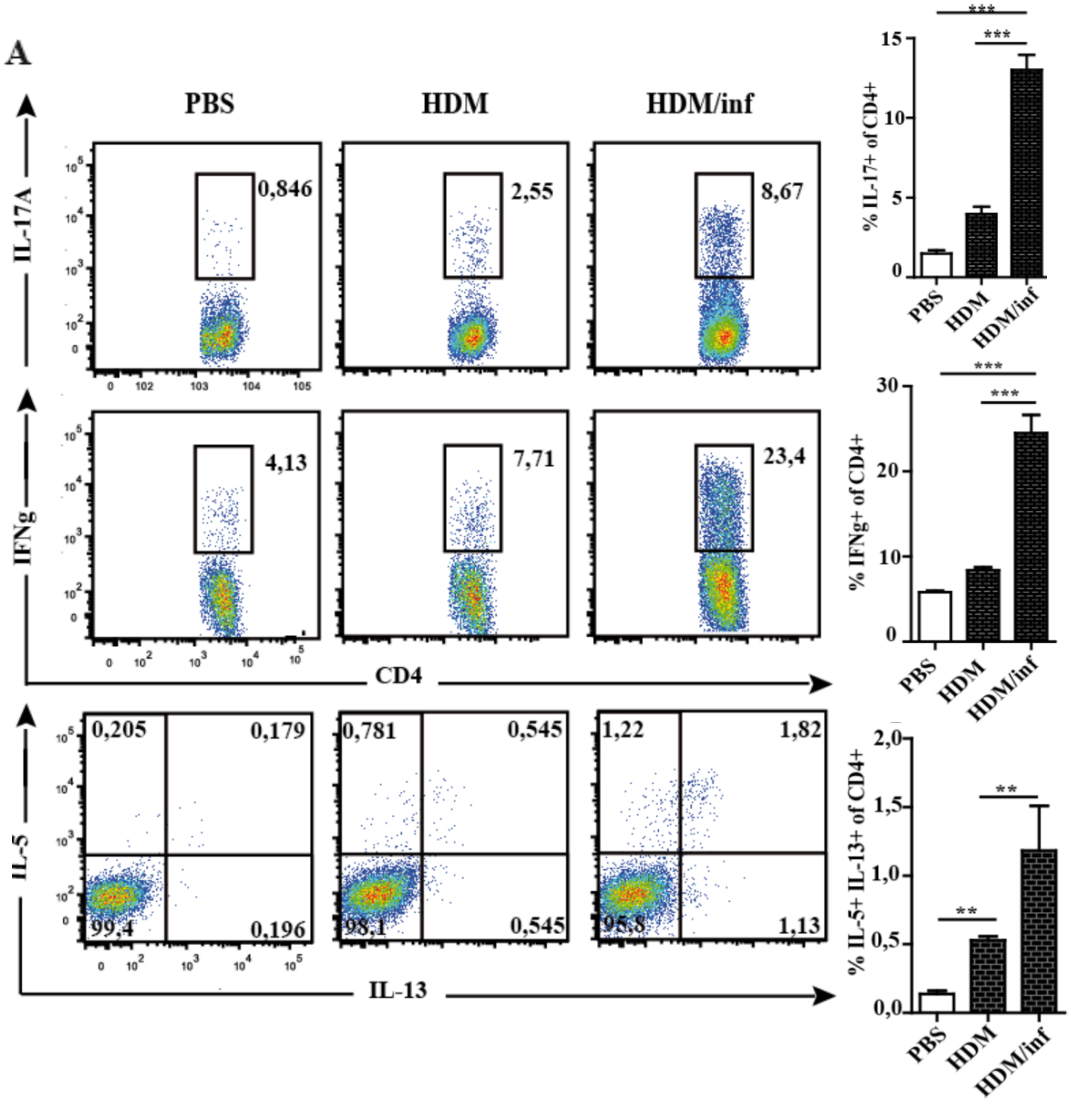


Figure 9 Frequency of T cells, BAL and Lung homogenates from infection during HDM challenge *(A) WT mice were infected with 200× 10^6 CFU M.catarrhalis. Lungs were harvested on day 23. Homogenised and the cells were stained for CD4, IFNγ, IL-17A, IL-5 and IL-13.*

*Numbers indicate in the percentage of positive cells located the respective quadrant. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

Accordingly, increased amounts of IL-17 and IFNγ in the lung and TNF-α in the BAL were found in HDM allergic mice previously infected with *M. catarrhalis* (Fig 10 A,B). Serum levels of HDM-specific IgG2c antibodies were also increased in the *M.catarrhalis* infection during HDM challenge as compared to the uninfected allergic HDM group Fig 10.

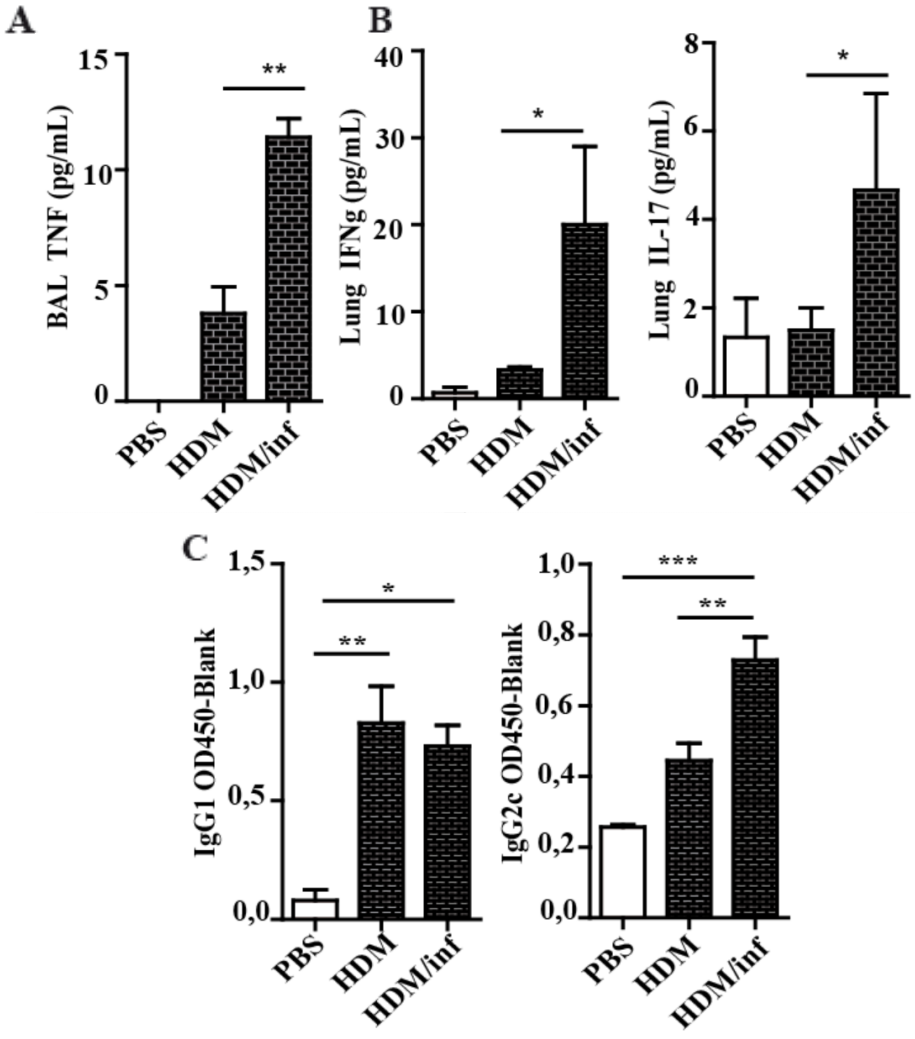


Figure 10. Cytokine measurement of BAL and lung *(A) TNF in BALF.(B)IFNγ and IL-17 in lung homogenates.(C)Measurement of the total amounts of IgG1, IgG2c titers in the serum at day 23. Data were from two independent experiments (n=6 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

5.6 *M.CATARRHALIS* INFECTION EXACERBATES AN ESTABLISHED HDM-INDUCED AIRWAY RESPONSES

We next wondered whether changing the time point of *M.catarrhalis* infection would lead to similar effect on allergic HDM asthma. Accordingly, we first infected mice 1 day after the final HDM challenge and analysed the BAL one week later to address the effect of the adaptive immune response (Fig.11 A). Inflammatory cell infiltration was increased in the airways of mice infected after HDM challenge (Fig.11 B). This inflammation was dominated by enhanced neutrophilic, and lymphocytic infiltrates (Fig.11 C).

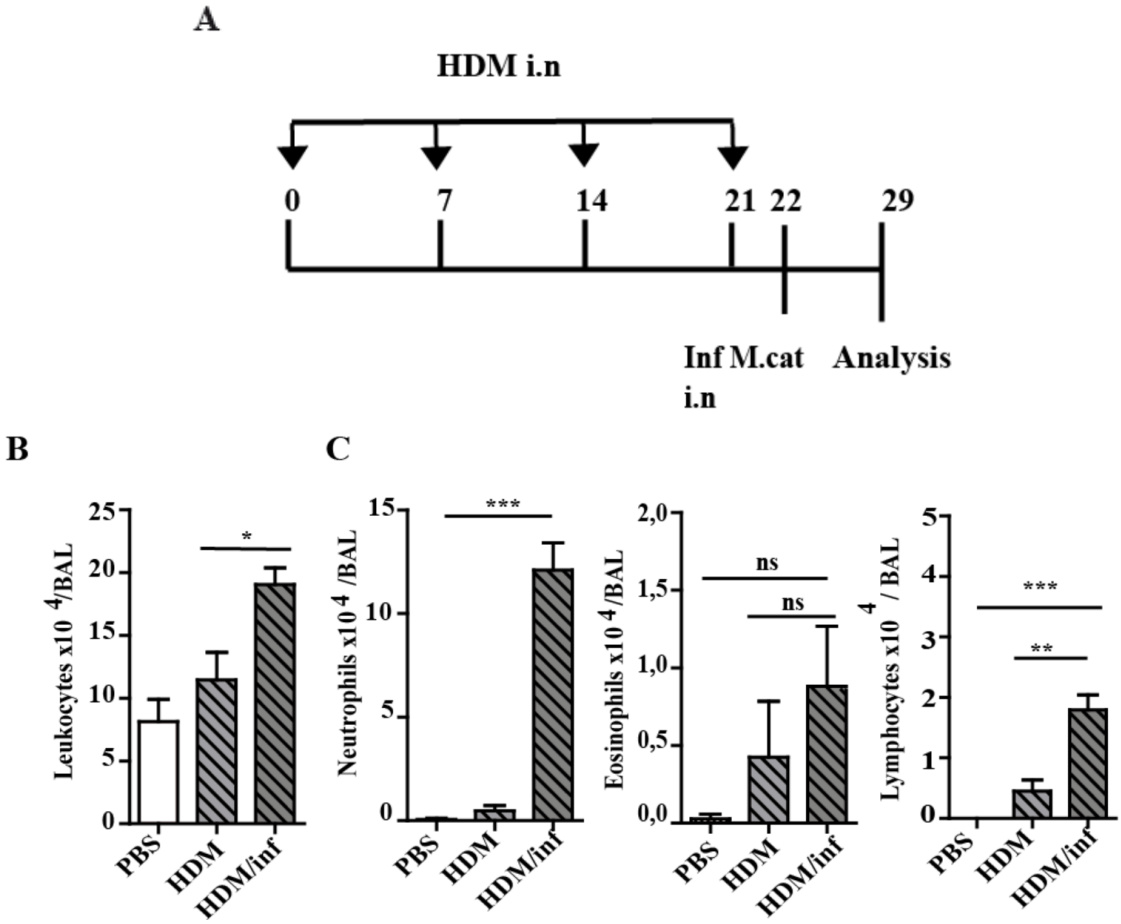


Figure 11. M.catarrhalis infection exacerbates an established HDM-induced airway responses
*(A) Animals were treated with HDM or PBS on days 0, 7, 14 and 21, analyses refer to day 29. When the mice were killed and samples were taken. (B,C) Bronchoalveolar lavage fluid (BAL) analysis in wt mice for cell infiltrates. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

Furthermore Lung slices were stained for mucus production by PAS staining and goblet cells. As seen by PAS staining, PBS control mice had healthy lungs whereas the HDM allergic mice showed mucus-producing goblet cells within the lung epithelium However, *M.catarrhalis* infection after HDM exposure showed significantly increased airway inflammation, goblet cell hyperplasia and mucus production from the lung compared to HDM allergic group (Fig.12 A, B, and C).

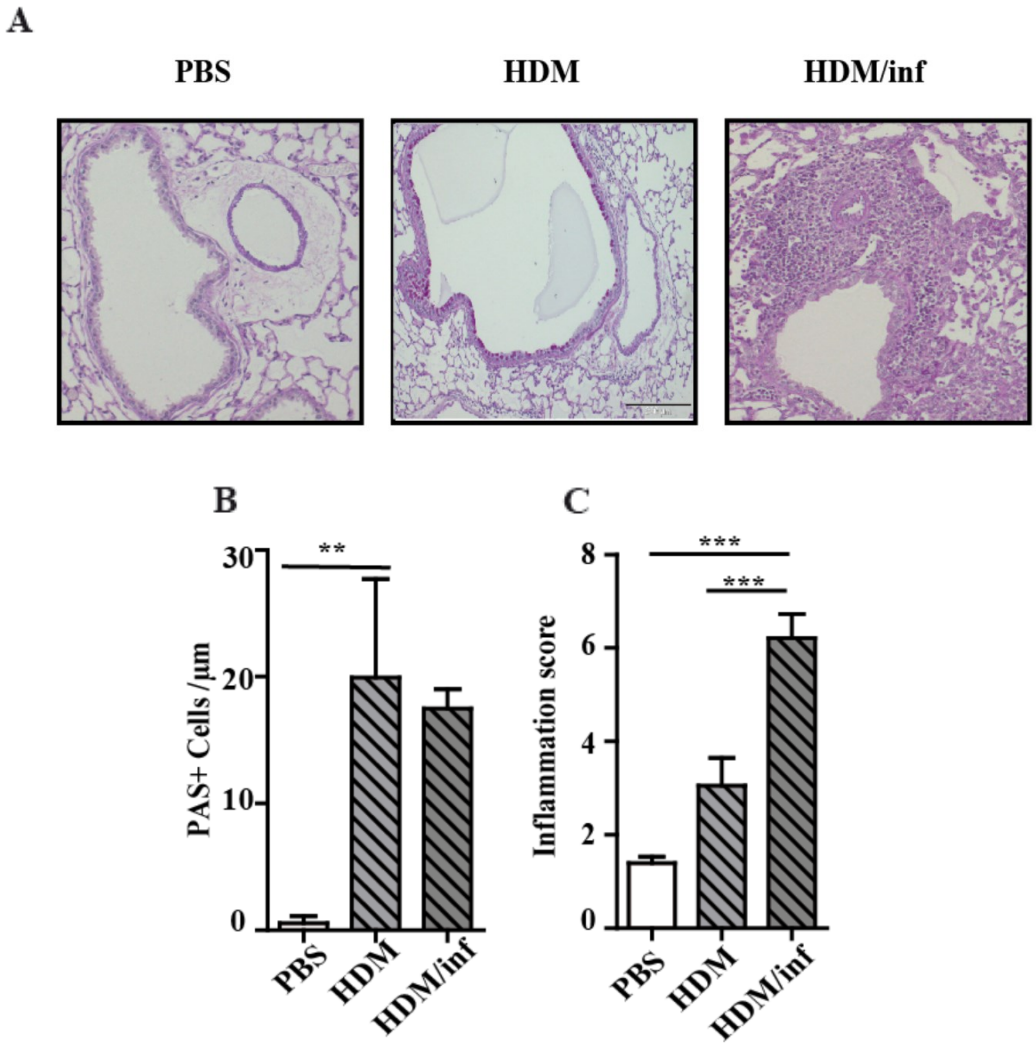
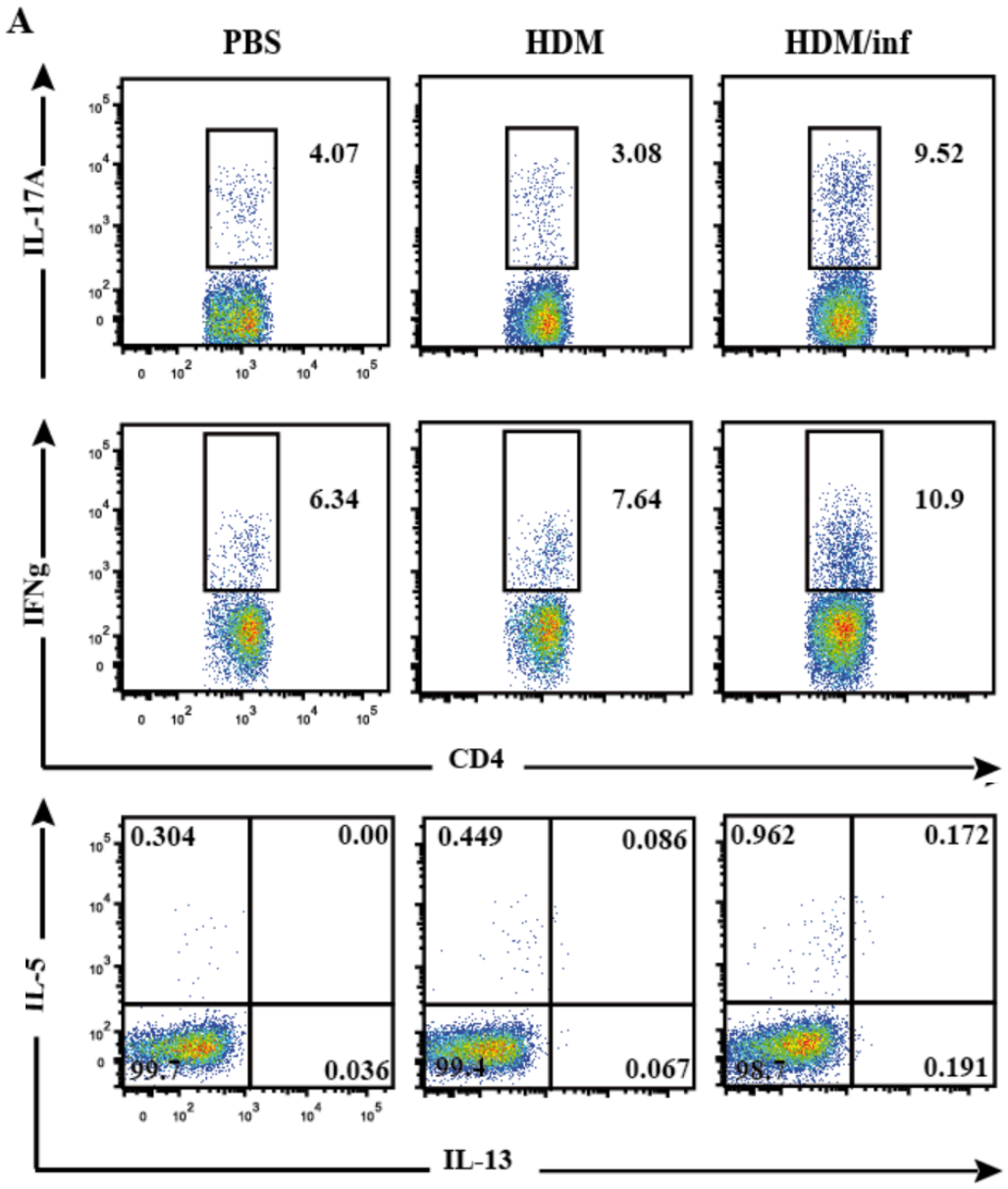


Figure 12. M.catarrhalis infection exacerbates an established HDM-induced airway responses
*Animals were treated with HDM or PBS on days 0, 7, 14 and 21, analyses refer to day 29. When the mice were killed and samples were taken. (A, B) Histological quantification of goblet cell counts, (C) infiltration of cell into lung tissue, inflammation score. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

In order to determine the cytokine profile of T cells, we directly restimulated lung cells for 4 hours with PMA and ionomycin in the presence of brefeldin A and stained immediately for CD4 and IL-17, IFNγ, IL-5 and IL-13 to determine the percentage of Th17, Th1, Th2 cells. In line with increased lymphocytic infiltrations, the frequencies of Th1, Th17 and Th2 were enhanced in lung tissue from mice infected after HDM challenge compared to the uninfected allergic HDM group (Fig.12 A).



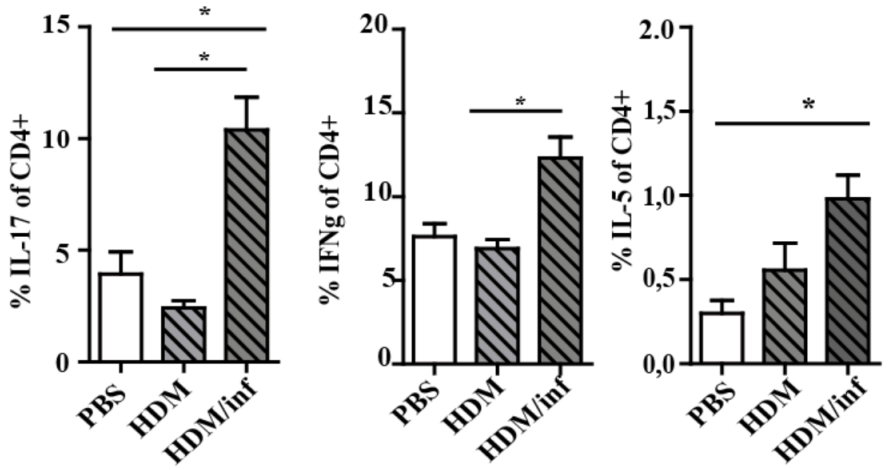
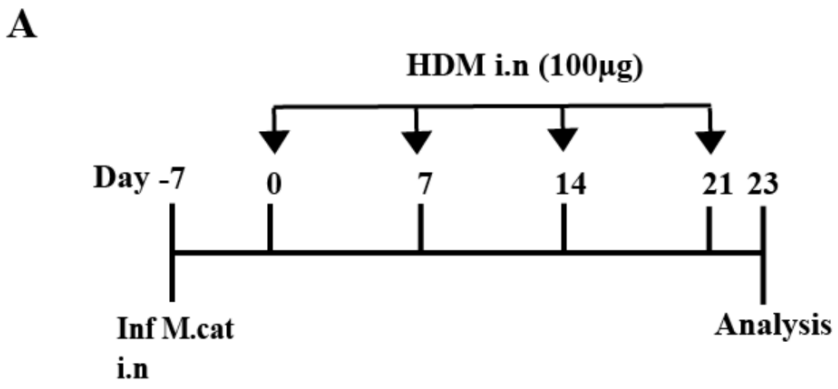


Figure 12. T cell profile from infection after an established HDM allergic airway response

*(A) Live lung cell from PBS, HDM and HDM with M.catarrhalis instilled mice were analysed for the frequency of IL-17A, IFNγ, IL-5 and IL-13 T cells (n= 8 mice per group). Statistical significance was determined with 1-way (ANOVA) *P=0.05, **P=0.01*

5.7 *M.CATARRHALIS* INFECTION PRIOR TO HDM SENSITISATION EXACERBATES ASTHMA VIA T-CELL IMMUNE RESPONSE

Further, we analysed whether a previous infection with *M. catarrhalis* that has already been resolved is still able to exacerbate HDM allergic reactions. Therefore, mice were infected with *M.catarrhalis* 7 days before first HDM exposure and analysed on day 23 (Fig.13 A). The results show that infiltrations with inflammatory cells were increased in the airways of mice infected after HDM challenge (Fig.13 B). Interestingly, under these conditions, inflammation was dominated by enhanced lymphocytic infiltrates (Fig.13 C)



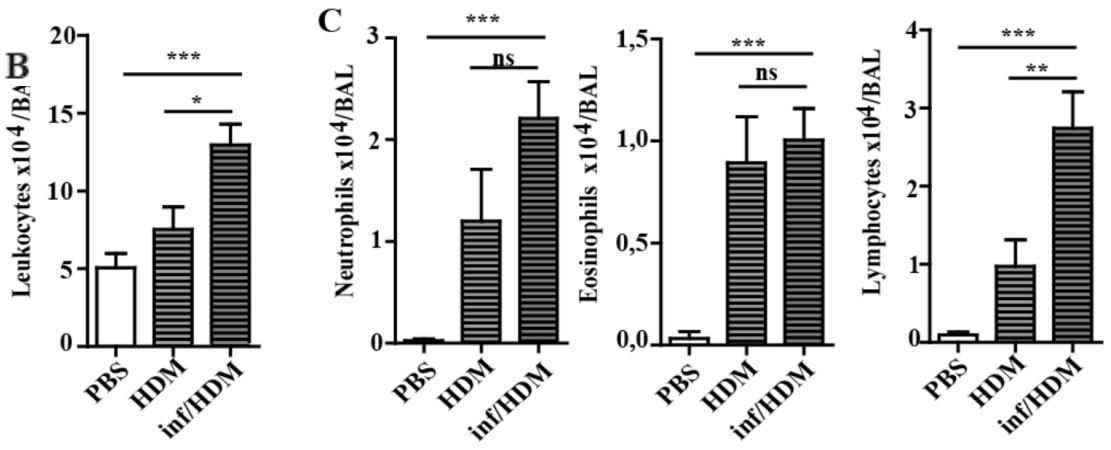
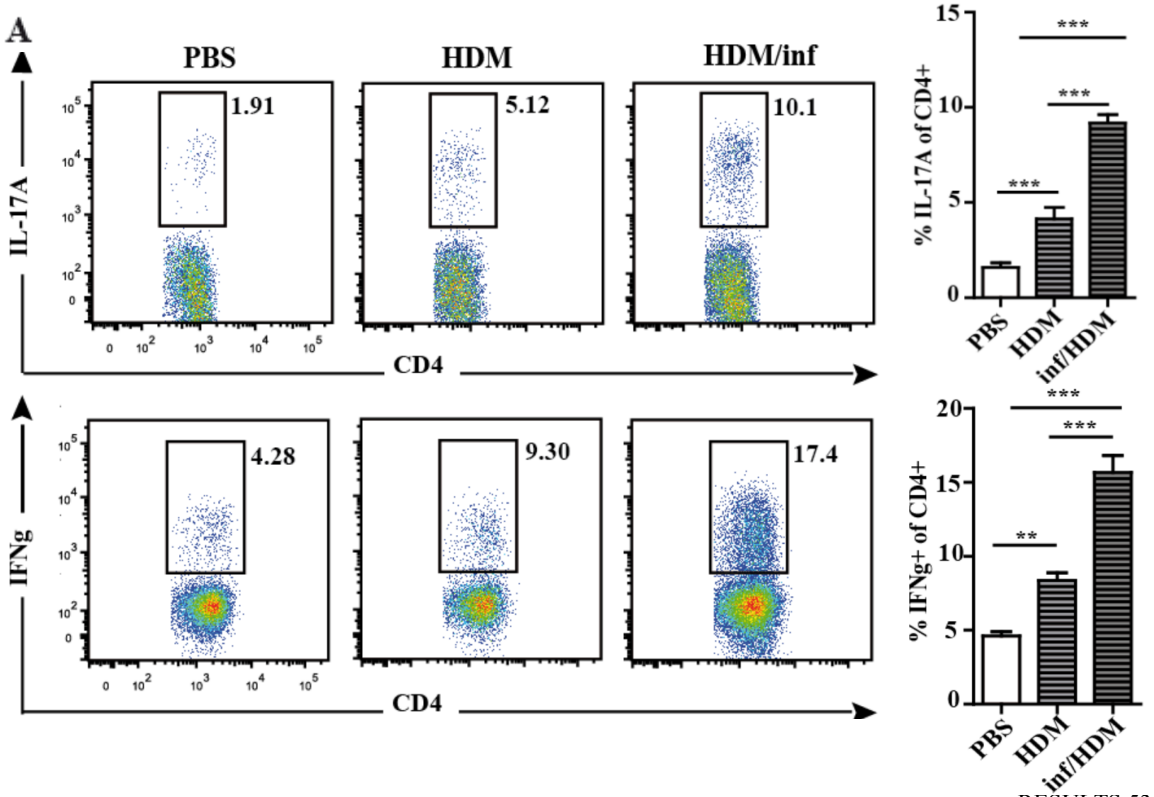


Figure 13. M.catarrhalis infection prior to HDM exposure exacerbates allergic airway disease via lymphocyte recruitment *Protocol for HDM-induced allergic airway inflammation. WT mice were given four intranasal (i.n) challenges with HDM extract, once per week. M.catarrhalis was given 7 days before the first HDM exposure intranasal. (B)Total BALF cell counts. (C) Differential cell counts. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

In contrast to the PBS - and HDM control groups, infection prior to HDM exposure induced Foxp3$^+$ CD25$^+$ regulatory T cells (Treg) in the lungs. Despite enhanced Treg frequencies, the amount of pulmonary Th17, IFN-γ and IL-5-secreting was still enhanced by a factor of two in the lung (Fig.14 D). Collectively these data suggest that exacerbation of HDM-allergic reactions is independent of the time point of infection and that IL-17 has a pathogenic role in asthma, enhancing the severity of HDM-induced allergic response.



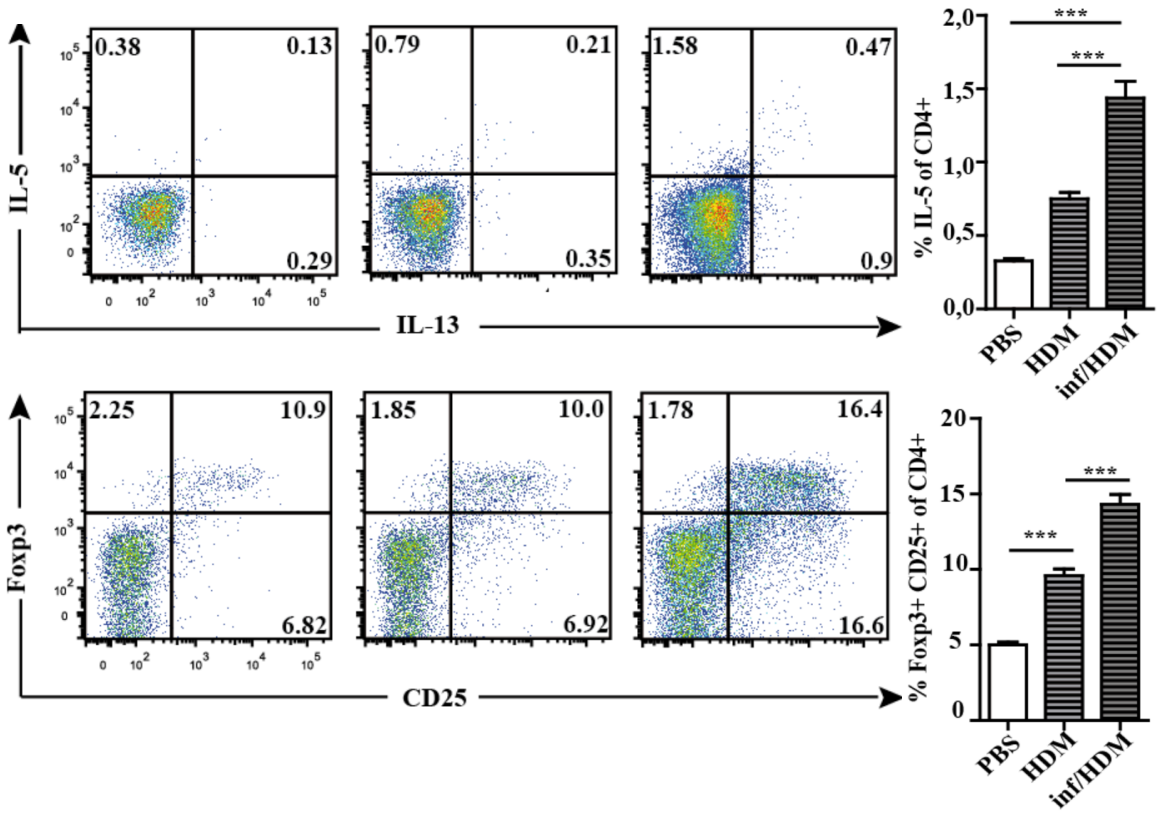


Figure 14. M.catarrhalis infection prior to HDM exposure exacerbates allergic airway disease via T-cell activation

Protocol for HDM-induced allergic airway inflammation. WT mice were given four intranasal (i.n) challenges with HDM extract, once per week. M.catarrhalis was given 7 days before the first HDM exposure intranasal. (A) Live lung cell from PBS, HDM and HDM with M.catarrhalis installed mice were analysed for the frequency of IL-17A, IFNγ, IL-5, IL-13, FoxP3 and CD25 T cells. Populations are sequentially gated on total live cells (n=12 mice per group).

5.8 IL-17 IS A KEY CYTOKINE IN DEVELOPMENT OF HDM-ALLERGIC AIRWAY INFLAMMATION

As IL-17 has been reported to be associated with the severity and pathogenesis of infection induced asthma (Bisgaard et al. 2014), we determined whether IL-17 enhances severity of allergen-driven AAD in the absence of bacterial infection. We employed IL-17 deficient mice to assess the role of this cytokine during development of HDM allergic airway responses. (Fig.15 A). In contrast to wt animals, IL-17 KO mice revealed significantly less inflammatory cell infiltration to the airways during HDM exposure (Fig.15 B). The inflammation was dominated by significantly less neutrophils, eosinophils and Lymphocytes in BAL fluid (Fig.15 C).

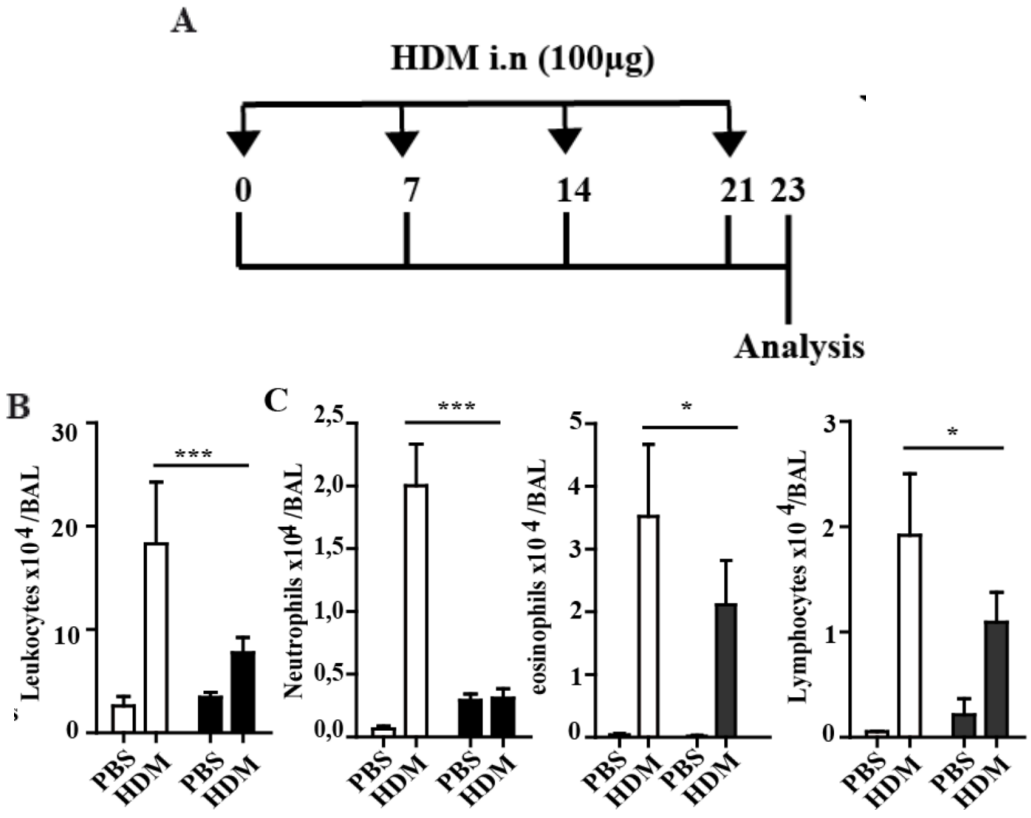


Figure 15. Reduced HDM-allergic airway inflammation in the absence of IL17

*(A) Protocol for HDM-induced allergic airway inflammation. WT and IL-17AF $^{-/-}$ mice were given four intranasal (i.n) challenges with HDM extract, once per week. (B)Total BALF cell counts. (C) Differential cell counts. Neutrophils, Eosinophils and Lymphocytes. (n= 8 mice per group). Statistical significance was determined with 1-way (ANOVA) *P=0.05, **P=0.01, ***P=0.001*

The predominant sign of allergic asthma is the lung inflammation accompanied with excessive mucus production. Therefore, histological analysis of the whole lung was the most important readout. Lung slices were stained for mucus production by PAS staining and the goblet cells were microscopically quantified in relation to the airway surface using the CellF software. The amount of goblet cells is a direct hint of disease severity in the lung. The graph (fig16. A) displays reduced goblet cell numbers per mm of the airways in IL-17 KO mice as compared to the wt mice. PAS staining could also quantify the inflammation score which displays the infiltration of cells to the lung tissue. The data show an increase in airway infiltration of cells in an HDM dependent manner, which was significantly reduced in IL-17AF$^{-/-}$ animals (Fig.16 B, C). However, the number of Th1, Th2 cells and HDM specific IgG1 titers were not decreased in IL-17AF$^{-/-}$ mice compared to wt animals (Fig.16 D, E).

Collectively, these data show that lack of IL-17 markedly reduced the severity of allergic asthma responses, demonstrating that this inflammatory cytokine significantly contributes to the full asthma phenotype in the HDM model.

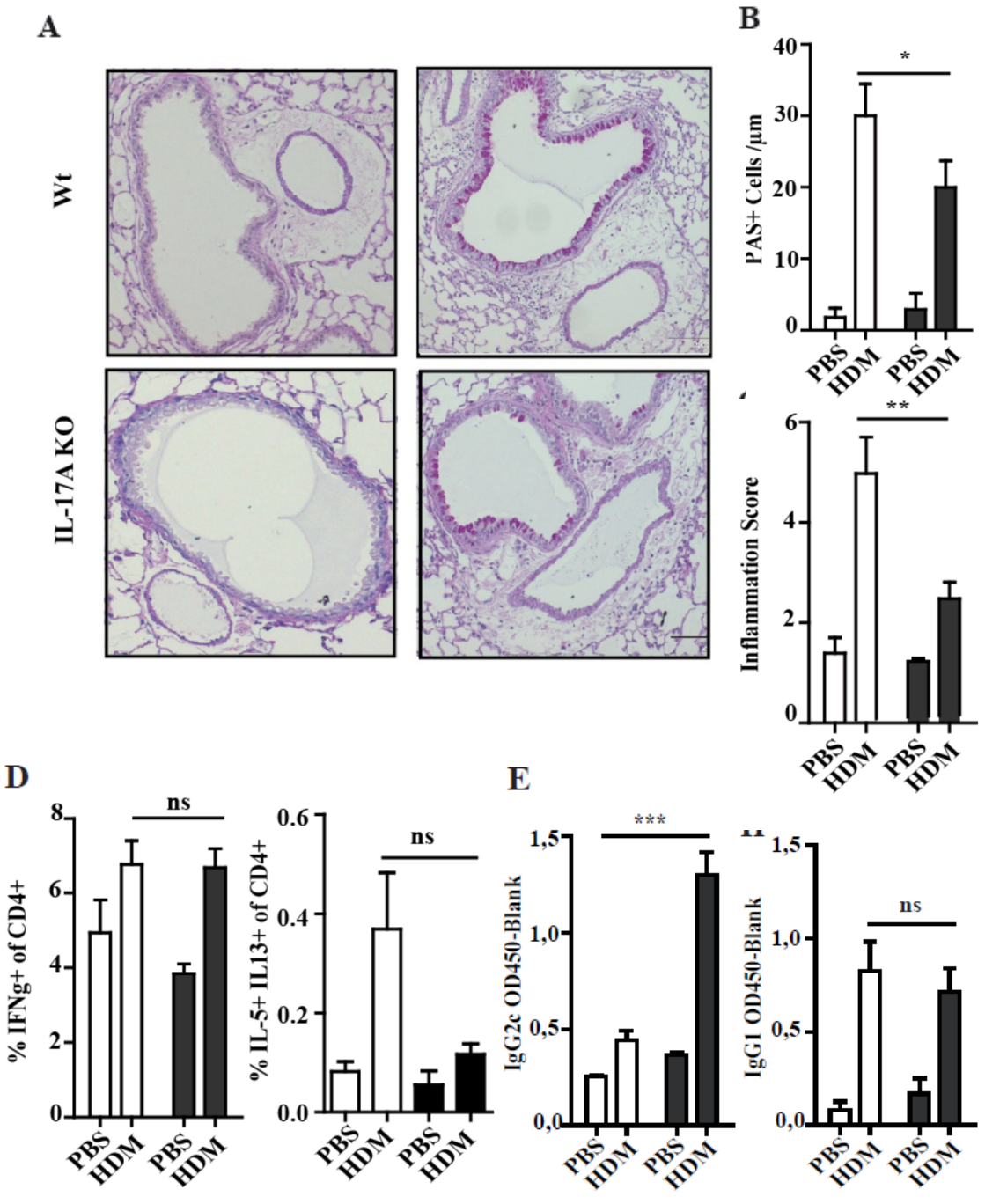


Figure 16. Reduced HDM-allergic airway inflammation in the absence of IL17

(A)Periodic acid Schiff (PAS) - stained for mucus producing goblet cells in PBS or HDM treated wildtype (WT) and IL-17AF-/- mice. (B) Histological quantification of goblet cell counts, (C) infiltration of cell to lung tissue, inflammation score. (D) Live lung cell from PBS, HDM installed mice were analysed for the frequency of IFNγ, IL-5 and IL-13 T cells. Populations are sequentially

gated on total live cells (n=12 mice per group). (E) Measurement of the total amounts of IgG1, IgG2c titers in the serum.

5.9 REDUCED INFLAMMATORY RESPONSE TO *M.CATARRHALIS* INFECTION IN IL-17 KO MICE

Neutrophil influx into infected tissues is a first-line defence mechanism predominantly controlled by IL-17. We wondered, whether IL-17 KO mice are able to control microbial airway infection and compared antibacterial resistance in wt and IL-17KO mice. Despite comparable amounts of *M. catarrhalis* two hours after infection, IL-17 KO mice cleared bacteria surprisingly more rapidly from lungs as compared to wt animals (Fig.17 A). Total cell counts were significantly reduced after 4 days of *M.catarrhalis* infection in the IL-17AF KO compared to WT mice. This reduction was mainly dominated by the reduction of neutrophils, and miner reduction of eosinophils, lymphocytes and macrophages (Fig.17 B, C)

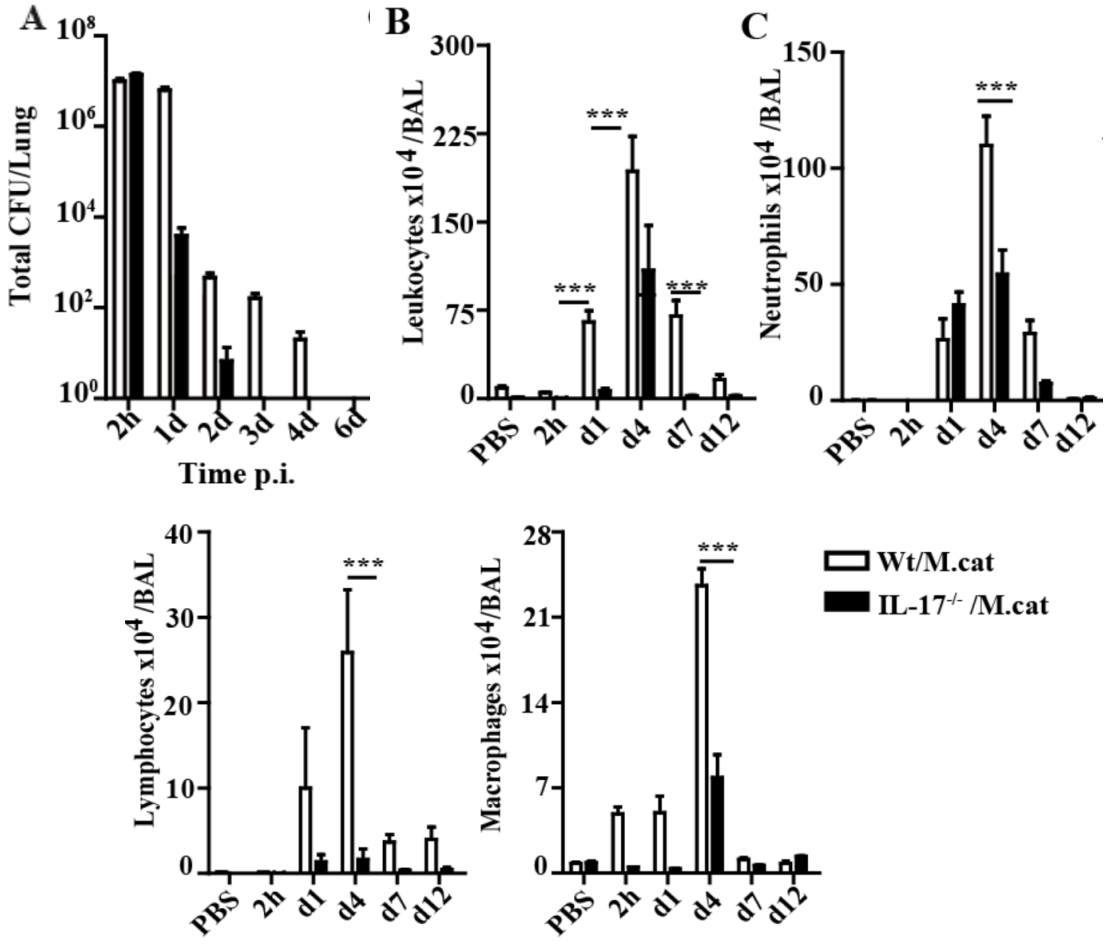


Figure 17. M.catarrhalis infection in WT and IL-17 KO mice

*IL-17 KO (black bars) and wt mice (white bars) were infected (i.n.) with M.catarrhalis. BAL and lung were harvested at indicated time points. (A) Bacterial recovery from lung homogenates (B) airway inflammation represented by BALF total cell counts (C) Differential cell counts. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

To determine the cause of faster clearance in IL-17AF KO mice we wondered whether early neutrophil influx is altered in IL-17AF KO mice after *M.catarrhalis* infection

However, cell counts in the BAL-fluid and lung tissue revealed 24h after infection comparable number of neutrophils in wt and IL-17 KO mice (Fig.17 A), Similar or enhanced levels of the CXCL1, 5 and 10 chemokines in IL-17 KO mice seems to compensate for early neutrophil attraction independently of IL-17 (Fig.17 B).

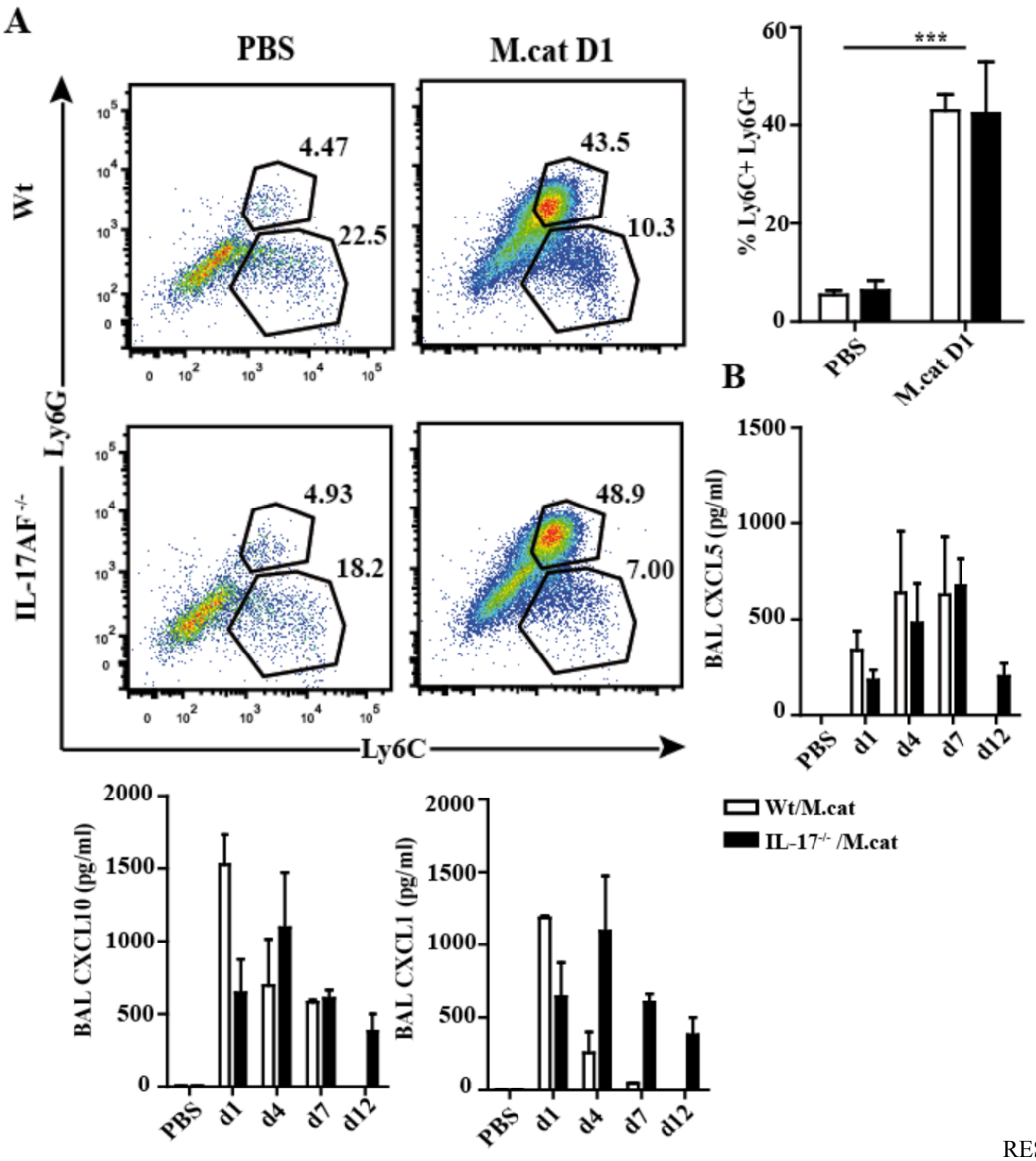
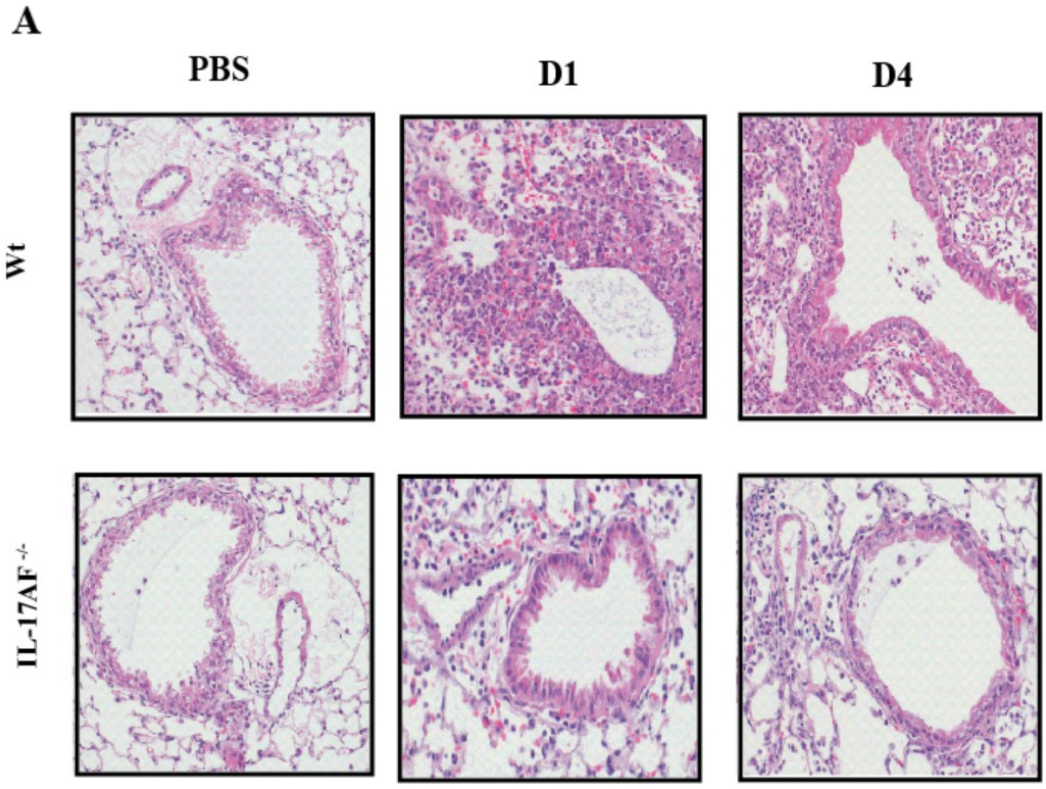


Figure 18. Neutrophils influx induced by M.catarrhalis infection in early phase is independent of IL-17AF

*(A)Lung cells measured by FACS for neutrophil cell which are Ly6C^{+} and Ly6G^{+}positive and Macrophages which are Ly6C^{+}Positive at day 1 after M.catarrhalis infection (B) BALF levels of CXCL1, CXCL5 and CXCL10 were assessed by using CBA assay. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

Furthermore, for examination of pathophysiological changes of the lung induced by allergic asthma, the lung sections were first stained with haematoxylin and eosin. The amount of infiltration cells is a direct hint of disease severity. The graph (fig.19 A) shows the number of infiltrating cells around the vessels of the airways in *M.catarrhalis* infected mice , which was significantly reduced in IL-17AF$^{-/-}$ mice compared to the WT mice in different time points.



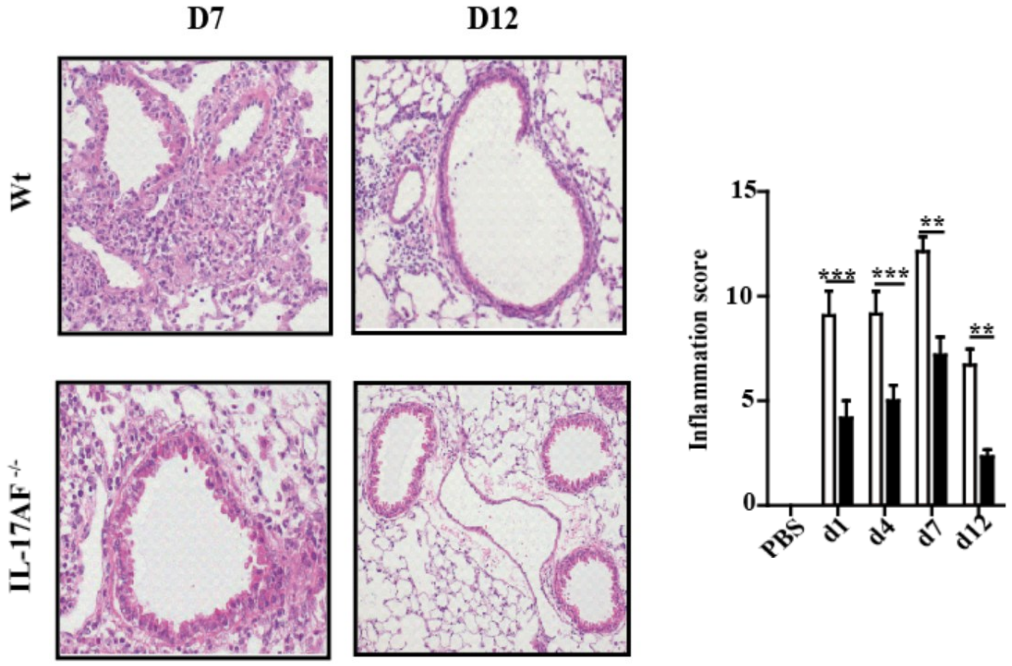


Figure 19. Histological analysis of the lung tissues after M.catarrhalis infection

*(A) Hematoxilin and eosin stained for infiltration of cells and pathophysiological changes and lung damage in PBS or M.catarrhalis in different time points in WT and IL-17AF$^{-/-}$ mice. (B) Inflammation Score. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

5.10 IL-17AF DEFICIENCY RESULTS IN REDUCED MORBIDITY AND MORTALITY OF *M.CATARRHALIS* INFECTION

As *M. catarrhalis* has been shown to induce a strong inflammatory immune response via activation of the epidermal growth factor receptor (Slevogt H et al. 2014), the amount of inflammatory cytokines was analyzed in serum and BAL. *M. catarrhalis* infection induced on day one significantly more IL-6 and TNFα in serum and IL-6, TNFα and IL-1ß were significantly induced in BAL WT animals compared uninfected mice. These inflammatory cytokines were massively reduced in infected IL-17 KO mice (Fig.20 B). To address whether this dramatic increase in proinflammatory cytokine production has any consequences on survival, we analyzed the responses to *M.catarrhalis* in mice of WT and IL-17AF KO mice. Despite the same infection dose (2×10^8 CFU), WT mice (days 1 till 7 after infection) died within 24-48 h, whereas all IL-17 KO mice survived (Fig.20 C).

Thus, these findings suggest that IL-17AF plays a role in the enhancement of morbidity and mortality of *M.catarrhalis* infection.

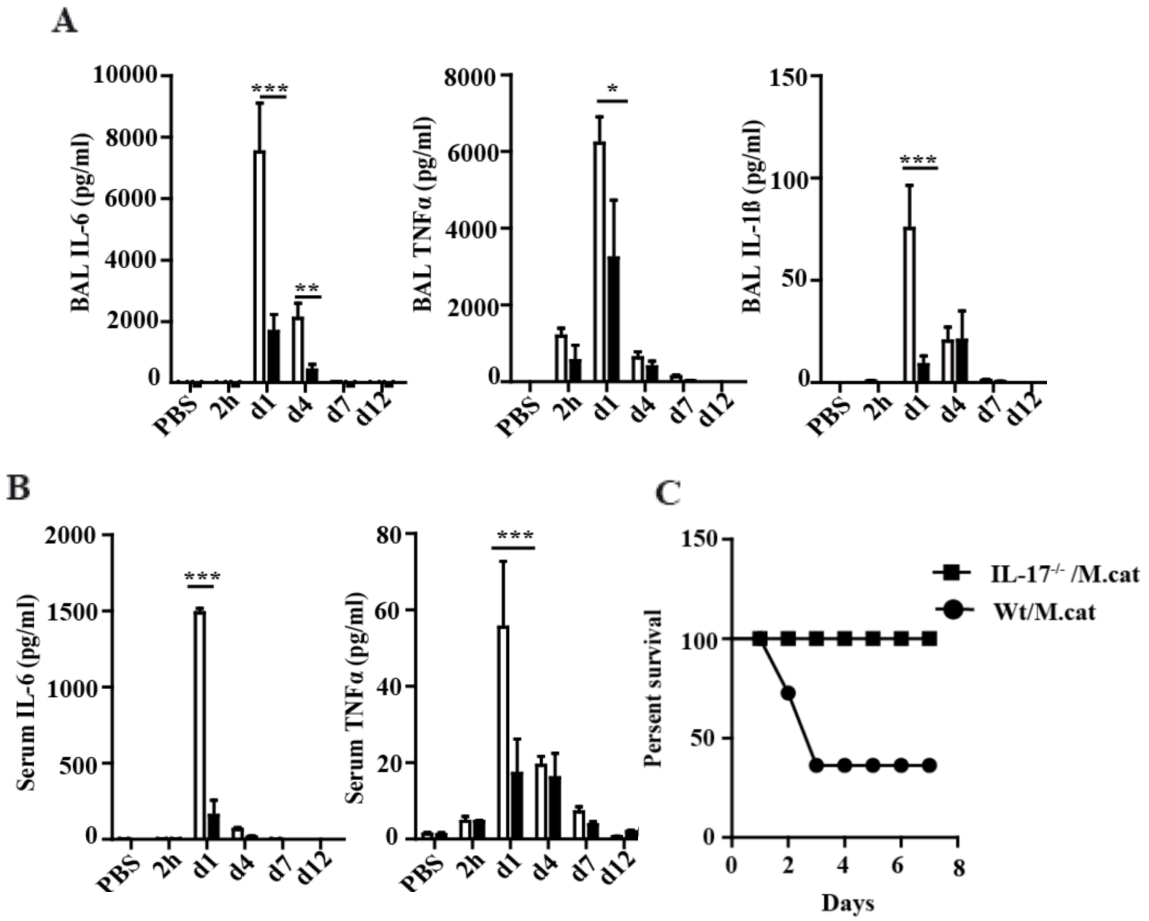


Figure 20.Reduced local and systemic pro-inflammatory cytokines in IL-17AF$^{-/-}$ mice upon M. catarrhalis infection

*WT and IL-17AF-/- mice were infected with 2×10^{8} CFU of M.catarrhalis infection (A,B) Proinflammatory cytokines were analysed by CBA flex assay for Pro-inflammatory IL-6, TNF-a and IL-1ß cytokines at different time points in BAL and Serum. (C) Survival curve. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

To determine whether Tregs and IFNγ were involved in the suppression or enhancement of airway inflammation, we quantified Tregs after infection in both Wt and IL-17KO mice. We observed that IL-17KO mice infected with *M.catarrhalis* increased the_numbers of Tregs, and IFNγ similarly to infected WT mice (Fig.21 A, B, C, D). Taken together, *M.catarrhalis* infection does not affect Treg cells nor Th1 cells in the absence of IL-17.

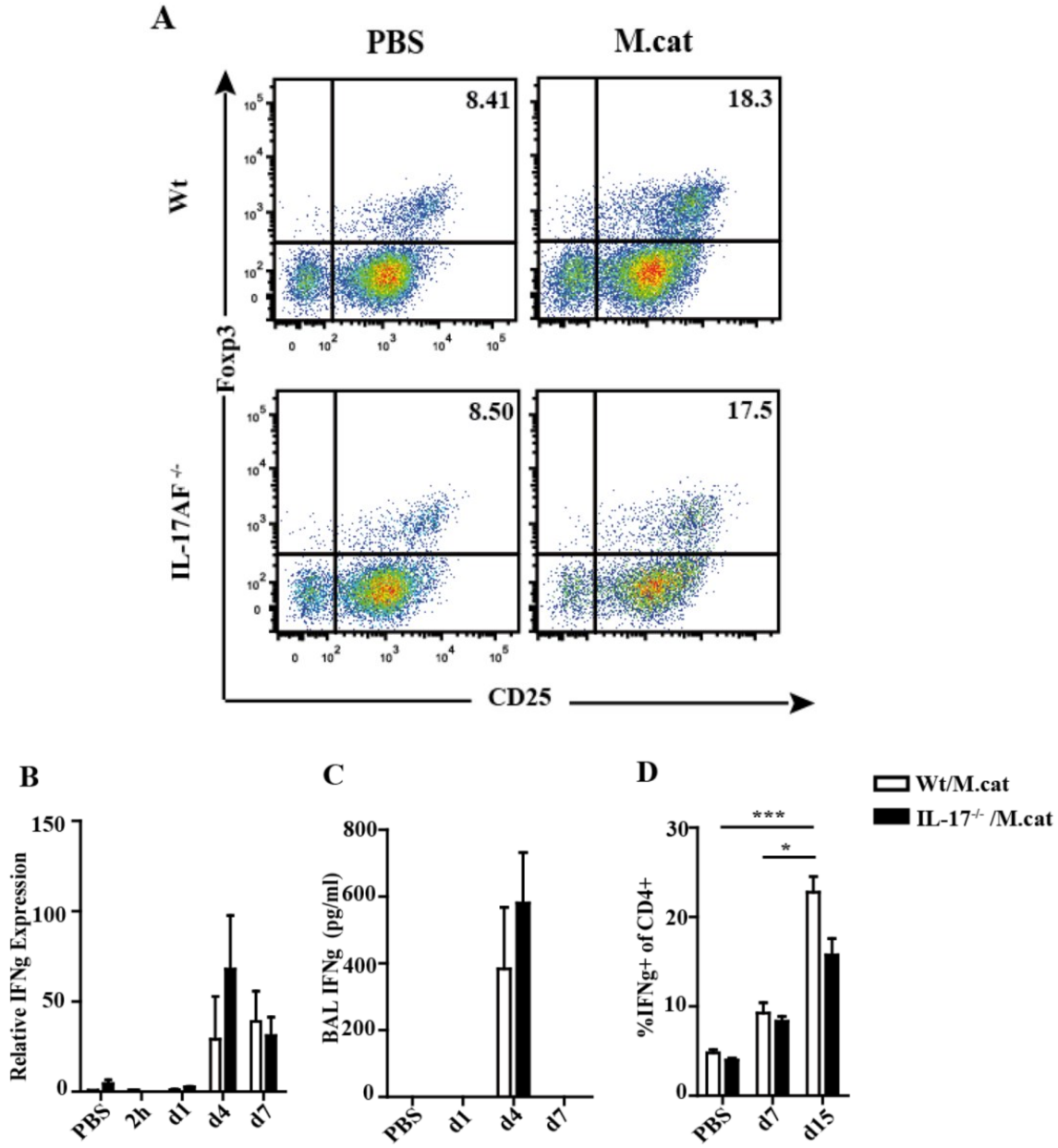


Figure 21. Analysis of CD25+, FoxP3+ T cells and IFNγ in the lung of WT and IL-17AF KO mice after M.catarrhalis infection

Treg numbers in the lung, IFNγ numbers in the BAL and lung at indicated time points (A)Tregs cells were analysed after 7 days of M.catarrhalis infection in Wt and IL-17AF$^{-/-}$ mice. (B) IFNγ measurement in the BAL (C) mRNA expression in lung tissue for IFNγ was assessed (D) frequency of IFNγ^{+} CD4^{+} T cells was analysed by flow cytometry.

5.11 *M.CATARRHALIS* EXACERBATION OF HDM-INDUCED ALLERGIC INFLAMMATION IS DEPENDENT ON IL-17

To further investigate whether IL-17 is also crucial for infection induced exacerbation of allergic airway disease (AAD), IL-17 KO mice were infected during HDM allergen

exposure and analyzed, as shown in (Fig.22 A). The data demonstrate that both total cellularity and the numbers of neutrophils, eosinophils and lymphocytes as well as CXCL1 expression in the BAL of infected IL-17 KO mice were comparable to the non-infected, HDM control group (Fig.22 B, C).

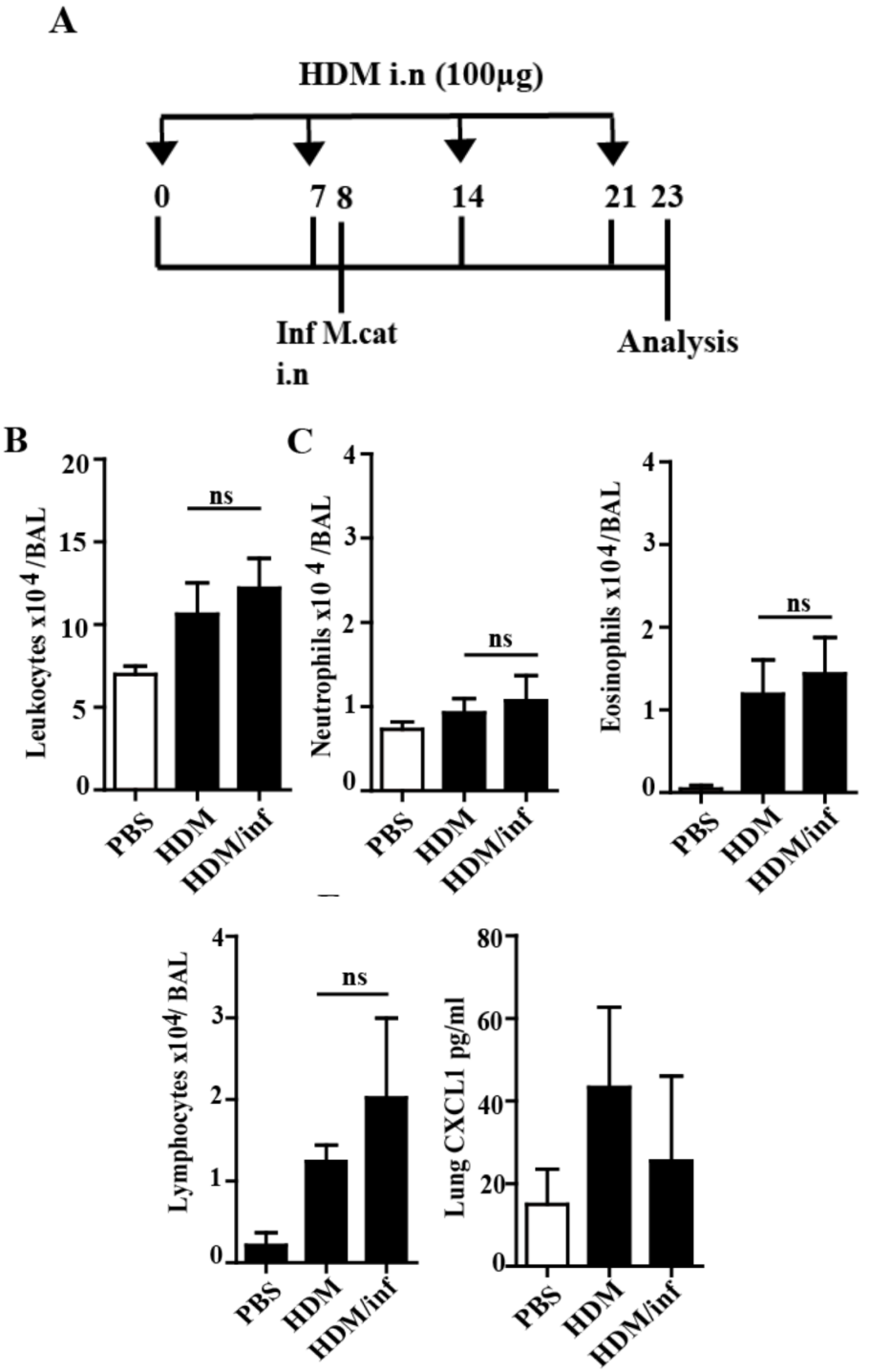


Figure 22. M. catarrhalis induced exacerbation of AAI is mediated by IL-17

C57Bl/6 animals were intranasally infected with 2x10^8 CFU M. catarrhalis after second HDM exposure. BAL and lungs were analyzed at day 23. (A) Protocol (B) Total cell counts in BALF. (C) Differential cell counts. (D) The secretion of CXCL1 chemokine in the BAL.

Furthermore, Lung slices were stained for mucus production by PAS staining and goblet cells. As visualized by PAS staining, PBS control mice had healthy lungs whereas the HDM allergic mice showed mucus-producing goblet cells within the lung epithelium of IL-17AF$^{-/-}$ mice. However, *M.catarrhalis* infection during HDM exposure showed no further increase of airway inflammation, goblet cell hyperplasia and mucus production from the lung compared to HDM allergic IL-17AF KO group (Fig.23, A, B).

Thus, similar numbers of mucus secreting cells and inflammation score suggests that IL-17 is a key cytokine for infection induced exacerbation of AAD (Fig.23 C).

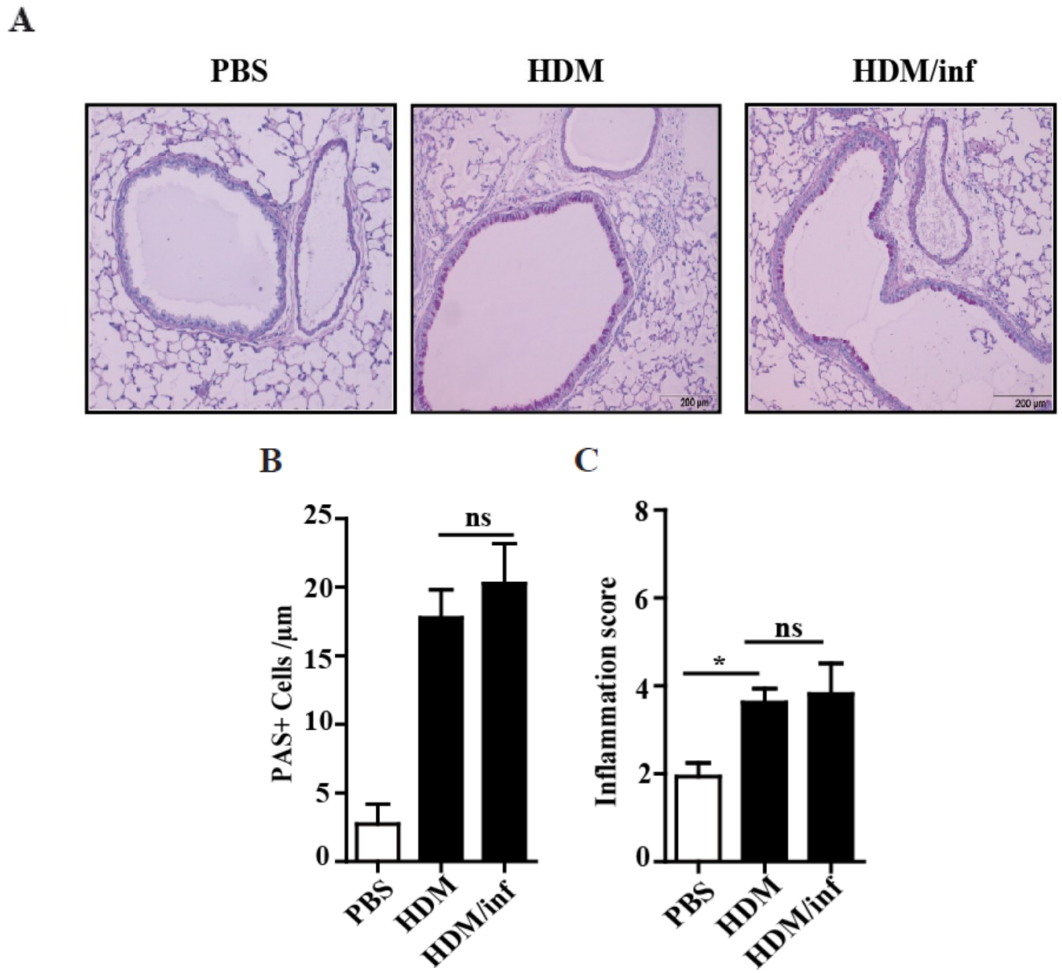


Figure 23. Histological lung analysis of M.catarrhalis infection during HDM induced AAD in IL-17AF$^{-/-}$

*Animals were treated with HDM or PBS on days 0, 7, 14 and 21, analyses refer to day23. When the mice were killed and samples were taken. (A) Representative PAS- stained airways. (B) Goblet cell counts in lung tissues. (C) Inflammation Score. . Data were from two independent experiments (n=8 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

However, it has to be mentioned that infection of HDM allergic IL-17 KO mice still enhanced the expression of pulmonary IFNγ as well as IL-5 and IL-13 but not TNF-α in the BAL (Fig 24 A, B). The absence of IL-17 did not completely abrogate HDM asthma, which suggested that other factors such as Th2 and Th1 drove the remaining allergic response (Fig.24 C, D). Serum levels of HDM-specific IgG1 and IgG2c antibodies were also not differed in the *M.catarrhlis* infection during HDM challenge as compared to the uninfected allergic HDM group (Fig.24 E).

Together, these findings show that IL17 similarly contributes to exacerbation of HDM-induced allergic responses by *M. catarrhalis* infection.

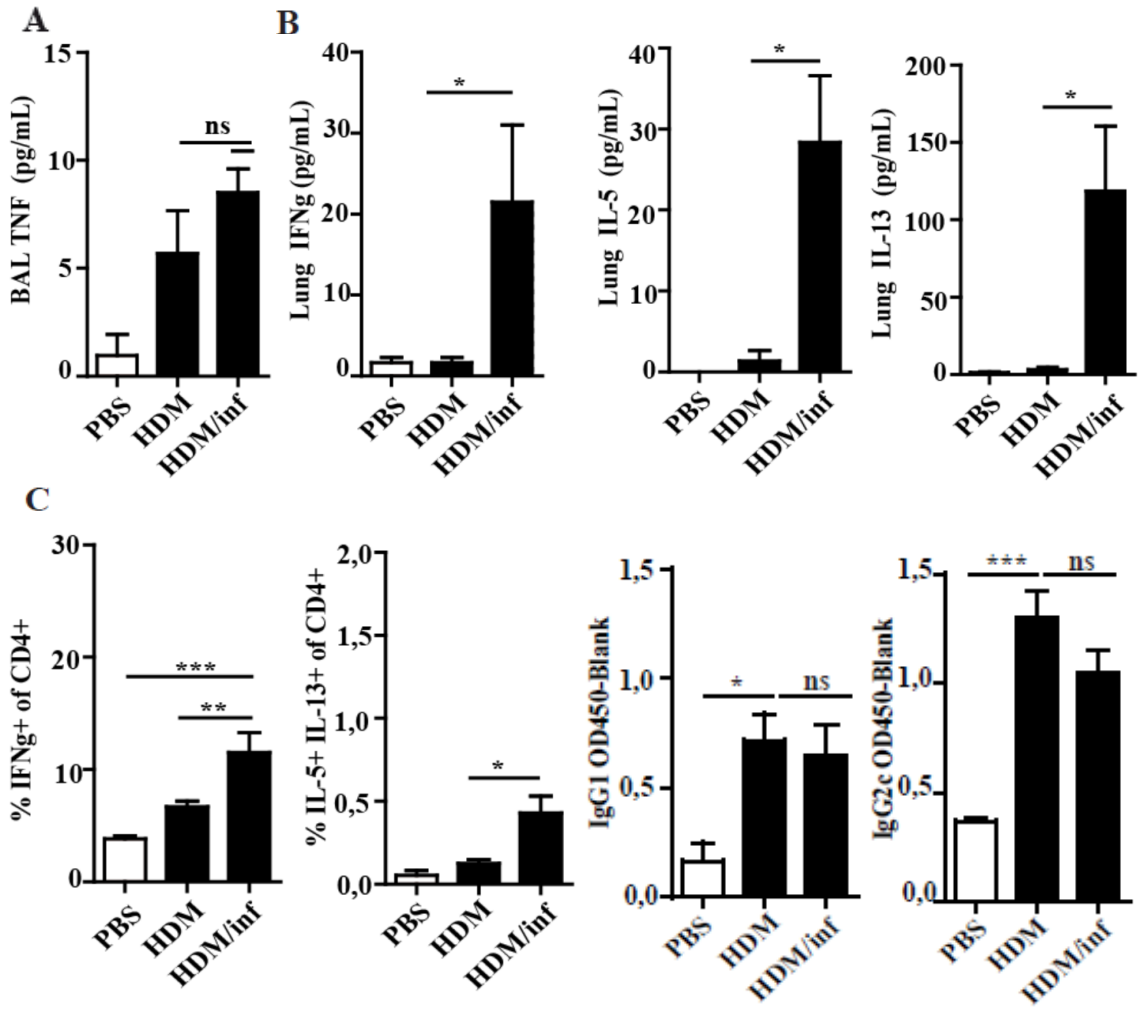


Figure 24. IL-17AF$^{-/-}$ mice are protected from M.catarrhalis induced asthma exacerbation

C57Bl/6 animals were intranasally infected with 2x10^{8} CFU M. catarrhalis after second HDM exposure. BAL and lungs were analyzed at day 23. (A) TNF-α in BALF (B) IFNγ, IL-5 and IL-13 in lung homogenates (C)Intracellular staining of CD4^{+} lung T cells producing IFNγ, IL-5 and IL-13. (D) Measurement of the total amounts of IgG1, IgG2c titers in the serum.Data were from two

*independent experiments (n=16 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

5.12 ANTI TNF-α TREATMENT ATTENUATES IL-17 DEPENDENT EXACERBATION OF INFECTION INDUCED AHR

Increased amounts of pulmonary TNF-α is found in children infected with pathogenic airway bacteria or patients suffering from severe, refractory asthma. Further, it known that TNF-α and IL-17 act synergistically in inflammation triggered recruitment of neutrophils (Griffin G, 2012 J.I.) We thus wondered whether TNF-α neutralization attenuates the inflammatory phenotype of infection induced exacerbation of allergic airway reactions. Briefly, HDM sensitized mice were treated with anti-TNF-α mAbs (100µg i.p.) four hours before intranasal infection with *M. catarrhalis* and analyzed at day 23 as depicted in Fig.25 A. Control groups included mice that received PBS or HDM alone as well as infected, HDM sensitized animals.

Differential cell counts of BAL cells revealed that predominantly neutrophils were reduced by anti-TNF-α treatment while the numbers of macrophages were increased when compared to HDM/infected controls. Increase of BAL macrophages after TNF-α neutralization seems to compensate for the low number of neutrophils for elimination of *M. catarrhalis*. No significant reduction after TNF-α treatment was seen in the number of eosinophils or lymphocytes (Fig.25 B, C).

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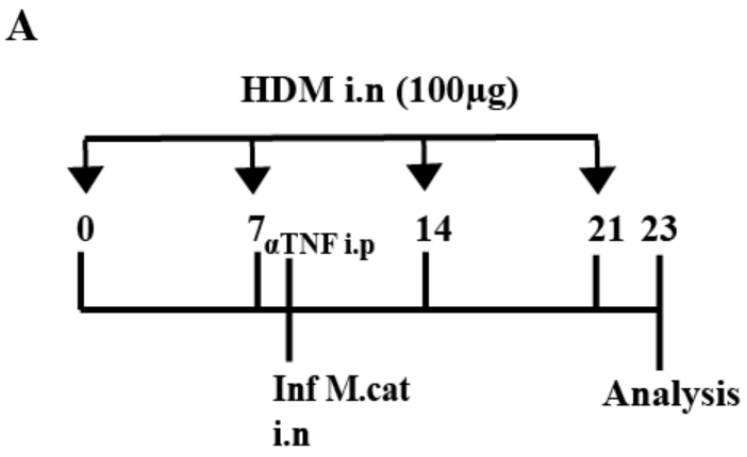


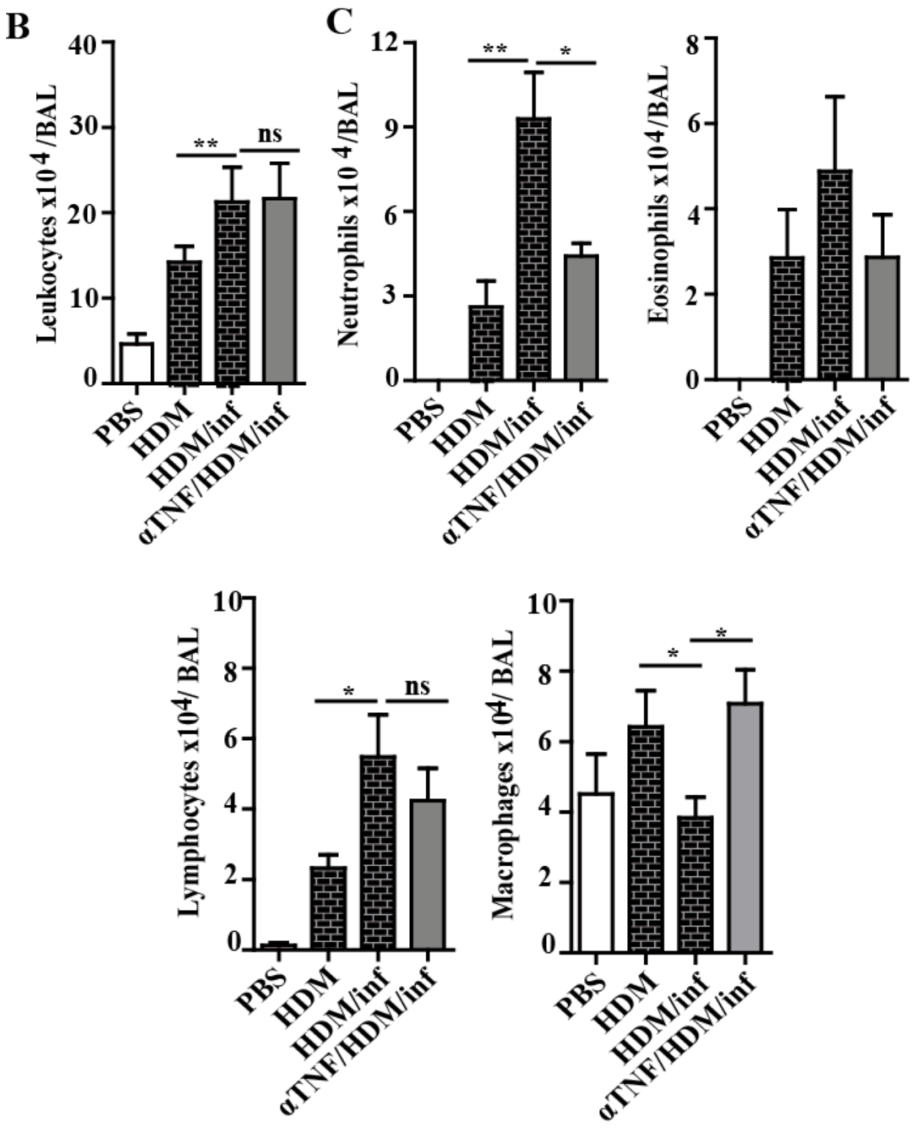


Figure 25. M.catarrhalis during HDM exposure augments allergic airway responses via TNF

*C57Bl/6 animals were i.n infected with 2x10^8 CFU M. catarrhalis after second HDM exposure. Administration of a rat anti murine TNF (100µg; MP6-XT22) or an IgG1 control antibody i.p intraperitoneal before 4 hours of M.catarrhalis infection. BAL and lungs were analyzed at day 23. (A)Protocol (B) Total cell counts in BALF. (C) Differential cell counts. . Data were from two independent experiments (n=6 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

Analysis of the lung tissue confirmed that anti-TNF-α treatment vastly neutralized infection induced inflammatory infiltrates of HDM allergic animals which was also reflected by the reduced number of PAS$^+$ cells and low inflammation score (Fig.26 A,B,C).

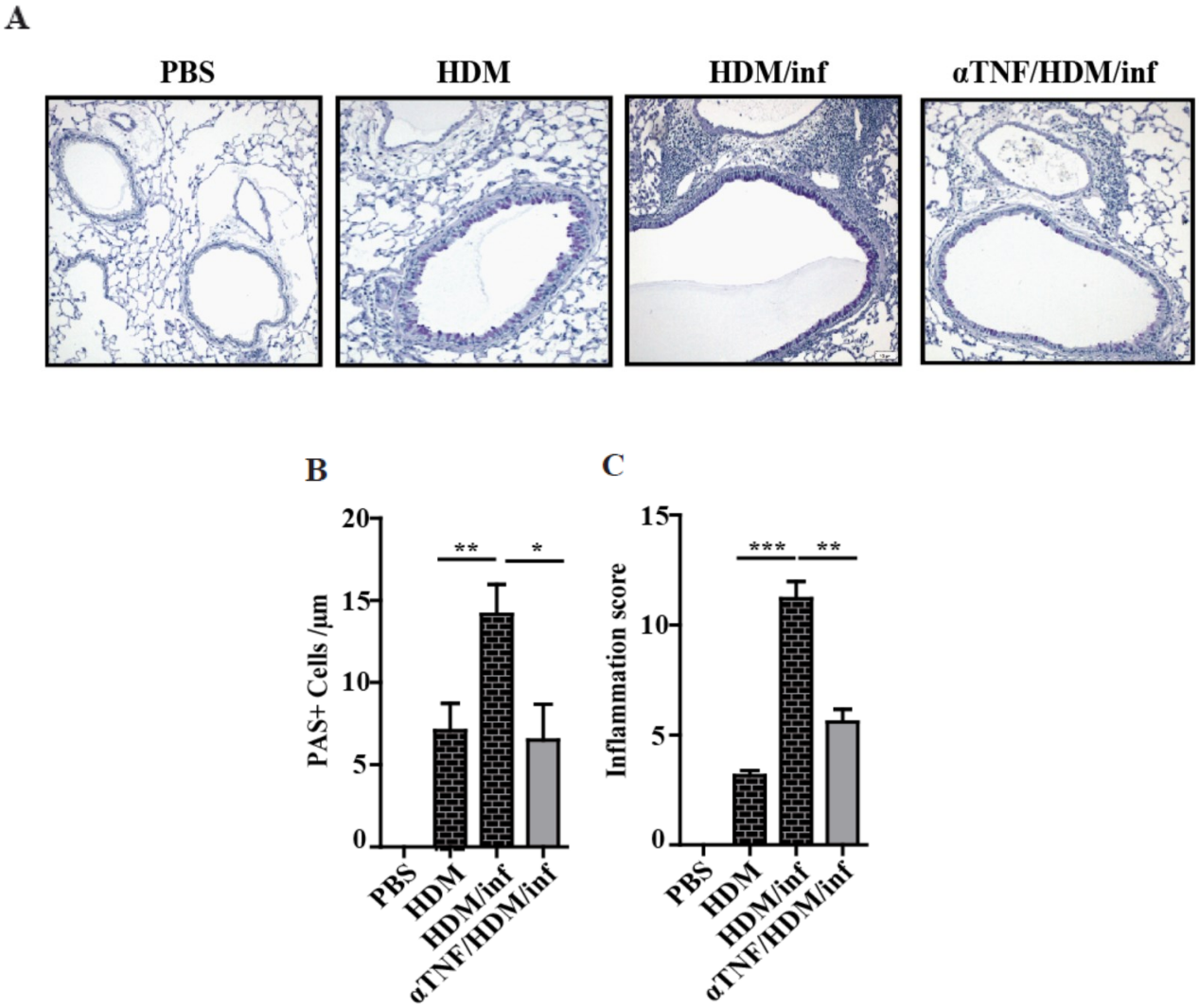
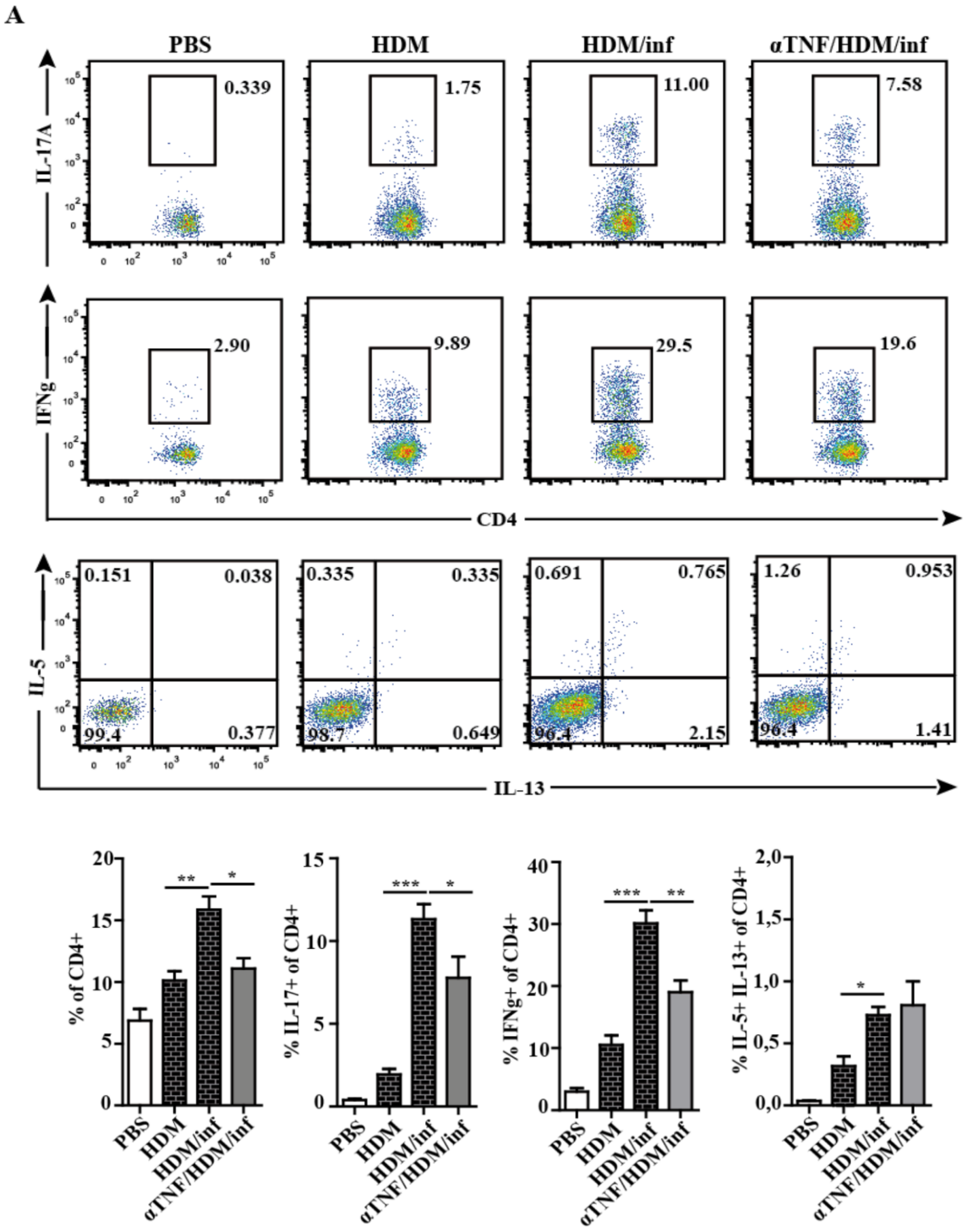


Figure 26. M.catarrhalis during HDM exposure augments allergic airway responses via TNF

*C57Bl/6 animals were i.n infected with 2x10^8 CFU M. catarrhalis after second HDM exposure. Administration of a rat anti murine TNF (100µg; MP6-XT22) or an IgG1 control antibody i.p intraperitoneal before 4 hours of M.catarrhalis infection. BAL and lungs were analyzed at day 23. (A) Representative periodic acid Schiff- stained airways. (B) Goblet cell counts in lung tissues. (C) Inflammation score. . Data were from two independent experiments (n=6 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

We next wondered whether TNF-α neutralization also affects pulmonary T cell activation. Analysis of lung Th1, Th2 and Th17 cytokine secreting CD4$^+$ T cells revealed that anti-TNF-α treatment slightly attenuated the activation of IFN-γ$^+$ and IL17$^+$ lymphocytes but had no influence on the low frequency of IL5/IL13 secreting T cells (Fig. 27 A,B). Together with the unaltered ratio of IgG1 and IgG2c antibody titers, the data show anti-TNF-α treatment does not affect the Th1/Th2 balance.

In summary, the experiments revealed that one application of anti-TNF-α mAbs is sufficient to hugely attenuate *M. catarrhalis* induced exacerbation of AHR



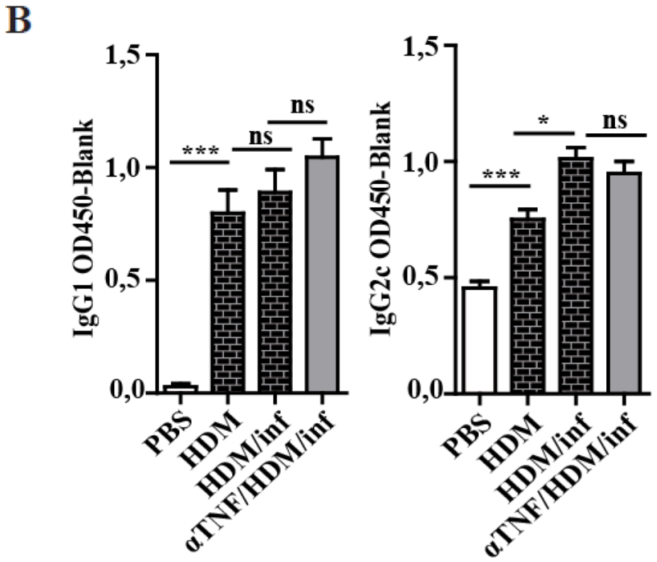


Figure 27. M.catarrhalis during HDM exposure augments allergic airway responses via TNF

*(A) staining of $CD4^+$ lung T cells producing IL17A, IFNγ, IL-5 and IL-13.(B) Measurement of the total amounts of IgG1, IgG2c titers in the serum. Data were from two independent experiments (n=16 mice per group). ***P=0.001, **P=0.01, *P=0.05 (1 way ANOVA).*

6. DISCUSSION.

Asthma is a complex syndrome with many clinical phenotypes and caused by a combination of complex and incompletely understood environmental and genetic interactions (Martinez et al. 2007). It has been recently shown that increased rates of asthma are due to changing epigenetics and changing living environment (Dietert RR et al. 2011). Many environmental factors have been associated with asthma development and exacerbation including allergens, air pollution, dust mites cockroaches and infections. Certain viral and bacterial respiratory infections, such as rhinovirus, *Haumophilus infuenzae* and *Moraxella catarrhalis* have been shown to increase the risk of the development of asthma and asthma exacerbation (Murray et al. 2010) (Bisgaard et al. 2007), while other microbial infections, such as *Acinetobacter lowffii,* seem to be associated with a reduced risk of asthma development *(*Debarry J et al. 2007*).*

These findings demonstrate that we are still lacking a deep understanding of the mechanisms that determine asthma protection versus induction or exacerbation by pathogens. It has been shown that *M.catarrhalis* is a common pathogen that causes middle ear infections and exacerbations of chronic obstructive pulmonary disease in infants and children and lower respiratory tract in adults in the USA (Murphy et al. 1998) (Herva E et al. 2001*)*. However, compared to *H.influenzae* and *S.pneumoniae*, the knowledge about *M.catarrhalis* induced host immune response is lacking. Thus, we were interested to investigate the pulmonary immune response after *M.catarrhalis* infection and the nature of the association between *M.catarrhalis* infection and allergic airway inflammation.

6.1 PULMONARY INNATE IMMUNE RESPONSE IN *M.CATARRHALIS* INFECTION

In this study we have established and characterized for the first time a murine model of *M.catarrhalis* infection with a strain isolated from humans. Since *M.catarrhalis* strain was isolated from human patients, we were wondering if it is able to colonize mice after intranasal instillation. Infection of C57Bl/6 mice revealed that bacterial titers of the lung were maximal at day 2 and then declined rapidly resulting in clearance by day 6 in WT mice.

In contrast, the previously described murine infection with *H. influenzae,* which is also associated with asthma exacerbation, revealed the course of infection is quite different to that of *M. catarrhalis*. Here bacterial numbers peaked at day 5 and clearance of the pathogen was observed by day 16 after infection *(Ama-Tawiah Essilfie*. et al. 2011). Moreover, bacterial clearance was mediated by early but surprisingly low amount of neutrophils in the airways. Compared to *H. influenzae*, *M. catarrhalis* infection induced with delayed kinetics high amounts of neutrophils which seem to be responsible for the fast clearance of this pathogen. Accordingly, high amounts of chemokine attracting neutrophils in the BAL coincide with the prompt appearance of neutrophils and may act as a feedback loop to further attract neutrophils and T cells.

While TNF stands as one of the most impactful cytokines in defence against a wide range of infections, it also plays a central role in the pathogenesis of an array of inflammatory diseases. Indeed, TNF blockade represents one of the most effective treatments for autoimmune diseases, such as rheumatoid arthritis, while increasing the risk of some infectious diseases. Despite the investigation for over four decades, the mechanism by which TNF provides resistance against infection remain unclear and has been attributed to many affects, such as enhancing the expression of cytokines, chemokines, inflammatory cell trafficking and activating phagocyte oxidase (Sedgwick et al, 2000; Strieter et al. 2002). Although previous studies has demonstrated that TNF- deficient mice are more susceptible to infections such as *K.pneumoniae* (Laichalk et al. 1996), it is still unclear what effector functions and TNF producing cells are induced.

A recent study has shown that *M.catarrhalis* induced activation of mitogen activated protein kinase (MAPK) is essential for the secretion of pro.inflammatory cytokine TNF-α (Ferdaus Hassan, Xin-Xing Gu et al. 2012). *DeVries* has also shown that TNF-α is upregulated after *M.catarrhalis* infection by human epithelial cells (DeVries et. el 2004).Similarly, our study confirmed that *M.catarrhalis* induces TNF-α after 24 hours of infection. To determine the source of TNF-α which could be produced by several cells such as monocytes, dendritic cells, B cells, $CD4^+$ T cells, neutrophils, mast cells and eosinophils, and stromal cells, ie, fibroblasts, epithelial cells, and smooth muscle cells. We found that TNF-α is produced by tracheal epithelial cells which are the cells that meet the infection for the first time and start an immune reaction against *M.catarrhalis*. These cells were isolated from WT animals infected with *M.catarrhalis* and analysed at 4 and 24 hours after infection.

One of the most striking discovery was that TNF is the mediator of endotoxin poisoning and thus plays an important role in septic shock. So we wondered whether TNF-α is the mediator of pulmonary inflammation and thus responsible for the lethality often seen after *M.catarrhalis* infection. Our study showed that TNF-α enhances IL-6, IL-17 and IL-1ß cytokines which are known to play an important role in many respiratory infections as pro or anti-inflammatory cytokines. Since *M.catarrhalis* infection resulted in enhanced mortality at the peak time point of these cytokines suggesting their involvement in infection induced pathology. We found that neutralization of TNF-α resulted in local and systemic reduction of IL-6, IL-1ß and IL-17 with subsequent protection and survival in *M.catarrhalis* infected mice. Collectively, in some acute infection cases, monoclonal anti-TNF antibodies could have a high therapeutic value (K.Thavarajah et al. 2009).

Next, to characterize the involvement of IL-6. Since IL-6 is one of the cytokines that were induced after *M.catarrhalis* infection together with the proinflammatory cytokines TNF-α and IL-1ß. We wondered whether the contribution of IL-6 during *M.catarrhalis* infection was similar to TNF-α. IL-6 is known to play an important role in the induction of acute phase reactions. Recent studies suggested that IL-6 plays an anti-inflammatory role during *Y. enterocolitica* and *S.pneumoniae* infection (Dube PH et al. 2004) (Albrecht LJ et al. 2016). Another study has shown that IL-6 plays a pro-inflammatory role during enteroviral infections (Khong WX et al. 2011). However, whether it plays a pro- or anti-inflammatory role in local or systemic responses after *M.catarrhalis* infection remains unclear. In order to address this, we neutralized IL-6 by injection of antibodies and observed increased local and systemic TNF-α as well as early IL-1ß and IL-17 in the BAL. Most surprisingly, anti-IL-6 treatment resulted in enhanced mortality demonstrating that IL-6 exerts an anti-inflammatory function during pulmonary *M.catarrhalis* infection by controlling the level of proinflammatory cytokines.

6.2 PULMONARY TH17 IMMUNE RESPONSE TO *M.CATARRHALIS* INFECTION

IL-17 is an important mediator of neutrophilic inflammation during infection and in disease states (Gibson PG et al. 2001) (Simpson JL et al. 2007). IL-17 is produced by several cells including Th17 cells (Hellings PW et al. 2003), γδ T cells (Lockhart E et al. 2006),

macrophages (Song C et al. 2008) and neutrophils (Ferretti et al. 2003) and has critical roles in host defence against bacterial infections(Ye P et al. 2001), suggesting a potential role in the pathogenesis of bacterial-induced neutrophilic asthma.

The importance of IL-17 in mediating protection against pulmonary bacterial infection was shown for several pathogens, including *Klebsiella pneumoniae*, *Mycoplasma pneumoniae, Bordatella pertussis* and *M.tuberculosis* after vaccination with BCG (Baraldo et al. 2007). Consequently, IL-17 receptor KO mice revealed an increased susceptibility for bacterial infections, due to decreased neutrophil and macrophage recruitment to the site of bacterial entry (Barnes 2008). But at the same time, IL-17 released into the airways recruits neutrophils and thus promotes neutrophilic asthma which is characterized by treatment resistance compared to other phenotypes (Larsen et al. 2014; Fahy 2009; Brightling et al. 2008). Most importantly, expression of pro-inflammatory IL-17 in the airways of asthmatics has been shown to correlate with disease severity (N'Guessan et al. 2014).

Since IL-17 has been described as key cytokine involved in the pathogenesis of *H.influenzae* infection triggered neutrophilic inflammation in allergic airway disease. Therefore we tested the amount, frequency and the origin of IL-17 production after *M.catarrhalis* infection. We could show that the majority of IL-17 secreting cells in the lung were conventional $CD4^+$ T cells which started to produce IL-17 at day 7 post infection in BAL and lung. However the role of IL-17 producing cells in *M.catarrhalis* infection was unknown. To investigate the contribution of IL-17AF after *M.catarrhalis* infection we used IL-$17AF^{-/-}$ mice versus wt. Despite comparable amounts of *M. catarrhalis* titers two hours after infection , IL-17AF KO mice cleared bacteria surprisingly more rapidly from the lung as wt animals. Cell counts in the BAL-fluid and lung tissue shortly after infection revealed comparable number of neutrophils in wt and IL-17 KO mice. This might be explained by similar or enhanced levels of the chemoattractant CXCL1, CXCL5 and CXCL10 in IL-17AF KO mice which might compensate for early neutrophil attraction independent of IL-17. However, later stages of infection, the amount of neutrophils was significantly lower in IL-17AF deficient mice, most likely other compensatory mechanisms which were not addressed in our work might be active. On the other hand, lack of IL-17 might well explain the reduced pathology which is seen in lung sections of *M. catarrhalis* infected IL-$17AF^{-/-}$. In accordance with our findings, a similar study has shown that infection of IL-$17^{-/-}$ mice with *K. menoumonia* did not reduce the recruitment of neutrophils into infected lungs (Xiong H et al. 2016). Thus, it remains

enigmatic why IL-17AF KO mice clear the infection faster and recruit similar amounts of early neutrophils after *M.catarrhalis* infection.

6.3 T CELLS IN *M.CATARRHALIS* INFECTION

Several studies have proposed that different kinds of infection such as *Streptococcus pneumoniae*, can promote the differentiation of Treg cells (Preston J.A 2010). Tregs are an important cell involved in immune tolerance and the suppression of inflammation. We wondered whether the protection affect in IL-17AF$^{-/-}$ infected mice was due to enhanced Tregs. *M.catarrhalis* infection induced CD25^{+} FoxP3^{+} CD4^{+} T cells with similar frequencies in wt and IL-17AF$^{-/-}$ mice. Although the regulatory function of Tregs have not been analyzed in detail, our findings suggest that *M.catarrhalis* induced Treg cells have a limited ability to control Th1 and Th17 responses. However, to make a final conclusion about the involvement of Tregs in infection triggered asthma responses, experiments with Treg deficient mice have to be performed.

Another important cytokine for antibacterial defence is IFNγ which is produced primarily by T cells and NK cells.It has been shown that IFNγ plays an important role in mediating macrophage activation in controlling a number of intracellular pathogens, including leishmanial major (Liew, F.Y et al. 1990), listeria monocytogenes (KIderlen, A.F, S.H. Kaufmann et al. 1984). IFNγ enhances the microbicidal function of macrophages by stimulating the synthesis of reactive oxygen intermediates and nitric oxide (Stark GR, IM Kerr et al. 1998).

So we wondered whether IFNγ plays a role in *M.catarrhalis* infection. In our study IFNγ was strongly induced after *M.catarrhalis* infection in wt, therefore we suggested if the protection of IL-17AF$^{-/-}$ mice was due to increased amounts of IFNγ that may compensate for the lack of IL-17. However, our results clearly revealed that WT and IL-17AF$^{-/-}$ mice produced similar amounts of IFNγ suggesting that IFNγ does not account for enhanced antibacterial protection of IL-17AF$^{-/-}$ mice.

6.4 *M.CATARRHALIS* INFECTION EXACERBATES ALLERGIC AIRWAY INFLAMMATION

M. catarrhalis has been shown to be an important risk factor for newborns to either develop asthma in later years or for adults with chronic pulmonary diseases to exacerbate disease symptoms (Jackson et al. 2011; Pelaia et al. 2006). While there is evidence that the frequency of respiratory infections rather than infection with a specific pathogen influences asthma development, it is currently still not known whether the time of pulmonary infections affects asthma, i.e. what is the influence of infection before, during and after contact with allergen (Bisgaard et al. 2007a). Therefore, we studied the immunological mechanisms of infection triggered asthma exacerbation at various time points and tested potential targets for their therapeutic efficiency. Since murine models of AAD have helped to reveal some of the potential mechanisms and thus play an important role in identifying the contribution of distinct immune reactions in development of allergen- induced inflammation and airway hyperresponsiveness. Today, several experimental models of asthma exist, with OVA and HDM model as the most prominent ones. While the OVA model of allergic inflammation shows several limitations with respect to the human disease (Taube C, et al. 2004), we used the House Dust Mite (HDM) model which is clinically more relevant to humans, as HDM is a common allergen associated with asthma. The model of HDM induced AAD is a mixed Th1/Th2 inflammatory phenotype with increased numbers of eosinophils and neutrophils. As we were interested to study the effect of asthma exacerbation by *M.catarrhalis* with special interest whether the time-point of infection with *M. catarrhalis* influences the outcome of asthma exacerbation. Infection during HDM allergen exposure massively increased the influx of neutrophils and eosinophils while infection after complete HDM sensitization caused asthma exacerbation primarily by neutrophils. In contrast, infection prior to HDM exposure predominantly triggered the expansion of pulmonary T lymphocyte populations. However, common to all three settings of infection is the induction a mixed pulmonary Th1, Th2, and Th17 immune response. Thus, our data suggest that bacterial infection irrespective of the time-point of allergen exposure influences the development or outcome of allergic airway reactions and reflects the situation in patients: Viral and/or bacterial infections in early life or during asthma have been associated with the development or exacerbation of asthma, respectively (Bisgaard et al. 2007b; Lambrecht und Hammad 2015; Vock et al. 2010b). Our experimental data show, that a non-persisting infection of the respiratory tract with *M.catarrhalis* has the potential to exacerbate lung allergic inflammatory responses,

independently of the time, i.e. before, during or after allergen contact. Infection-triggered exacerbation of HDM-allergic reactions was due to the enhanced influx of neutrophils, eosinophils and Th1, Th2, and Th17 cells together with enhanced secretion of inflammatory IL-17, TNF-α, IFN-γ and the Th2 cytokines IL-5 and IL-13.

However these murine experimental findings have to be set in the context of the human situation. Hans Bisgaard has demonstrated that the association between *M.catarrhalis*, *H.influenzae* and *S.pneumoniae* and acute severe exacerbation of wheeze was time specific. This would show that our findings are not of clinical relevance. Bisgaard has shown that newborns infected with *M.catarrhalis* are more prone to asthma at later life and patients with established asthma will develop exacerbation of the disease.
Probably most interesting is the fact what happens if we were infected with *M.catarrhalis* a long time before allergic sensitization. Under such conditions, the effect of pathogen-specific memory T cells on asthma development or exacerbation can be tested. With respect to our experimental setup, this might have been too early.

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6.5 IL-6 AND TH1 IN ASTHMA

IL-6 has been considered as promising target to reduce pathogenesis of asthma and COPD, particularly those of eosinophilic/neutrophilic phenotype (Bonnelykke et al. 2015; Green et al. 2002). On the other hand, IL-6 is known to mediate pro-inflammatory and anti-inflammatory immune functions, which are mediated via trans- and classical signaling, respectively (Folsgaard et al. 2013). Our data however show, that anti-IL6 treated, non-allergic animals were highly susceptible to *M. catarrhalis* infection with high amounts of local and systemic TNF-α that we did not further consider IL-6 as appropriate target for treatment of *M. catarrhalis* triggered asthma exacerbation.

The typical Th1 cytokine IFNγ has been shown to play an important role in the development of AHR and in aggravating inflammatory events in asthma. Moreover, studies could not show airway hyperresponsiveness after adoptive transfer of OVA specific Th1 cells, other groups were able to induce AHR (J.Cui, SPazdziorko et al. 2005). However, Th1and Th2 responses may even act in combination to augment each other's activity to induce airway inflammation

and AHR (J.G.Ford et al. 2001).Nevertheless, more studies are required to fully elucidate the role of Th1 cells and especially of IFNγ in asthma pathology. (Christina Vock et al. 2010). Since *M.catarrhalis* infection induced significantly Th1 cells during allergic airway disease. Therefore we were interested to investigate the effect of Th1 cell in our model. Since IL-17AF$^{-/-}$ mice were protected against *M.catarrhalis* induced AAD we wondered whether the protection affect in IL-17AF$^{-/-}$ infected mice was due to altered Th1 cells . *M.catarrhalis* infection induced IFNγ^{+} CD4^{+} T cells with similar frequencies in wt and IL-17AF$^{-/-}$ mice. Although the function of Th1 cells have not been analyzed in detail, our findings suggest that *M.catarrhalis* induced Th1 cells have a limited ability to control Th2 and Th17 responses. However, to make a final conclusion about the involvement of Th1 in infection triggered asthma responses, experiments with Th1 deficient mice have to be performed. Whether these cells play a role in this protection.

6.6 IL-17 IN ASTHMA EXACERBATION

Although asthma is classically associated with eosinophilia and Th2 cytokines, some patients show a neutrophil-predominant disease with an absence of Th2 cytokines. In particular, patients with more severe forms of asthma seem to have neutrophilic inflammation with less reversible airway obstruction and a mixed Th1 and Th17 cytokine milieu (mckinley et al. 2008). The role of IL-17 and Th17 cells in allergic asthma has been fully elucidated, as this cytokine has been shown to sometimes be involved in protection and in other cases (most cases) with favours disease development. Thus, IL-17 can be pro or anti-inflammatory cytokine depending on the time point of neutralization or administration of IL-17 for example, IL-17A plays a protective role in asthma only during the challenge phase (schnyder-candrian et al. 2006). Another study showed that high exposure to diesel exhaust particles is associated with both exacerbated asthma symptoms and increased serum concentrations of IL-17A in children with atopic asthma (Brandt, E.B et al. 2013). In mice and humans, IL-17 can also cause direct contraction of bronchial smooth muscle cells and thus cause bronchial hyperreactivity (BHR) in the absence of neutrophilic inflammation.

It has been previously demonstrated that IL-17A can exacerbate IL-13 induced AHR (Lajoie S, et al. 2010). Other study suggested a contribution of IL-17A to Diesel exhaust particle exacerbation of HDM-induced allergic responses (Brandt Eric B et al. 2013). To study whether IL-17A is involved in the pathology of allergic airway disease, we examined the effects of HDM treated IL-17AF KO mice by a protocol of four times of sensitization and challenge periods. Interestingly, IL-17AF KO mice had significantly less AAD than WT animals which were identically treated. Moreover, we found that the mixed phenotype of neutrophilic/eosinophilic airway inflammation in HDM challenged WT mice switched to an attenuated, almost exclusive eosinophilic response in the absence of IL-17. Moreover, infection during allergic immune response did not lead to exacerbation of pulmonary inflammation in IL-17 KO mice. However, IL-17AF$^{-/-}$ mice did not completely abrogate HDM-induced AAD, which suggested that other factors such as Th2 cytokines, drove the remaining allergic airway response. Together, our experimental findings indicate that pulmonary infection is an essential trigger for IL-17-induced, severe, neutrophilic asthma which is refractory to conventional treatment.

6.7 TNF-α IN ASTHMA

The likelihood that TNF-α contributes to the inflammatory response in asthmatic airways is supported by observations that TNF-α mRNA (Ying et al. 1991a) and protein levels were increased in the airways of patients suffering from asthma. Importantly, inhalation of recombinant TNF-α to normal individuals led to the development of AHR and airway neutrophilia (Thomas et al. 1995b; Thomas und Heywood et al. 2002). The mechanisms driving TNF-α induced AHR have not been fully understood. In addition to its effects on AHR, TNF-α is a chemoattractant for neutrophils and eosinophils, increases the cytotoxic effect of eosinophils on endothelial cells, is involved in activation of T cells, and increases epithelial expression of adhesion molecules such as intracellular adhesion molecule (ICAM) 1 and vascular cell adhesion molecule (VCAM)1.The high amounts of TNF-α produced after airway infection with *M.catarrhalis* together with previously reported beneficial effects with TNF-α blockers in animal models and patients with severe asthma (Curtis und Way et al. 2009) (Zhang et al. 2009a) prompted us to study the effect of TNF-α neutralization under the condition pulmonary bacterial infection and consequent exacerbation of allergic airway

inflammation.. Our data show that TNF-α neutralization was able to largely prevent infection induced exacerbation of HDM-allergic reactions despite mild effects on pulmonary infiltrations of Th1, Th2 and Th17-producing T cells. While the involvement of TNF-α in the development of AHR has been supported by several observations (Jatakanon et al. 1999; McKinley et al. 2008) (Wang und Wills-Karp et al. 2011), the mechanisms by which this cytokine may acts in asthmatic airways are unclear: Besides its effect to induce Rantes and IL-6 in human airway smooth muscle cells (Linden et al. 2001), TNF-α has also been shown to recruit neutrophils and eosinophils (de Vries, Stefan P W et al. 2013a), to enhance T cell responses (Essilfie et al. 2012), to mediate glucocorticoid resistance and finally to be involved in airway remodelling (Chu et al. 2015). Although, repeated anti-TNF-α treatment in our model might have led to even lower numbers of pulmonary infiltrates, previous studies have shown that despite some clinical benefit, prolonged anti-TNF-α therapy was also associated with severe infections or development of metaplastic malignancies (Zhang et al. 2009b; Rincon und Irvin 2012; Scheller et al. 2011).

Further, we identified IL-17 and TNF-α as main mediators of *M. catarrhalis* triggered exacerbation of AHR. Accordingly, ablating or neutralizing of one of the two cytokines was sufficient to prevent *M. catarrhalis* induced exacerbation. However, there is also a complex interaction between Th17 cell driven asthma and tumor-necrosis factor (TNF). Pulmonary and systemic amounts of TNF are increased in patients with severe steroid-resistant asthma, although some studies have not confirmed this (Manni et al. 2014; Berry et al. 2006c).
Moreover, we are aware that both treatment strategies bear risks, as IL-17 and TNF-α have been shown to be crucial for the defence against several human pathogens, including *Streptococcus pneumoniae* (Dejager et al. 2015), *Pseudomonas aeruginosa* (Berry et al. 2006b), *Klebsiella pneumonia* (Ying et al. 1991b), *Mycobacterium tuberculosis* (Bradding et al. 1994) and even rhinovirus infections (Thomas et al. 1995a) .

SUMMARY

In this study we have demonstrated in a murine model of allergic airway inflammation that intranasal infection with the human pathogen *M. catarrhalis* exacerbate pulmonary inflammation.

The aim of the study was: i) to establish an animal model of infection induced exacerbation of lung allergic reactions, ii) to study the pulmonary immune responses of HDM allergic animals with and without infection and iii) to identify potential targets to prevent exacerbation of lung inflammation.

The essential findings are that the human pathogen, *M. catarrhalis,* is able to colonize murine lungs for approximately 6 days and thus can be used as a model germ for acute respiratory infection. We further could show, that infection with *M. catarrhalis* exacerbates allergic airway inflammation (AAI) via innate and adaptive immune responses, depending on the time of infection, i.e. before, during or after established allergic sensitization. While *M. catarrhalis* infection in a non-allergic environment induced high amounts of IL-6 and TNF-α but moderate amounts of T cell derived IFN-γ and IL-17, infection triggered exacerbation of AAI mainly by IL-17 and TNF-α but not IL-6.

Further, we could show that lack of IL-17 or neutralization of TNF-α but not IL-6 is able to prevent *M. catarrhalis* induced exacerbation of AAI.

In conclusion, infection with *M. catarrhalis* has the potential to exacerbate allergic airway inflammation at any time of infection and temporary treatment with anti-IL-17 and/or anti-TNF-α antibodies may be appropriate to prevent aggravation of this lung disease.

ZUSAMMENFASSUNG

Im Rahmen dieser Arbeit konnte anhand eines Mausmodells für allergische Atemwegsentzündung (AAE) gezeigt werden, dass intranasale Infektionen mit humanpathogenen *M. catarrhalis* pulmonale Entzündungsreaktionen verschlimmern kann.
Ziel der Arbeit war i) die Etablierung eines Tiermodells für infektionsinduzierte Exazerbation von pulmonalen, allergischen Entzündungsreaktionen, ii) die Untersuchung pulmonaler Immunantworten von HDM-allergischen Tieren mit und ohne Infektionen und iii) die Identifikation potentieller Zielstrukturen, um eine Exazerbation pulmonaler Entzündungsreaktionen zu verhindern.
Die wesentlichen Erkenntnisse sind, dass der humanpathogene Keim *M. catarrhalis* Lungen von Mäusen infizieren kann und daher als Modellkeim für akute Atemwegsinfektionen dienen kann.
Weiterhin konnten wir nachweisen, dass *M.catarrhalis*-Infektionen allergische Atemwegsentzündungen abhängig vom Infektionszeitpunkt entweder über angeborene und adaptive Immunantworten verschlimmern können. Während eine Infektion mit *M.catarrhalis* in einem nicht-allergischen Umfeld hohe Mengen IL-6 und TNF-α, aber moderate Mengen an T-Zell produziertem IFN-g und IL-17 hervorruft, induziert die Infektion die Verschlimmerung einer AAE hauptsächlich durch IL-17 und TNF-α, aber nicht durch IL6.
Zusätzlich konnten wir zeigen, dass ein Mangel an IL-17 oder die Neutralisation von TNF-α, aber nicht eine IL-6 Neutralisation, die *M. catarrhalis* induzierte Exazerbation von AAE verhindern kann.
Zusammengefasst hat eine *M. catarrhalis*-Infektion das Potential allergische Atemwegsentzündungen zu jedem Zeitpunkt der Infektion zu verschlimmern. Eine temporäre Behandlung mit anti-IL17 und/oder anti-TNF-α Antikörpern könnte geeignet sein, um eine Verstärkung dieser Lungenerkrankungen zu verhindern.

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My academic teachers include the Professors and Privatdozents from:

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