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Mechanistic studies on the allergy protective effect of *Acinetobacter lwoffii* in the mouse

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Bilal Alashkar Alhamwe

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"DER ERSTE SCHRITT IN DER WISSENSCHAFT IST DAS SCHWEIGEN, DER ZWEITE DAS ZUHÖREN, DER DRITTE DAS VERSTÄNDNIS, DER VIERTE IST DIE NUTZUNG IHRES WISSENS UND DER FÜNFTE IST DIE PUBLIZIERUNG"

Moustafa Lutfi Manfalutie

CONTENT

1. INTRODUCTION

1.1. Asthma-allergy-atopy and farm environment

Allergic diseases, such as asthma, allergic rhinitis and atopic dermatitis, whose prevalence have dramatically increased in the last decades, are a major public health issue in industrialized countries. Asthma is a chronic inflammatory airway disease characterized by recurrent episodes/attacks of breathlessness and wheezing, chest pain coughing especially in the night or in the morning (Kudo, Ishigatsubo and Aoki, 2013).

Asthma attacks occur when those symptoms are at their peak, which might begin suddenly and can range from mild to severe. The swelling of the airway can then completely block the oxygen supply through the lungs thus preventing the oxygen from reaching the vital organs, which can be fatal and requires urgent hospitalization (Mims, 2015).

According to the original hypothesis by Strachan made in 1989, the small family size and good hygiene could increase the prevalence of atopic diseases such as asthma in early childhood. This hypothesis provides us with initial views on how the environmental factors such as microbes, pollutants and life style or diet during early childhood could underlie the dramatic increase in the incidence of allergic diseases, especially asthma in industrialized countries (Brooks, Pearce and Douwes, 2013). Considering the low numbers of reported asthmatic patients in the rural or farm areas, living in a farming environment during early childhood was significantly associated with a decreased risk of atopy and allergic diseases during childhood which was also found to be able to extend into the adulthood. Various regulatory pathways are standing behind the protective effects of the farming environment (Burbank *et al.*, 2017). It has been found that, the farm milk (raw milk), farm animals or farm bacteria in early childhood may protect from the development of allergic dieses such as asthma during childhood (Sozańska, 2019) (Schuijs *et al.*, 2015).

The protective effects of farming environment against atopy and asthma have been reported in many cohort studies to be related to the differences in the internal components of the exposome such as environmental microbes between children growing up in farming environment and their peers living in more urban environments (Pekkanen *et al.*, 2018). Such an example is the farm homes' dust microbiota which was defined with very high microbial richness and low-abundance compared to the

non-farm homes' microbiota, for instance, a high availability of *Bacteroidales*, *Clostridiales* and *Lactobacillales* orders in farm environment which could mediated the protective effects against asthma development(Kirjavainen *et al.*, 2019). On the other hand, the non-farm homes showed higher proportions of other microbes such as the *Streptococcaceae* family and *Staphylococcus* genus which may represent a risk factor for asthma development (Frati *et al.*, 2019). Recent studies have demonstrated the role of cowshed bacteria like *Acinetobacter lwoffii* (*A. lwoffii*) or *Lactococcus lactis* (L. *lactis*) in modulating the allergic reactions by changing the capacity of DCs (dendritic cells) to polarize either Th-1 or Th-2 responses. Those bacteria were able to shift the adaptive immunity from Th-2 toward Th-1 reactions in response to a specific allergen and have been shown to prevent the development of allergies or asthma development in experimental mouse model; these findings strongly support the hygiene hypothesis (Debarry *et al.*, 2007b).

1.2. Asthma symptoms and diagnose

Due to the heterogeneity of asthma, the peak of symptoms is quite different from patient to patient which leads to different treatment strategies (Bostantzoglou *et al.*, 2015).

The most common symptoms are wheezing, shortness of the breath and coughing, children with asthma show the same symptoms as in adults, moreover, the pattern of symptoms is also differed leading to a better diagnosis, some of the symptoms occurred in the night or early in the morning, some of them during or after the exercise or in some seasons or after expose to specific triggers/allergens and so on ("Childhood Asthma | AAAAI" USA).

Based on the disease severity, asthma can be classified into four different types, including the intermittent asthma if the symptoms occur less frequently than twice a week, mild persistent asthma when the symptoms persist two or more days a week, moderate persistent asthma if the symptoms are present at least every day, and severe persistent asthma if the symptoms are repeated during the day (Oksel *et al.*, 2018) **(Table. 1)**.

The physical examination is not enough to confirm the diagnose or the type of asthma, therefore, different lungs function tests are to be used, including spirometry, peak airflow, FeNO tests (exhaled nitric oxide) and provocation to measure the breathing pattern (Pavord *et al.*, 2018).

	Frequency of symptoms		% predicted	Variability
	Day	Night	FEV_1/PEFR	PEFR%
Intermittent	<1/wk	≤2/m	≥80%	<20%
Mild persistent	≥1/wk <1/day	>2/m	≥80%	20-30%
Moderate persistent	Daily	≥1/wk	60-80%	>30%
Severe persistent	Daily	Frequent	≤60%	>30%

Classified according to the Global Initiative for Asthma [GINA] guidelines
Intermittent: 1 point
Mild persistent: 2 points
Moderate persistent: 3 points
Severe persistent: 4 points

Table 1. Classification of asthma severity (Cheng *et al.*, 2010)

1.3. Epidemiology of asthma

The recent substantial increase in the burden of asthma among children and adults has been reported worldwide **(Figure 1.)**. Because the symptoms of asthma are not very specific to the disease, it makes difficult to differentiate between asthma and other respiratory diseases, particularly, in later life, chronic obstructive pulmonary disease (COPD). Therefore, there is no widely recognized single instrument that can be used to identify asthma. In epidemiologic studies, only questionnaires are applicable for statistical information (“Asthma vs. COPD | AAAAI” USA).

Based on the statistical evaluation report published by the European Respiratory Society (ERS), in the whole of Europe, about 30 million children and adults under 45 years of age suffer from asthma. The incidence of asthma has dramatically increased between 1950 and 2000 (“Epidemiology - ERS”). In Germany, 50% of the population reported with allergic sensitization. Furthermore, the survey conducted in children and adolescents between 2003 and 2006 through interviews and questionnaires (Children and Adolescent Health Survey; Kinder- und Jugend-Gesundheitssurvey, KiGGS) reported that 4.6%, 10.7%, and 13.2% of participants to be diagnosed with asthma,

hay fever, and atopic dermatitis, respectively. The fellow-up of this study between 2009 and 2012 confirmed the figures from the past, with 6.3%, 12.6%, and 14.3% study participants to be diagnosed with asthma, hay fever, and atopic dermatitis, respectively (Karl-Christian Bergmann et al. 2015).

On the other hand, the Centers for Disease Control and Prevention (CDC) reported that more than 25 million Americans are suffering from asthma, corresponding to 7.7% of adults and 8.4% of children. The incidence of the disease has been increasing since the early 1980s in all age, sex and racial groups (“Asthma Surveillance Data | CDC” USA). Currently, there are 6.2 million children under the age of 18 having asthma in USA (“Asthma Facts | AAFA.Org” USA).

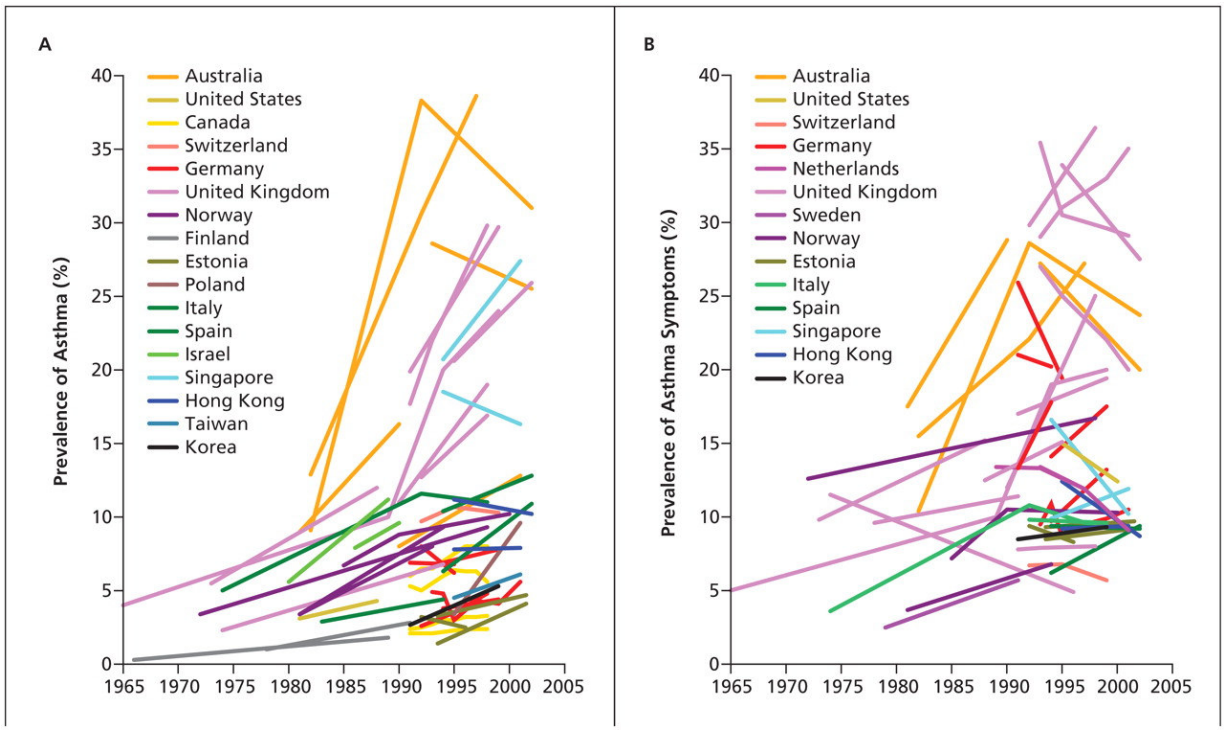


Figure 1. Changes in prevalence of diagnosed asthma (A) and asthma symptoms (B) over time among children and young adults (Subbarao, Mandhane and Sears, 2009).

1.4. Asthma risk factors

A comprehensive multifactorial list of most common risk factors proposed to account for the increasing prevalence of asthma in childhood consistently reported among different studies including age, sex, gene-by-environment interactions, smoking, exposure to pollutions, allergens, and infections and microbial substances is graphically illustrated in **(Figure 2.)** (Beasley, Semprini and Mitchell, 2015).

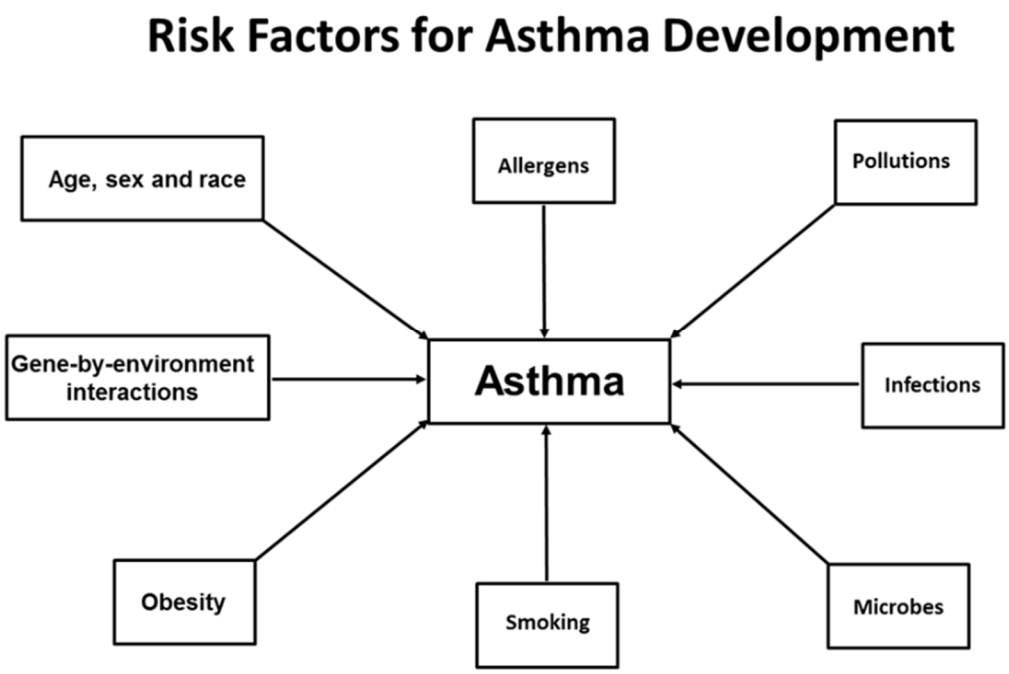


Figure 2. Risk factors for asthma development

1.4.1. Gene-by-Environment

The heterogeneity of asthma makes it a complex genetic disorder. Gene-Environment interactions are factors that influence or shape the responses of immune system, thus modulating the clinical symptoms or manifestation of the disease and associated significantly to the inception, severity and the treatment of asthma (Vercelli, 2010).

The response to the environmental changes is also different between genotypic backgrounds, with the investigations for novel genes in asthma among the genome-wide studies suggested many other genes with minor roles rather than few genes with strong effects contribute to asthma development. Besides, based on those studies, it appears that genetics influences the susceptibility to allergic disorders but does not define any single genes directly responsible for (and/or genetic markers of) disease development (Holloway, Yang and Holgate, 2010).

Among genes that have been consistently shown to be associated with asthma, its clinical features, or responses to the treatment include among others, genes involved in cytokine signaling related to Th-1 and Th-2 cell differentiation, such as interleukins 13, 4 and 5 (IL-13, IL-4 and IL-5), interleukin 4 receptor (IL-4RA), interferon gamma (IFNG), STAT6, GATA3, TBX21, and the beta2 adrenergic receptor gene(Ortiz and Barnes, 2015).

1.4.2. Tobacco smoke

Tobacco smoke is a complex mixture of over 5,000 different harmful chemicals. Nicotine, hydrocarbons, carbon monoxide, volatile organic compounds and other compounds are primary materials produced from tobacco (Talhout *et al.*, 2011).

The major effects of tobacco smoke occur locally, on the lungs, reaching however all immune and epithelial cells of the respiratory tract and causing an increase in epithelial permeability and weakening mucociliary clearance through increase in the release of IL-6, chemokine 8 (CXCL8) and other pro-inflammatory cytokines from the epithelial cells(Higham *et al.*, 2018) (Spira *et al.*, 2004). Besides, tobacco smoke has ability to suppress the Th-1 response and enhance Th-2 response, which disrupts Th-1/Th-2 balance locally and systemically (Brown *et al.*, 2016).

Among studies a positive association was reported between smoking and an increased risk of asthma development in both children and adolescents (Mitchell *et al.*, 2012). Exposure to maternal smoking during pregnancy was associated with the changes in the immune functioning through epigenetic modulations caused by nicotine; those changes were considered as significant triggers for the development of asthma among children later in life (Zacharasiewicz, 2016).

Interestingly, in some countries like United Kingdom, a marked reduction in childhood asthma hospitalization was associated with a public smoking ban, suggesting a casual relation between smoking and asthma morbidity(Millett *et al.*, 2013).

1.4.3. Pollutants

Over the last three decades and despite the attempts to reduce air pollution, exposure to indoor and outdoor air pollution still remains a significant risk factor for both asthma development and exacerbation. The most comment indoor pollutants are secondhand tobacco smoke (SHS), nitric oxide (NO2), and airborne particulate matter (PM) such as dust and diesel particulate matter (Burbank and Peden, 2018). Moreover, it has been shown that the concentration of indoor pollution is much higher in urban than in the non-urban homes which could also explain the high prevalence of asthma in children living in the cities compared to those living in the rural areas (Hulin, Caillaud and Annesi-Maesano, 2010).

There is evident support for the hypothesis that early exposure to air pollution is an important predictor of respiratory morbidity among children. A systematic review and meta-analyses showed that early or lately exposures to traffic-related air pollution were

associated with the development of asthma in children, and asthma incidence was increased with age (Khreis *et al.*, 2017) (Orellano *et al.*, 2017). In addition to air pollution, other multiple environmental factors such as pollens or fungal spores could modify the response to traffic-related air pollution (Reinmuth-Selzle *et al.*, 2017). The same we find under the influence of socio-economic factors, therefore, living in more polluted areas caused an increased in the incidence of asthma (Norbäck *et al.*, 2018). Due to the complexity of the disease, more studies are required to fill the gap of pollutants exposure-age relation currently present in the literature.

1.4.4. Obesity

Obesity is defined by a body mass index (BMI) of 30 or higher. Over the past four decades, mean BMI and obesity have dramatically increased, especially among children living in urban areas. Overall, the clinical and epidemiological studies have clearly demonstrated a significant association between obesity and asthma development. It is most likely that obese patients suffer worse symptoms and are less able to control their asthma than non-obese asthmatic patients with an increase in asthma severity and worse quality of life (Peters, Dixon and Forno, 2018) (Jiang *et al.*, 2019). Due to chronic low-grade systemic inflammation, obese patients are more likely to suffer from asthma and asthma exacerbation. Low grade of inflammation in obese individuals have been accompanied with increase in the production of inflammatory mediators such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), interleukin 18 (IL-18), C-reactive protein (CRP) etc. (Carpaij and van den Berge, 2018).

In addition, the adipocytes can also contribute to the production of IL-6 and TNFα constitutively, particularly in patients with abdominal obesity. High levels of IL-6 and TNF-α in obese patients with asthma could influence the expression of IL-4 and IL-5 which consequently stimulate the eosinophils and mediate IgE production (Bolus and Hasty, 2018) (Liu *et al.*, 2016). In more detail, both leptin and adiponectin are the hormones released by adipocytes of obese asthmatic patients. Leptin acts as a pro-inflammatory mediator and it has the same structure as in IL-6. Increased the levels of leptin in serum were associated with enhancement of airway hyperresponsiveness. In contrast, adiponectin is an insulin sensitizing hormone released by adipocytes which enhances the interleukin 10 (IL-10) production by macrophages (Saltiel and Olefsky, 2017).Giving adiponectin to mice attenuates the allergen-induced airway inflammation and hyperresponsiveness (Shore *et al.*, 2006).

Increasing evidence of a relation between obesity and asthma eventually brings up these conditions together more frequently than before. Further research is needed in this field.

1.4.5. Microbes

Early sensitization is an important factor for the development of asthma later in life (Anderson and Jackson, 2017). Studies among the interactions between the host and microbes (viruses, bacteria, and fungi) give somehow explanation to both development and progression of asthma. According to the findings reported by the Copenhagen Prospective Study on Asthma in Childhood (COPSAC) high-risk birth cohort, early life airway asymptomatic bacterial colonization with Moraxella catarrhalis, Haemophilus influenzae, or Streptococcus pneumoniae in one-month-old infants was associated with development and exacerbation of asthma in childhood. Those pathogenic asymptomatic bacteria are also accused of increasing the severity of respiratory illness such as asthma exacerbations attributed to rhinovirus infection (Rahman Fink *et al.*, 2018).

In another cohort study, the Childhood Asthma Study (CAS), which analyzed nasopharyngeal samples from infants, it has been shown that early colonization with Streptococcus was associated with asthma development at age of 5 (Teo *et al.*, 2015). Sigurs et al. 2010 and Jackson et al. 2012 were also able to confirm the significant association between Respiratory Syncytial Virus (RSV) or Human Rhinovirus (HRV) infection in early life and the development of asthma later in life. Data showed that in HRV infection, the parental atopy and allergic sensitization of the child is a key factor to predict asthma development later in life. Similarly, RSV infection in the first year of life was associated with greatest risk of asthma development(Saglani, 2013) (Beigelman and Bacharier, 2013) (Jackson and Johnston, 2010).

On the other hand, the early contact with farm-related microbes has been linked to protection against asthma and allergic diseases later in life, which strongly supporty the hygiene hypothesis. A number of bacterial species isolated from cowsheds such as A. lwoffii and L. lactis were able to significantly reduce the number of the eosinophils in the lung of the mice subjected to mouse model of experimental asthma using ovalbumin (Debarry et al. 2007).

1.5. Treatment

The main goals of asthma treatment are reducing symptoms, maintaining normal pulmonary function, and minimizing the risk of future exacerbations (Papi *et al.*, 2018). Understanding the molecular pathway and mechanism are required in order to determine new molecular targets which are amenable to both small-molecule and biological interventions. Inhaled corticosteroids, short- and long-acting B2-adrenoceptor agonists (SABAs and LABAs) are now the major treatment option used for asthma management; some other options could be helpful as well such as phosphodiesterase inhibitors, anti-IL-5 and anti-histamines ("GLOBAL INITIATIVE FOR ASTHMA A GINA Pocket Guide For Health Professionals 2018) ("Short-Acting Beta Agonists (SABAs) | AAAAI" USA) (Tang, Sun and FizGerald, 2018).

1.5.1. Corticosteroids

Corticosteroids diffuse across the cell membrane to bind to the glucocorticoid receptors (GR) in the cytoplasm, and then such complex is translocated into the nucleus where it binds directly on the DNA and interferes with gene expression (Ferrara *et al.*, 2019). Corticosteroids work through the inhibition or activation of the expression of cytokines, chemokines and adhesion molecules. For example, corticosteroids inhibit the expression of nuclear factor-κB (NF-κB) and activator protein 1 (AP1). Moreover, some inflammatory cytokines like IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-13 and IL-15, TNF-α, and GM-CSF are also inhibited (Ferrara *et al.*, 2019).

On the other hand, corticosteroids could activate the expression of anti-inflammatory cytokines such as IL-10 and IL-12. At the cellular level, inhaled corticosteroids reduce the levels of eosinophils, mast cells, dendritic cells and other cells involved in asthma exacerbation (Liberman *et al.*, 2018).

Patients with severe asthma are less responsive to the high doses of inhaled corticosteroids (ICS) due to corticosteroid resistance. In children, corticosteroid resistance might be related to the allergen exposure via IL-2- and IL-4-dependent mechanisms (Barnes and Bush, 2012). Besides, subjects actively or passively exposed to cigarette smoke show high levels ICS resistance. Many studies demonstrated that also obesity could be a cause for steroid resistance (Bhatt *et al.*, 2018).

1.5.2. β 2-agonists

Beta (2) adrenergic agonists are used widely as bronchodilators together with inhaled corticosteroids for better control of asthma symptoms. It has been clarified that β2-receptor activation is mediated by an increase in intracellular cyclic mono phosphate cAMP levels enhanced by the stimulation of adenylate cyclase that catalyzes the conversion of adenosine triphosphate into cAMP.It is not fully understood how cAMP leads to airway smooth muscle cell relaxation but it is believed that it catalyzes the activation of protein kinase A (PKA), which in turn phosphorylates key regulatory proteins involved in the control of muscle tone. Moreover, cAMP inhibits calcium ion (Ca2+) release from intracellular stores which subsequently leads to relaxation of the airway smooth muscles and that relieves bronchoconstriction in asthma (Cazzola *et al.*, 2013) (Billington, Penn and Hall, 2016).

The most common long acting beta 2 agonists (LABA) are formoterol and salmeterol, which induce bronchodilation for at least 12 hours. Besides, it has been observed that adding LABA to asthma treatment protocol increases the efficacy of ICS treatment in asthmatic patients (Buhl, FitzGerald and Busse, 2018).

1.5.3. Phosphodiesterase inhibitors

Theophylline is a bronchodilator used to treat asthma by antagonizing the bronchoconstriction. It has both, the activity of a phosphodiesterase (PDE) inhibitor and the properties of an adenosine-receptor antagonist. Furthermore, it has been noticed that theophylline has also some anti-inflammatory effect. Recently, its usage in asthmatic patients appears to be limited due to cardiac side effects (Barnes, 2013) (Mahemuti *et al.*, 2018).

1.5.4. Leukotriene modifiers (LM) or leukotriene antagonists

Leukotriene antagonists are medicines which have been used in addition to ICS as a useful approach for uncontrollable asthma treatment. LM cause a greater improvement of the level of airway inflammation and pulmonary function in asthmatics patients when they are used as adjuvant therapy to ICS. It is not very clear how LM improve the pulmonary functions and airway liability but it seems to be mediated through the RANTES-pathway (Montuschi, 2010).

Zafirlukast, montelukast, and zileuton are leukotriene modifiers available for asthma treatment, all of which are used to decrease the inflammatory chemicals released in

the lungs after the cells are encountering an allergen or allergy trigger (Sharma, Hashmi and Chakraborty, 2020).

1.5.5. Antihistamine

Histamine is an important mediator in allergic disease. It is elevated in the airway of asthmatic patients and involved in the etiology and pathophysiology of asthma (Yamauchi and Ogasawara, 2019). H1-antihistamines can bind to the histamine (H1) receptors mainly on the mast cells in the airway and block its activity (Thangam *et al.*, 2018). It could be an option to prevent asthma, especially the allergen-induced asthma. Furthermore, it has been proved that antihistamines such as azelastine, cetirizine, desloratadine, and fexofenadine have beneficial effects on the improvement of asthma symptoms and pulmonary function. Antihistamine could be used in a combination with other an asthmatic medications such as LM or ICS. Studies on asthmatic patients demonstrated beneficial effects of these combination in asthma prevention or treatment (Mener and Lin, 2015) (Scichilone *et al.*, 2015).

1.6. Asthma mechanism and pathophysiology

Chronic airway inflammation and airway hyperresponsiveness (AHR) characterizing asthmatic disease lead to irreversible obstruction of the airway (Holgate, 2012). This mechanism of asthma is mediated by type 1 and type 2 immune response paradigm which is mainly regulated by subpopulations of CD4+ T cells underlying T helper 1 (Th-1) and (Th-2) immune response. The type 2 immune reactions mediated by Th-2 cells play a major role in the development of asthma (Lloyd and Snelgrove, 2018) (Fahy, 2015).

Generally, most of asthma starting in the childhood or in the adulthood is associated with sensitization to the environmental allergen such as house dust mites, cockroaches, animal, fungi, and pollens. Thus, although adult and child asthma have different presentations their environmental triggers are similar (Trivedi and Denton, 2019). Simply, when the allergen is taken up by dendritic cells (DCs), they present it to the naïve T helper cells and subsequently, allergen-Th-2 cells reaction occurrs (Gill, 2012).

This sequence of events stimulates proliferation of Th-2 cells which consequently produce high amounts of interleukins 4, 5 and 13 (IL-4, IL-5 and IL-13). These in turn recruit other cells, mainly the eosinophils and neutrophils. In addition, those cytokines could influence the release of other potent chemical mediators from inflammatory cells in the airway as it is shown in **(Figure 3.)**.

All those cellular and molecular players together shape the etiology and heterogeneity of the disease (Ling and Luster, 2016).

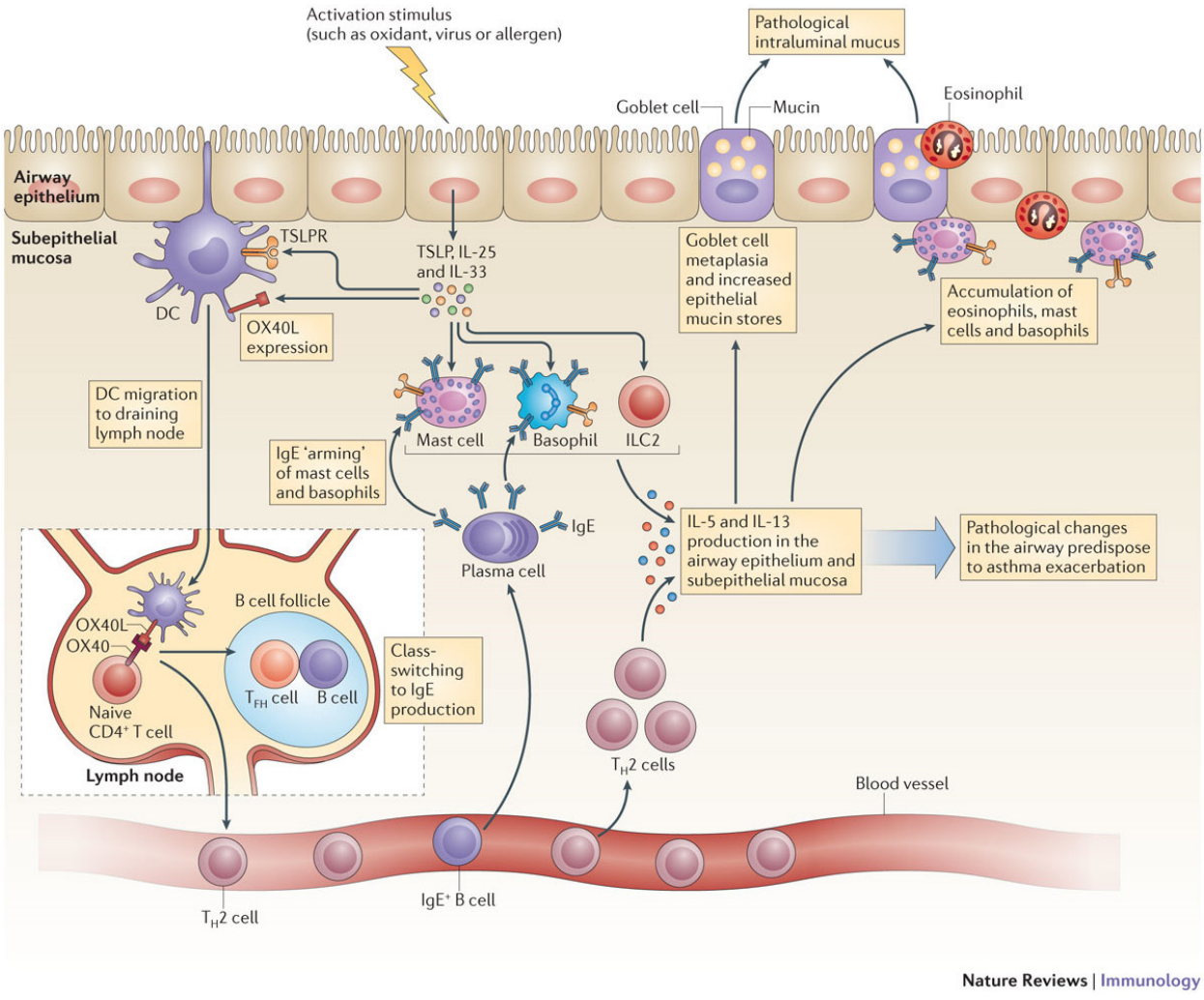


Figure 3. Type 2 immune responses in asthma (Fahy, J, 2015).

1.6.1. Cell types involved in asthma

The immunohistopathologic analysis of asthmatic airways shows the involvement of many types of inflammatory cells such as epithelial cells, dendritic cells, eosinophils, neutrophils, lymphocytes, and mast cells.

1.6.1.1. Epithelial cells

Airway epithelial cells are the first line of defense against exposure of the airway to triggers or allergens. Epithelial cells activation is one of the asthma features which are significantly associated to allergic sensitization. As a part from the innate immune function in the lungs epithelial cells exhibit mucociliary escalator by taking up or removing the pathogens through the movement of cilia on the top of the epithelial cells (Whitsett and Alenghat, 2015).

Epithelial cells are also very important player in the regulation of adaptive and innate immune cells by expression of soluble chemokines, cytokines and cell-surface molecules, which contributes to the eradication of the pathogens. Activation of the epithelial cells alters the function of DCs, T and B cells in the airway (Kim, 2012).

Several studies have demonstrated the role of airway epithelial-derived cytokines, such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) in asthma pathogenesis and the recruitment or activation of inflammatory cells (Mitchell and O'Byrne, 2017). Furthermore, the disruption of the junctional complex of airway epithelial cells allow the allergens to enter to the respiratory tract submucosa which can influence the inflammatory process by directing dendritic cells (DCs) towards T helper 2 (Th-2) responses(Hammad and Lambrecht, 2015).

Therefore, studying the regulatory function of the airway epithelial barrier can be considered as a critical checkpoint to understand the innate and adaptive immune responses associated with asthma and asthma exacerbation (Gon and Hashimoto, 2018).

1.6.1.2. Eosinophils

Eosinophils (EOSs) are granulocytes circulating in the healthy status at low levels, up to 3% of white blood cells. These cells are the major inflammatory cells recruited to the inflammatory site; their effects in asthma are related to the production of different toxic granule proteins, reactive oxygen species (ROS), cytokines, and lipid mediators (Nakagome and Nagata, 2018).

EOSs are major contributors to the pathogenesis of asthma, such as airway epithelial cell damage, mucus hypersecretion, airway remodeling, and airway hyperresponsiveness (AHR) and dysfunction (HOLGATE, 2008).

EOSs can also release different types of cytokines and of pro-inflammatory mediators, such as IL-5, IL-1, IL-2, IL-6, IL-8, and TNF-α. Those cytokines are mainly contributing to the hypersensitivity reaction triggered by allergens, with subsequent IgE production

and B-cell class switching. In addition, EOSs considered as a major defense against parasites (McBrien and Menzies-Gow, 2017).

1.6.1.3. Dendritic cells

Among the cells involved in asthma pathophysiology, dendritic cells (DCs) are considered as a primary antigen presenting cells (APCs). They engulf the antigens, process and present them to the T cells lymphocytes. Different types of DCs are capable of polarizing CD4+ T lymphocytes towards Th-1, Th-2, Tregs or tolerance to antigen(Fedulov and Kobzik, 2011).

The crosstalk between CD4+ T lymphocytes and DCs at different molecular levels through cytokines and chemokines induces and shapes the adaptive immune responses at the inflammatory site. In airway inflammation, together with epithelial cells, DCs process the type 2 immune response in which predominantly polarized Th-2 type cells are involved. A various pro-inflammatory cell participate such as eosinophils, neutrophils, mast cells, basophils, plasma cells, and epithelial cells. These cells are able to produce different cytokines such as TSLP, IL-4, IL-5, IL-6, IL-9, IL-13, IL-17, IL-25, IL-33 and chemokines such as eotaxin, IL-8, Monocyte chemoattractant protein-1 (MCP-1), other pro-inflammatory mediators are also involved such as histamine, prostaglandins and leukotrienes (Lloyd and Snelgrove, 2018). Furthermore, the differences in the type of DC responses may result from the genetic and epigenetic factors which could regulate the immune hyperresponsiveness in allergic asthma. The human DCs are divided into two major type, the myeloid and plasmacytoid dendritic cells. These DCs have no precis location but they are different in the markers expressed on their surface (Collin and Bigley, 2018) **(Table 2.)**.

Human lung DCs	Function	Markers
Myeloid DC (CD103+/BDCA3)	**Take up the antiges and present them to naïve Tcells**	**CD11c+/int, BDCA3+ (CD141), and HLA-DR**
Myeloid DC (CD103+/BDCA1)	**Take up the antiges and present them to naïve Tcells**	**CD11c+/hi, BDCA1+ (CD1c), and HLA-DR+**
Plasmacytoid dendritic cells	**Take up the antiges and present them to naïve Tcells, block Th-2 response through Foxp3+Tregs and induce tolerance.**	**CD123+ (IL-3 receptor), CD11c−, BDCA2+ (CD303), BDCA4+ (CD304), HLA-DR+**

Table 2. Lung DCs in human. Their functions vary depending upon their respective protein expression.

1.6.1.4. Mast cells

Mast cells are considered as key players involved in asthma pathogenesis. Their chemical mediators such as histamine and bioactive lipids are observed in the acute phase of allergic reaction. In human, mast cell progenitors which observed to be predominantly highly in blood circulation of asthmatic patients have been developed by the influence of inflammatory stimuli into lung resident mast cells. In asthmatic patients, an activation of mast cells occurs via crosslinking of high affinity IgE receptors (FcεRI) with allergens (Méndez-Enríquez and Hallgren, 2019). Studies demonstrated that accumulation of activated mast cells in the lung functionally contributes to the etiology of the disease (Carroll, Mutavdzic and James, 2002a). The lung mast cells have been found to be increased together with EOSs in the airway smooth muscle of asthmatic patients, suggesting a major role of these highly activated lung mast cells in asthma exacerbation (Carroll, Mutavdzic and James, 2002b).

1.6.1.5. Neutrophils

Like EOSs, neutrophils are important plyers in asthma pathogenesis. Persistent increase in the lung neutrophils occurs mostly in uncontrolled or corticosteroid-resistant asthma. High levels of interleukine-8 (IL-8), matrix metalloproteinase-9 (MMP-9), leukotrienes-4 (LTB-4), and platelet-activating factor (PAF) in asthmatic patients have been found to be involved in the promoting the migration of neutrophils to the site of inflammation (Ito *et al.*, 2008). Unlike eosinophilic asthma which is associated with Th-2 type of immune response, the neutrophilic asthma is most likely to correlate with the presence of Th-17 cells. Increase in the levels of interleukin -17 (IL-17) production promotes the differentiation of CD34 progenitor cells into neutrophils, thus increasing the mucus production and resulting in airway remodeling process (Doe *et al.*, 2010).

Neutrophil elastase which is released from activated neutrophils can result in airway hyperresponsiveness, bronchospasm, gland hypersecretion, and airway remodeling, the latter leading to irreversible changes in airway structure (MacDowell and Peters, 2007). In addition, neutrophils are considered as the first line of contact against respiratory pathogens such as microbes or viruses. Cross-talk between lymphocytes and neutrophils play a curial role in shaping adaptive immune responses (Han Gao et al. 2017).

1.6.1.6. Lymphocytes

Lymphocytes are considered the major cell type in the pathogenesis of allergy and asthma **(Figure 4.)**. Type 2 immune response which is provoked by allergens is characterized by the differentiation of naïve CD4+ T cells into Th-2 cells, which is associated with increasing levels of IgE, and activation of EOSs, mast cells and neutrophils. In asthmatic individuals, when DCs get in contact with soluble allergens, they move toward the draining lymph nodes, where antigens are presented to the naïve T cells and, through particular chemokines and other chemokine receptors expressed on the surface of Th-2 cells such as CCR3, CCR4, and CCR8 and the PG D2, polarize them into either adaptive Th-2 cells or T follicular cells (Tfh). Th-2 cells migrate to the site of inflammation in lungs to amplify the type 2 immune response by producing Th-2 cytokines. The key cytokines which are involved in the allergic reaction include interleukin IL-4, IL-5, IL-9, and IL-13 (Caminati *et al.*, 2018) (Friedmann *et al.*, 2020).

Accumulation of Th-2 cells in the lungs is required for the initiation and persistence of airway inflammation. Together with high IgE levels, those cytokines are the main orchestrators of type 2 adaptive immunity (Froidure *et al.*, 2016). IL-4 and IL-13, which are located on genomic locus called Th-2 cytokine locus, are considered as major cytokines highly expressed in type 2 inflammatory responses, both triggered by parasite or allergen. Along with Th-2 cells, basophils, eosinophils mast cells and innate lymphoid cells type 2 (ILCs2) are able to produce IL-4 and IL-13 at the inflammatory site. Moreover, IL-4 is able to regulate Th-2 differentiation and B-cell IgG1 and IgE class switching. In contrast, IL-13 is regulating the contractility of smooth muscle and the secretion of gel-forming mucins in the respiratory tract through the epithelial goblet cells (Junttila, 2018) (Bao and Reinhardt, 2015) (Bagnasco *et al.*, 2016).

Despite possible role of other cytokines in eosinophilic asthma such as IL-13, IL-4 and IL-9, IL-5 seems to be the key cytokine involved in the recruitment, proliferation, activation and maturation of eosinophils. In a mouse model, the absence of IL-5 was able to abolish tissue eosinophilia, which shed light on the importance of IL-5 in the survival and maintenance eosinophilic asthma development(Kips, 2001) (Mould *et al.*, 1997). In spite of the fact that Th-2 cells are mostly predominant in allergic disease, other types of T cells are also intended to be important especially to the severity of the disease. Th9 cells are a distinct helper T cell subset that secretes IL-9 as a signature cytokine. IL-9 has been demonstrated to be involved in asthma development in murine

models of asthma; adoptive transfer of Th-9 cells to recipient mice was able to induce airway inflammation by recruiting eosinophils and increasing the mast cell numbers and serum levels of IgE (Jones *et al.*, 2012).

Th-9 cells themselves were able to enhance Th-2 cytokines production from Th-2 cells (Temann, Ray and Flavell, 2002). Mice treated with anti-IL-9 antibody demonstrated significant decreases in the inflammatory features (Staudt *et al.*, 2010). Altogether, these findings indicate the role of Th-9 and IL-9 production in maturation of mast cells and eosinophil accumulation leading consequently to enhance Th-2 inflammatory response, AHR and mucus production in the lungs. Regarding the light of other types of T cells involved in asthma pathogeneses, Th-17 cells are a subset of CD4+ T cells characterized mainly by secretion of IL-17, a cytokine that was associated with the more severe phenotypes of asthma characterized by resistance to the treatment with inhaled corticosteroids(Banuelos *et al.*, 2017).

Th-17 asthmatic phenotype is positively correlated with high numbers of neutrophils in sputum (Barczyk, Pierzcha and Sozañska, 2003).

Moreover, Th-17 responses have been associated with airway inflammation triggered with viral infection. Further studies are needed to fully understand the role of Th-17 immunity in the allergic airway inflammation.Regulatory T cells (Tregs) represent a major subtype of CD4+ T cells which play an important role in suppressing allergic inflammation and inducing peripheral tolerance (Holt *et al.*, 2008). IL-10, the anti-inflammatory mediator, is the major cytokine expressed from both innate and adaptive immune cells in which it is markedly suppressed IgE and Th-2 cytokines production in the lungs promoting tolerance against repeated exposure to the allergens(Lloyd and Hawrylowicz, 2009). Inhaled transfer of IL-10 has failed to influence pulmonary homeostasis in human asthmatic patients but not in asthmatic mouse models (Ali *et al.*, 2004).

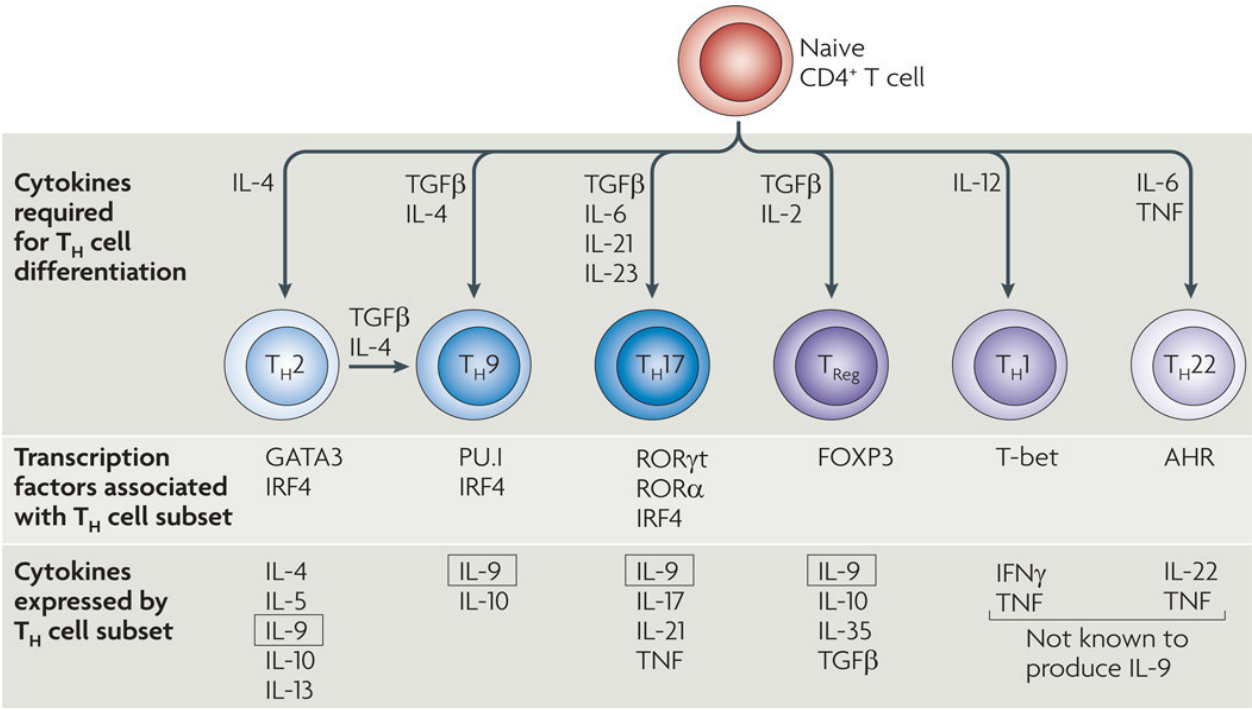


Figure 4. The classical monolithic view: lineages and master regulators (Noelle and Nowak, 2010).

1.6.2. Epigenetic modifications

The term epigenetics refers to inheritable or non-inheritable phenotypic changes in the cell that lead to the modified gene expression without alteration of the DNA sequence. There have been remarkable advances in describing of the “central dogma” of the transcription machinery transferring the genetic information embedded in DNA to RNA and subsequently to proteins (Lacal and Ventura, 2018).

In the human genome, 80% of the DNA is packed into nucleosomes and the rest is providing linkers between nucleosomes. The nucleosomes are further packed to into three-dimensional (3D) structure so called chromosomes (Albert A et al. 2008). The core components of the nucleosome are the histones, which are accessible to different types of posttranslational modifications (PTMs) such as acetylation, methylation, phosphorylation, sumoylation, and ubiquitination. PTMs are able to change the accessibility of the DNA to transcription factors, especially in regulatory genomic regions such as enhancers or promoters, which is subsequently associate with active, poised, or silenced transcriptional status **(Figure 6.)** (Alaskhar Alhamwe *et al.*, 2018).

DNA methylation is another type of the epigenetic change, in which a methyl (CH3) group is enzymatically added by DNA methytransferases (DNMTs) onto the cytosine rings of DNA.

This molecular change can be also reverted by during DNA demethylation, which is mediated by ten-eleven translocation methylcytosinedioxygenase (TET) family proteins. The external or environmental stimuli can disturb the balance between DNA methyltransferases and DNA demethylases which lead to DNA methylation or demethylation at the promoter regions of the gene that can further suppress or activate the expression of the respective locus **(Figure 5.)** (Potaczek *et al.*, 2017a) (Alhamwe *et al.*, 2019).

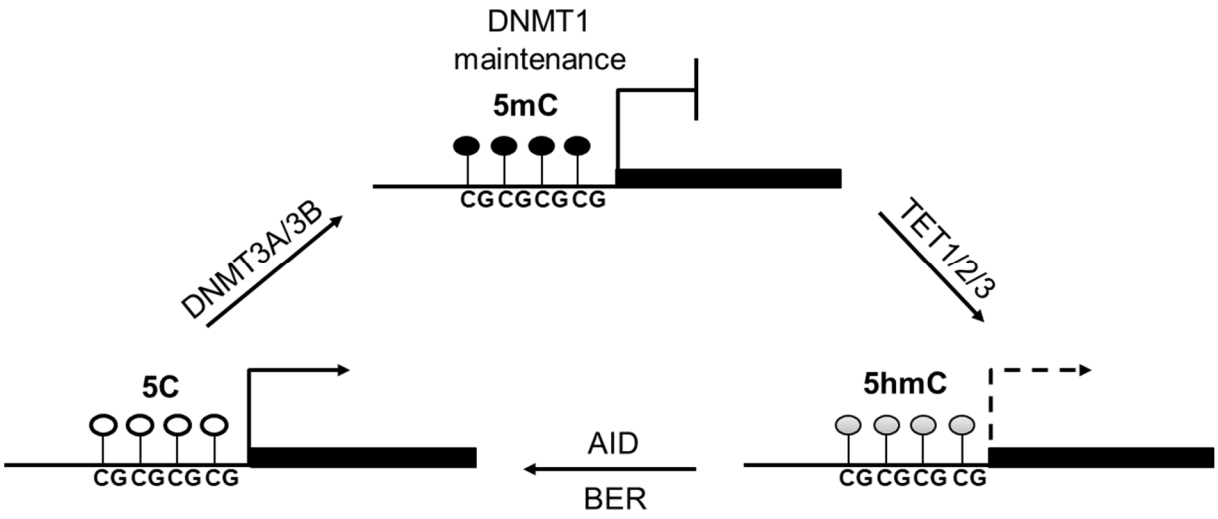


Figure 5. DNA de/methylation mechanism (Alhamwe *et al.*, 2019)

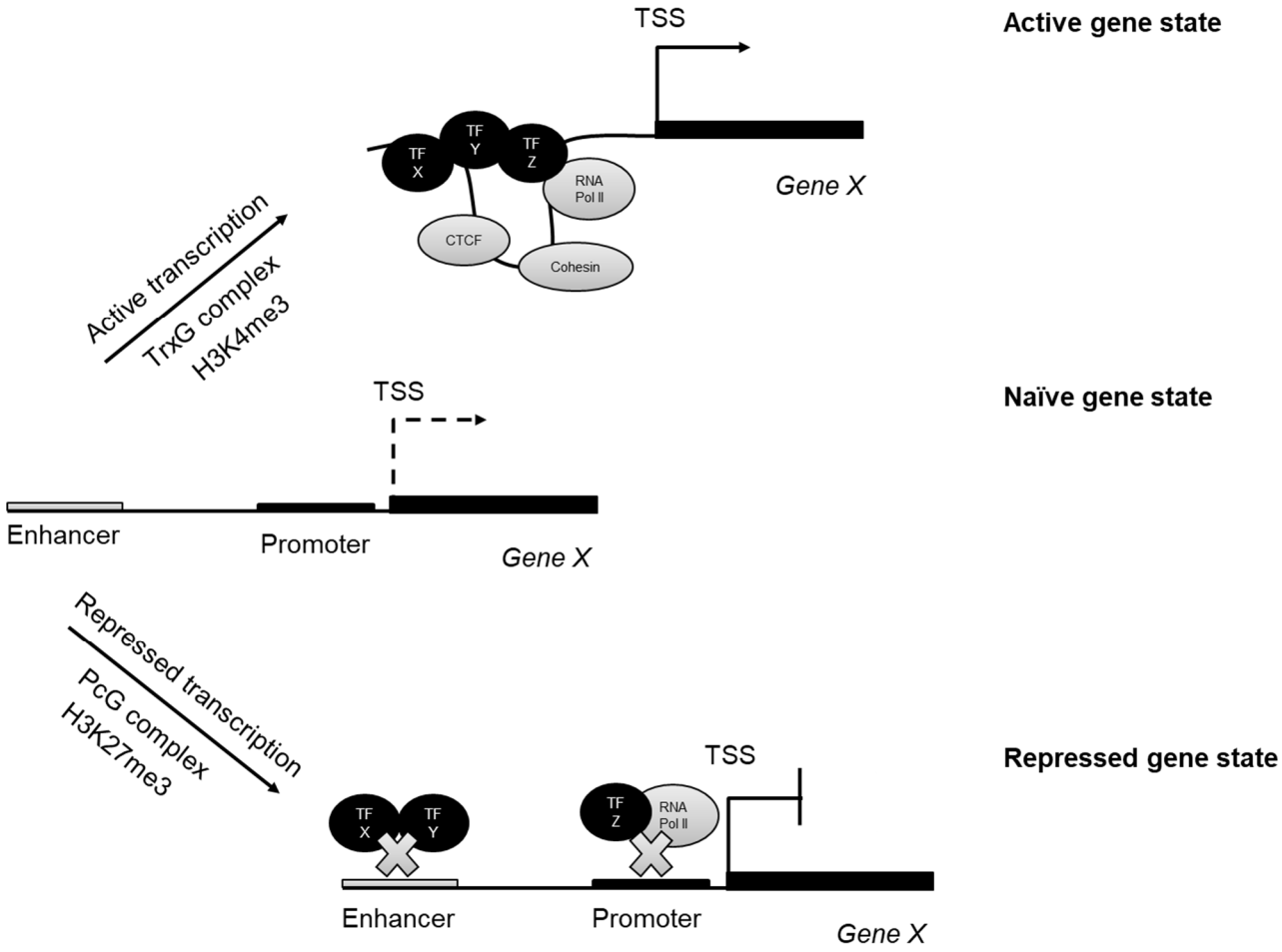


Figure 6. Histone modifications mechanism (Alhamwe *et al.*, 2019)

1.6.2.1. T- Cell epigenetics

Naïve CD4+ T cells differentiation in allergic disease such as asthma is strictly controlled by epigenetic modifications **(Figure 7.)** (Potaczek *et al.*, 2017b). Gene-environment interactions might be associated positively or negatively with the development of asthma (Pfefferle and Renz, 2014). In other words, these environmental exposures are considered as risk-increasing or risk-reducing factors (Harb *et al.*, 2016). Moreover, naïve CD4+ T cells differentiation is triggered by defined cytokine environment at the site of inflammation, which influences the expression of lineage-specific transcription factors (TFs) (Alaskhar Alhamwe *et al.*, 2018). Furthermore, the crosstalk between the innate immune cells such as epithelial or dendritic cells is also an important driver towards a specific subpopulation of naïve CD4+ T cells such as Th-1, Th-2 (and Th-9), regulatory T cells (Treg cells), and Th-17 (Kabesch, Michel and Tost, 2010) (Fontenot, Gavin and Rudensky, 2017) (Kaplan, Hufford and Olson, 2015).

In Th-1 cells which differentiated from naïve CD4+T cells under the influence of interferon-γ (IFN-γ) together with IL-12, the expression of T-box 21, an essential TF of Th-1 cell subpopulation development, so called Th-1 master transcription factor, occurs (Chen *et al.*, 2000) (Leung *et al.*, 2010).

In contrast, the transcription factor GATA binding protein 3 (GATA3) is required for the differentiation of naïve CD4+ T cells into Th-2 lineage in the presence of IL-4. GATA3 binds to the Th-2 cytokine gene locus to stimulate the expression of different Th-2 cytokines such as IL-4, IL-5 and IL-13. These cytokines are subsequently essential for the maintenance of Th-2 differentiation and further activation/stimulation of the asthma-involved cells such as eosinophils, neutrophils and mast cells(Nemtsova *et al.*, 2019) (Tumes *et al.*, 2017) (Ling and Luster, 2016). Besides, they are involved in the inflammatory clinical and pathophysiological hallmarks of asthma like mucus production, AHR, ILC2 survival, and B cells class switching into IgE (Suarez-Alvarez *et al.*, 2012) (Lambrecht and Hammad, 2015).

There is no master transcription factor for Th-9 development; both IL-4 and transforming growth factor-β (TGF-β) are required for the naïve T cell in order to develop Th-9 phenotype. However, two TFs are also needed for Th-9 development such as interferon regulatory factor 4 (IRF4) and PU.1 but none of them is considered

as master regulatory TF (Goswami and Kaplan, 2011) (Kaplan, 2013). On the other hand, under the influence of TGF-β together with IL-6, naïve CD4+ T cells can develop Th-17 phenotype. RAR related orphan receptor C isoform 2 (RORC2) is considered as a master TF for Th-17 differentiation. Th-17 cells are capable of producing high amounts of IL-17A/F, which is typical for the neutrophilic asthma phenotype (Cosmi *et al.*, 2011). TGF-β alone can induce the expression of the forkhead box protein 3 (FOXP3), a master TF which differentiates naïve CD4+ T cells towards IL-10 producing Treg cells. IL-10 plays an important role in the suppression of inflammation and induction of airway remodeling in asthma (Sharma and Rudra, 2018) (Sharabi *et al.*, 2018).

The classical epigenetic changes such as DNA methylation and histone modifications play a crucial role in determining the T cell lineage (Potaczek *et al.*, 2017b). For instance, DNA demethylation of IFNG gene is essential in the development of Th-1 cells but this is not required in Th-2 or Th-17 development (Aune, Collins and Chang, 2009). In addition, DNA hypomethylation of IL-4 and IL-13 gene locus is required in Th-2 cells development. In Treg cells FOXP3 gene undergoes demethylation in order to develop Treg cells, while RORC remains methylated (Hirahara *et al.*, 2011)(Tumes *et al.*, 2017). In an opposite manner, demethylation of RORC and methylation of the FOXP3 locus are known to be required for Th-17 development. Not much is known on epigenetic regulation of Th-9 cells development. Histone modifications such a methylation and acetylation are also an important factor controlling the development of T cell lineage. Histone modifications in Th-1 and Th-2 cell differentiation have been well analyzed at the Th-1 (IFN-γ) and Th-2 (IL-4, IL-5 and IL-13) genes cytokine loci (Hirahara et al. 2016.). Positive correlation between histone modifications, such as acetylation of H4 (H4Ac) and H3K4me3 have been observed at the IFNG locus and IFNG expression, while the opposite manner has been reported for the histone modifications silencing the expression of the Th-2 cytokine (IL4 and IL13) gene loci (Kondilis-Mangum and Wade, 2013) (Koyanagi *et al.*, 2005).

Furthermore, it has been observed that histone methylase SUV39H1 is involved in the trimethylation of H3K9 (H3K9me3), which is able to silence Th-1 gene loci and stabilize/trigger Th-2 cytokine expression (Allan *et al.*, 2012). Generally, high levels of H3K4me3 and, to a lesser extent, of H3K27me3 were demonstrated at the promoter regions of IL4 in Th-2, IFNG in Th-1, IL17A and IL17F in Th17, RORC in Th-17, and

FOXP3 in Treg cells (Chang and Aune, 2007) (Wei *et al.*, 2009) (Rodriguez, Lopez-Larrea and Suarez-Alvarez, 2015).

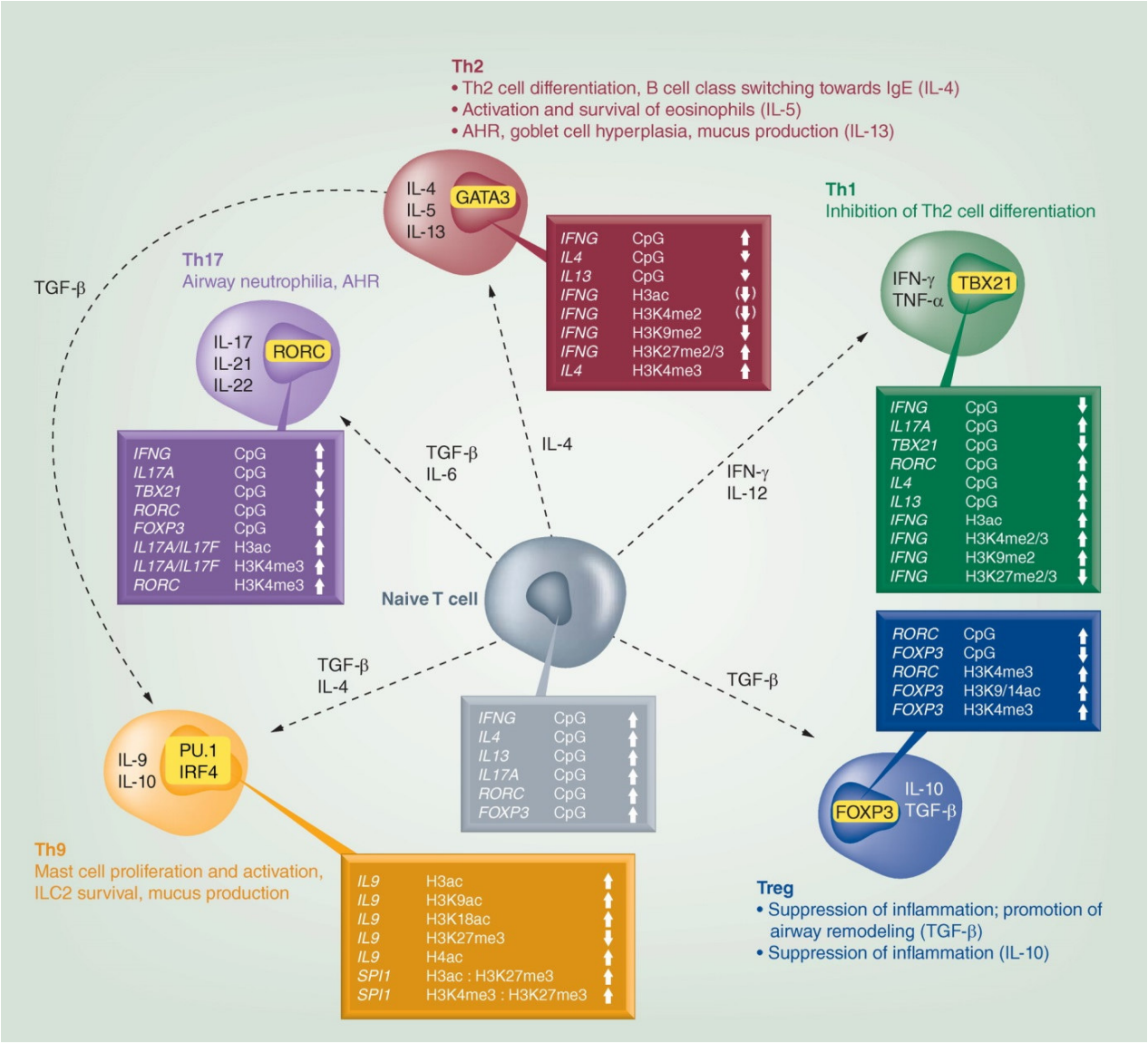


Figure 7. Major types of Th cells, their differentiation and its epigenetic regulation, and their crucial allergy-related functions (Potaczek *et al.*, 2017b).

1.7. Microbiome

Human microbiome, especially the gut microbiota, were found to be essential for the human beings. This microbiota are considered as major player in catalyzing or mediating many chemical and biological processes regulating human metabolism or mediating the epithelial and mucosal development. They are also crucial for shaping and educating innate and adaptive immune system.

Over the past decades microbiome has drawn an increasing amount of attention, tremendous amounts of evidence have strongly correlated the human microbiota to the development of diseases through complex mechanisms. Changing the microbiome diversity has a specific impact on the development of diseases; many factors could influence those changes in the gut microbiota, which is summarized in **(Figure 8.)**.

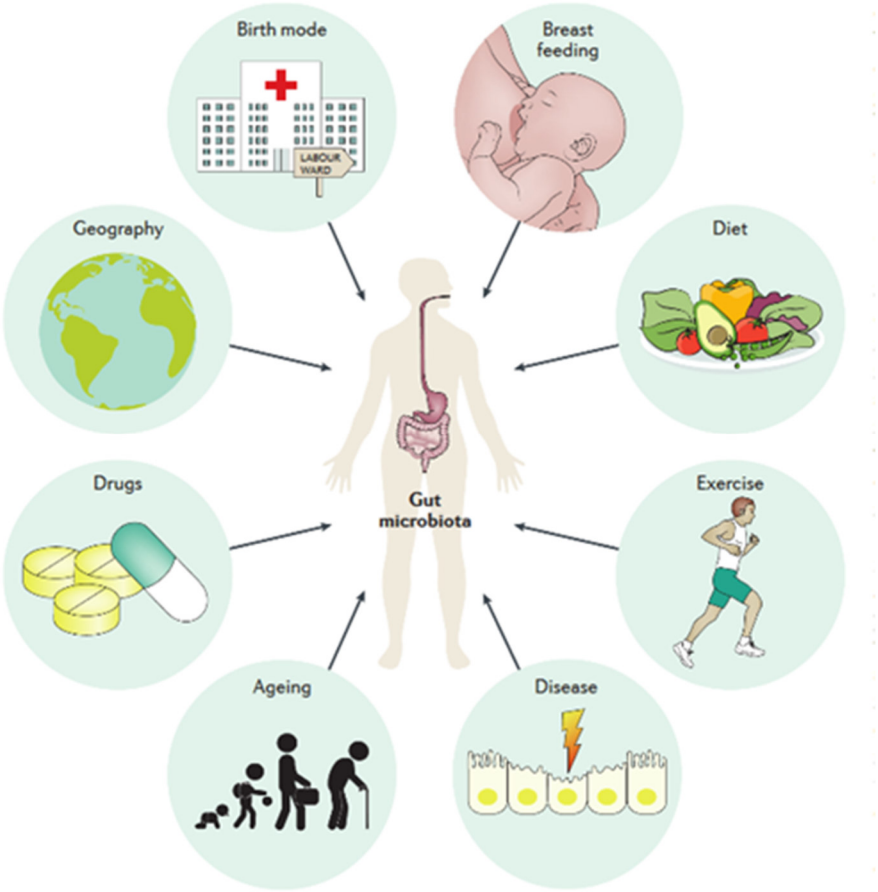


Figure 8. Factors influnce the gut microbiota changes (Quigley, 2017)

Large number of microbes with high alpha-diversity colonizes the mammalian gut, with most of them being *Firmicutes* and *Bacteroidetes* (Ley *et al.*, 2008). Furthermore, three genera, specifically *Bacteroides, Prevotella* and *Ruminococcus*, have been identified to be the major genera colonizing the human gut. Of importance, the same genera were also identified in the gut microbiota of mice (Arumugam *et al.*, 2011) (Hildebrand *et al.*, 2013).

In addition to their role in metabolisms, gut microbiota are able to modulate the immune system via different pathways, for example, the gram negative bacteria which are able to produce high amounts of lipopolysaccharide (LPS). LPS has been demonstrated to be a strong stimulator of the innate immune response; a positive correlation was detected between the LPS in plasma and the levels of serum C-reactive protein and this correlation negatively associated with the survival rates (Ghoshal *et al.*, 2009) (Kwan *et al.*, 2013).

Small chain fatty acids (SCFAs) such as butyric acid, propionic acid and acetic acid are also produced by gut microbiota via the fermentation of the dietary fibers. These SCFAs can bind to the G protein-coupled receptors (GPCRs) on the surface of epithelium, and thus translocate them into the host cells where they are able to inhibit the activity of histone deacetylases (HDACs). Inhibition of HDACs enhances the foxp3 expression and promotes the priming of the naïve T cells into Treg cells(Vinolo *et al.*, 2011) (Chang *et al.*, 2014). Increased number and function of gut regulatory Treg cells can initiate an immune tolerance and maintain the epithelial barrier function by increasing mucus productions by intestinal goblet cells. The SCFAs can transfer through the blood into brain and lung where they can decrease inflammatory responses that are associated with neuroinflammation and allergic airway disease, respectively (Furusawa *et al.*, 2013) (Smith *et al.*, 2013).

1.7.1. Lung microbiome and asthma

It has been indicated that the composition and diversity of the microbiota were changed in samples collected from asthmatic compared with healthy individuals (Abdel-Aziz *et al.*, 2019). The severity of asthma also correlated with this dysbiosis. Airway bacterial studies have shown that *Bacteroidetes* and *Actinobacteria* are more common in healthy controls and *Proteobacteria*, particularly *Haemophilus* species, are more common in asthmatic patients (Lee *et al.*, 2019). The diversity of lung microbiota seems to be higher in asthmatic patients compared with the healthy controls; some studies observed, however, no differences in microbiome diversity of lung or gut microbiome between asthmatics and non-asthmatics (Depner *et al.*, 2017) (Pang *et al.*, 2019).

Not only airway inflammation could influence the diversity of the lung microbiome. Inhaled corticosteroids are also able to influence the composition and diversity of the airway microbiota by suppressing the immune system and decreasing the mucus production which allows for some of the airway bacteria to overgrowth, thus increasing the diversity of the lung microbiota in asthmatics compared to healthy controls (Jung *et al.*, 2016). Furthermore, the lung microbiota are differentially clustering between eosinophilic and neutrophilic/non-eosinophilic asthma. It has been reported that *Proteobacteria* phylum, especially *Moraxella* and *Haemophilus*, are more abundant and *Actinobacteria* phylum is less abundant in neutrophilic/non-eosinophilic asthma compared with eosinophilic asthma. Another important study showed that, compared to those from more urbanized Finnish Karelia, children from less westernized Russian Karelia had higher overall bacterial diversity and abundance of genus *Acinetobacter* and were less likely to have allergic disorders including asthma (Ruokolainen *et al.*, 2017). These findings seem to be in line with the protective effects of *A. lwoffii* against asthma and allergic disease development observed in asthmatic mouse model (Debarry *et al.*, 2007a). Infections can also modulate the microbiota of the lungs; infants infected at 1 month of age with *Streptococcus pneumoniae*, *Haemophilus influenzae* or *Moraxella catarrhalis* showed an increase in the risk for recurrent wheeze and asthma at 5 years of age (Bisgaard *et al.*, 2007). In conclusion, there is no distinct lung or gut microbiome profile for healthy individuals since these the microbiota might be clustered in different ways based on different factors such as age, drugs and diet.

1.7.2. Gut-lung / lung- gut axis and *A. lwoffii* protective effects against asthma

The studies have shown that intestinal microbiome plays a role in influencing and shaping immune functions. The underlying inflammation and the serious problem of breathing disorder in asthma appear to be related to the composition or diversity of lung or intestinal microbiota (Frati *et al.*, 2019) (Pascal *et al.*, 2018). The interaction between different mucosal barriers, including the effect of the intestine on lung immunity (the gut-lung axis), is likely to be mediated by local microbes and circulating immune cells. For example, some intestinal bacteria are known to produce SCFA that modulate the development of T cells and thus allergic and asthmatic responses (Anand and Mande, 2018).

Although the lung intestinal axis has been much less studied, it is also possible that environmental bacteria such as *A. lwoffii*, which enter the human organism via the respiratory tract, may affect the microbial composition of the lung, which in turn would affect the composition of the intestinal microbiome. The changes in the intestinal microbiome would affect the immunity of the lung and the development of asthma. It has also been reported that the dysbiosis in the respiratory microbiota resulting from the intra-tracheal single dose of lipopolysaccharide leads to the movement of lung bacteria into the bloodstream. This leads in turn to an increase in the bacterial load in the intestine and thus to a disturbance of the microbial intestinal community. This dysbiosis could also be due to the interaction between translocated lung immune cells and intestinal microbiota(Sze *et al.*, 2014).

2. Hypothesis and aims

Early childhood or even prenatal exposures to farm bacteria such as *Acinetobacter lwoffii* have been demonstrated to predict a decreased incidence of allergies and asthma later in life. Studies in mouse models demonstrated that intranasal application of *A. lwoffii* stimulates local and systemic innate immunity as reflected by increased levels of pro-inflammatory cytokines, especially IL-6 levels in lungs and serum.

The goal of the study was to understand the underlying molecular mechanism of the *A. lwoffii* protective effect against asthma and to prove the hypothesis listed above, we aimed in our investigation to address the following points, (1). The potential role of IL-6 in *A. lwoffii*-induced protection against asthma/allergy development. (2). The first contact happened up on *A. lwoffii* intranasal application. (3). To check whether the activation of innate immune system up on contact with *A. lwoffii* can skew the function of the adaptive immune system towards mechanisms know to favor lower asthma and/or allergy susceptibility. (4). Further assessments in mouse model to reveal the role of other cytokines which secreted from T cells after culturing the naïve T cells with supernatant from *A. lwoffii*-exposed macrophages, such as IL-17 and IL-10 KO mice. (5). Moreover, to figure out whether the microbiota might contribute to the protective effects induced by *A. lwoffii* in the IL-6 KO mice and WT littermate via a possible interaction between IL-6 and the changes in the taxa abundance of the cecum microbiome, thus we speculated that *A. lwoffii* pretreatment might induce changes in the cecum microbiome which is mediated the asthma protective effect through a (Lung-Gut axis).

3. MATERIALS AND METHODS

3.1. Materials tables

BALB/c WT	Jackson Laboratory, USA
BALB/c *Il17af*$^{-/-}$	Prof. Steinhoff lab BMFZ
Backcrossed BALB/c *Il6*^{tm1Kopf}/J	Prof. Manfred Kopf, Zürich
BALB/c *il10*$^{-/-}$	Prof. Francis Crick, London
Fresh *Acinetobacter lwoffii* F78	BMFZ, Marburg.
Lyophilized *Acinetobacter lwoffii*	Prof. Dr. Holger Heine Lab, Borstel

Table 3. Animal experiment

Aceton	Roth, Karlsruhe, D
Albumin bovine Fraction V (BSA)	Serva, Heidelberg, D
Anti-mouse-CD3e	clone 145-2C11, BioLegend, USA
Anti-mouse-CD28	clone 37.5, BioLegend, USA
Brefeldin A	eBiosciences, San Diego, USA
CASY® Ton	Schärfe Systems, Reutlingen, D
CellFix	BD, Heidelberg, D
Complete Protease-Inhibitor Tablette	Roche, Mannheim, D
DAPI	BioLegend, San Diego, USA
Diff-Quick®-Solution	Dade-Behring, Marburg, D
Dithiothreitol (DTT)	PJK GmbH, Kleinblittersdorf, D
Dimethylsulfoxid	Sigma-Aldrich, Taufkirchen, D
EDTA ethylenediaminetetraacetic acid-disodium salt (Na2EDTA)	Roth, Karlsruhe, D
Eosin G	Merck, Darmstadt, D
Ethanol	Roth, Karlsruhe, D
Ethidium bromide soluation	Roth, Karlruhe, D
FACS Clean	BD, Heidelberg, D
FACS Flow TM	BD, Heidelberg, D
FACS Rinse	BD, Heidelberg, D
FCS Gold	PAA LaboratoriesGmbH, Cölbe, D
Hematoxilin II nach Gill	Merck, Darmstadt, D

HEPES	Sigma, Taufkirchen, D
Ionomycin Calcium Salz	Sigma, Taufkirchen, D
Ketamin	Inresa, Freiburg, D
L-Glutamin	PAA LaboratoriesGmbH, Cölbe, D
Natrium pyruvate	PAA LaboratoriesGmbH, Cölbe, D
Non essential amino acids	PAA LaboratoriesGmbH, Cölbe, D
PanColl Mouse	PAN Biotech, Aidenbach, D
Paraformaldehyde	Merck, Darmstadt, D
PAS staining kit	Merck, Darmstadt, D
PBS Dulbeccos (1x) ohne Ca. u. Mg.	PAA LaboratoriesGmbH, Cölbe, D
Penicillin/streptomycin	PAA LaboratoriesGmbH, Cölbe, D
Phorbol 12-myristate 13-acetate (PMA)	Sigma, Taufkirchen, USA
Polyethylene glycol 6000	Sigma-Aldrich, Taufkirchen, D
QuantitTectTMSYBR®Green PCR MasterMix	Qiagen, Hilden, DE
Recombinant murine IL-6	Peprotech Inc, Rocky Hill, USA
Recombinant murine IL-2	Peprotech Inc, Rocky Hill, USA
GM-CSF proteins	BMFZ (Homemade)
M-CSF proteins	BMFZ (Homemade)
FLT3L proteins	BMFZ (Homemade)
RNAse free water	Eppendorf, Hamburg, D
Rnase ERASE	MP Biomedical, Illkirch, F
Rompun 2% (Xylazin)	Bayer Health Care, Leverkusen, D
ROTI®Agarose ultra-quality	Roth, Karlruhe, D
Roticlear (Xylolersatz)	Roth, Karlsruhe, D
RPMI 1640 (1x) ohne L-Glutamin	PAA LaboratoriesGmbH, Cölbe, D
Saponin	Sigma-Aldrich, Taufkirchen
Sulphuric acid 95-97% (H2SO4)	Merck, Darmstadt, D
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe, D
Sodium pyruvat	PAA LaboratoriesGmbH, Cölbe, D
Streptavidin-peroxidase	Sigma, Taufkirchen, D
Streptavidin	Promega, D
Tris-HCl-solution 1M	Invitrogen, Karlsruhe, D

Trypan blue	Gibco, Karlsruhe
Tween®20	Roth, Karlsruhe, D
β-Mercaptoethanol	Roth, Karlsruhe, D
Ketamin 10%	Medistar GmBH, Germany
OVA (ovalbumine)	grade VI, Sigma, Germany
Micro 1.3ml lithium heparin tubes	Sarstedt, Germany
2 % agar	Carl Roth, Germany
Histocrom	Thermo Fisher, USA
Histomount	Merck, KGaA, Germany
LPS	Sigma, Germany
3 % Brewer thioglycollate	Brewers, Sigma-Aldrich, Germany
Heat-inactivated fetal bovine serum	PAA Laboratories, Germany
RBCs lysis buffer	Sigma, Germany
Pre-separation filters (30 µm)	Miltenyi Biotec, Germany
CD4+T Cell Isolation Kit mouse	Miltenyi Biotec, Germany
LS Columns	Miltenyi Biotec, Germany
CD62L MicroBeads	Miltenyi Biotec, Germany
MS Columns	Miltenyi Biotec, Germany
2-mercaptoethanol	Roth, Germany
RLT buffer	RNeasy Mini Kit, Qiagen, Germany

Table 4. Chemical materials

FITC -anti mouse CD45	0,5 mg/mL
APC-anti mouse F4/80	1 mg/mL
FITC -anti mouse CD45 isotype	0,5 mg/mL
APC-anti mouse F4/80 isotype	0,2 mg/mL
APC anti-mouse CD4	0,5 mg/mL
PE anti-mouse CD62L	0,5 mg/mL
APC-anti mouse CD4 isotype	0,5 mg/mL
PE-anti mouse CD62L isotype	0,2 mg/mL
anti-MHCII	0,5 mg/mL –dilution factor 1/300
anti-CD11c	0,5 mg/mL –dilution factor 1/300
anti-B220	0,5 mg/mL –dilution factor 1/300

Table 5. FACS-Reagents- All FACS reagents are from BioLegend or Thermo Fisher, San Diego, USA

IL-1β Duo Set	R&D Systems, Wiesbaden, D
TNFa Duo Set	R&D Systems, Wiesbaden, D
IL-6 Duo Set	R&D Systems, Wiesbaden, D

Table 6. ELISA-Reagents

CD4+ T Cell Isolation Kit, mouse	Miltenyi, USA
DNeasy PowerSoil HTP 96 Kit	Qiagene, Germany

Table 7. Kits

24-well-plate (steril)	Nunc, Roskilde, Dänemark
48-well-plate (steril)	Nunc, Roskilde, Dänemark
96-well-plate, Opaque plate	Costar, Cambridge, USA
96- well-plate black, opaque plate	Costar, Cambridge, USA
96- well-microtiter plate Maxisorp-Flat bottom	Nunc, Roskilde, Dänemark
Casy® Cups	Schärfe Systems, Reutlingen, D
Eppendorf Research 10 pipette	Eppendorf, Hamburg, D
Eppendorf Research 100 pipette	Eppendorf, Hamburg, D
Eppendorf Research 1000 pipette	Eppendorf, Hamburg, D
FACS-tube BD Falcon™	BD, Heidelberg, D
Filter tips 0,1-10 µL	Sarstedt, Nümbrecht, D
Filter tips 2-100 µL	Sarstedt, Nümbrecht, D
Filter tips 1000 µL	Sarstedt, Nümbrecht, D
Pasteur pipettes	Brand, Wertheim
Pipettes 5 mL, 10 mL und 20 mL	Greiner bio-one, Frickenhausen, D
Eppendorf Tubes (0,5 mL)	Biozym, Hess. Oldendorf, D
Eppendorf Tubes (1,5 mL)	Eppendorf, Hamburg, D
Eppendorf Tubes (2 mL)	Eppendorf, Hamburg, D
Nylon mesh 100 µm	BD Falcon, Bedford, USA

Table 8. Consumed materials

BD FACS Canto™ II	BD, Heidelberg, D
CASY®Model TT (Cell-Counter)	Schärfe Systems, Reutlingen, D
Counter AC-8	Assistent, Sondheim, D
Freezer	Liebherr, Ochsenhausen, D
Centrifuge- BIOFUGE fresco	Kendro, Langenselbold, D
Microscope Olympos BX51	Olympus, Hamburg, D
Micro plate reader- Sunrise	Tecan, Crailsheim, D
Neubauer-Cell chamber	Assistent, Sondheim, D
Rotator	LaBinco BV, Breda, NL
Vibrational incubator GFL 3031	GFL, Burgwedel, D
Thermo cell Cooling & Heating Block	Biozym, Hess. Oldendorf, D
Vortexer- MS1 Mini	IKA, Staufen
Water bad	GFL, Burgwedel, D
Centrifuge- Megafuge 1.0R	Heraeus, Osterode, D
Cytocentrifuge Cytospin 3	Shandon, Frankfurt, D
Bioruptor® Pico sonication	Diagenode, Denamark
Cytometric Bead Array-CBA	Bio-Plex® 200, Bio-Rad, USA
Fluorescence microscopy	Zeiss Axio, Germany
Microscope-Olympus IX81, Jaban	Hamburg, Germany

Table 9. Instruments

Magellan	Tecan, Crailsheim, D
BD FACS Diva6	BD, Heidelberg, D
CellF Imaging	Olympus, Hamburg, D
GraphPad Prism®	GraphPad Software, La Jolla (CA),USA
QIIME 2 pipeline	USA

Table 10. Software

3.2. Buffers and medium

β-Mercaptoethanol (ME) for cell culture

35 µL 2-Mercaptoethanol in 50 mL PBS solution (10 mM), steril filters

1% β-Mercaptoethanol in RLT buffer for RNA isolation

500 µL β-Mercaptoethanol in 50 mL RLT buffer

ELISA-Coating buffer:

4,2 g $NaHCO_3$ 0.1 M auf 500 mL H_2O, pH 8,3

ELISA-blocking und dilution buffer

PBS, 1 % BSA (m/v)

ELISA-wasch buffer:

PBS, 0,1 % Tween 20 (v/v)

Lung digestion medium

Each10 mL medium: 10 mL cell culture medium + 100 µL Na pyruvat + 200 µL DNAse.

Cell culture medium:

RPMI 1640, 10% (v/v) FCS, 1 % L-Glutamin (v/v), 1 x NEAA, 100 mg/mL Penicillin G, 60 mg/mL Streptomycin, 50 µM β-Mercaptoethanol

3.3. *In vivo* experiments

3.3.1. Animals

Pathogen-free 6- to 8-weeks-old female mice were used throughout the study. All mice were on BALB/c genetic background. Wild-type, *Il17af−/−, il6−/− and il10−/−* mice were generated locally in the animal facility of the Faculty of Medicine of the Marburg-University. *Il17af−/− C57BL/6* was provided by Prof. Ulrich Steinhoff then we backcrossed them into *BALB/c* genetic background. The *C57BL/6.129S2-Il6tm1Kopf/J* were originally mutated by Manfred Kopf, ETH Zurich and then they were backcrossed locally into *BALB/c* genetic background. The *BALB/c il10−/−* mice were provided by Prof. Anne O'Garra, the Francis Crick Institute, London, with approval by Prof. Anne O'Garra. All mice were kept under specific pathogen-free housing conditions and they were supplied with water and an OVA-free diet ad libitum. All animal experiments were performed in accordance with German and international guidelines and were approved by local authorities (Regierungspräsidium Gießen).

3.3.2. Bacteria

Gram-negative, facultative pathogenic bacterium *Acinetobacter lwoffii* F78 (*A. lwoffii*) isolated from cowshed dust samples by Prof. H. Heine, Borstel. Thereafter, it has been cultured by our microbiology facility (BMFZ) and delivered in PBS at 4°C to be used for the pretreatment of the animal as a potential allergy-protective agent. For *in vivo* experiments, 10^8 CFU of *A. lwoffii* were reconstituted in 50 µl PBS. For the *in vitro* experiments, 10^6 CFU of *A. lwoffii* was used for each well. The lyophilized bacteria were got it from Prof. Dr. Holger Heine Lab Borstel.

3.3.3. Pre-treatment, sensitization and challenge

Wild-type (WT), *Il17af*$^{-/-}$, *il6*$^{-/-}$ and *il10*$^{-/}$ knockout mice were anesthetized with 36 mg/kg of ketamine (a low dose; Ketamin 10% Medistar GmBH, Germany) plus 4 mg/kg rompun (Rompun 2% Bayer, Germany). The mice received intranasally 10^8 CFU of *A. lwoffii* F78 in a final volume of 50 µl PBS, or PBS as a negative control for 17 times every second day. The treatment was stopped one day prior to the first sensitization. After the completion of *A.lwoffii* pretreatment, the mice were sensitized to ovalbumin (OVA) by three adjuvant-free subcutaneous (s.c.) injections of 10 µg OVA (grade VI, Sigma, Germany) on days 36, 43 and 50 of the experimental protocol.

Then, on days 62, 63 and 64, the mice were challenged through inhalation of aerosolized OVA (1% wt/vol diluted in PBS) for 20 minutes. The mice were sacrificed after 48 hours from the last aerosol challenge. For the *A. lwoffii* treatment the mice were anesthetized with a low dose of ketamine and rompun. To sacrificy the mice a high dose of ketamine 180 mg/kg (Ketamin 10% Medistar GmBH, Germany) plus rompun 20 mg/kg (Rompun 2% Bayer, Germany) were given, the experimental plan was clarified in **(Figure 10. A) (Figure 12. A) (Figure 17. A) (Figure 18. A)**.

3.3.4. Serum samples

After scarifying the animal, blood samples were collected from the axillary vessels in Micro 1.3ml lithium heparin tubes (Sarstedt, Germany) and then gently mixed up and down at room temperature. The samples were centrifuged for 30 min at 2000x g at 4 °C and, afterwards, the serum supernatants carefully collected and stored at -20 °C for further analyses.

3.3.5. BAL preparation and differential cell counts

BAL was collected after 48 h from the last challenge with aerosolized OVA. The mice were sacrificed at day 67 and a tracheal cannula was used in order to collect the bronchoalveolar lavage (BAL). BAL was performed once using 1 ml PBS containing 1× protease inhibitor cocktail (Roche, Germany). The BAL was centrifuged for 10 min at 350x g; the cell-free BAL supernatant was removed and stored at -20 °C for further cytokines or microbiome analysis. The pellet was re-suspended in 1 mL PBS plus 1% BSA (Bovine serum albumin, Sigma, Germany) and total leukocytes were counted with an automated cell counter CASY®ton (Casy TT; Schaerfe Systems). For cytospine measurement, 50 µl of the BAL cells in PBS/1 % BSA was pipetted into the cell funnel of the cytocentrifuge (Cytospine 3, Shadan, Germany). After centrifugation for 5 min at 225x g at room temperature, the cells were distributed on a slide and the liquid was absorbed through a filter paper. The slides were dried for 1 hour at room temperature and then stained with Diff-Quick solution (Merz & Dade AG). The differentiation of the cells was performed under the light microscope (Olympus microscope CX23, Japan) at 400x magnification using the standard morphological criteria of the individual cell. Cell subtypes was identified. One hundred cells were counted per cytospin.

3.3.6. Lung histology

In order to examine mucus production and infiltration of the airways by the inflammatory cells, lungs were used for histology analysis. After isolating the trachea from the heart, to get red from the blood, the lungs were washed by pushing 5 ml of 0.9% normal saline into the tracheal cannula directly after the BAL. Then lungs were fixed using 10% formalin (Paraformaldehyde, Carl Roth, Germany) at room temperature; the fixed lungs tissue was moved into warm solution of 2% agarose (Agarose NEEO, ultra-quality, Carl Roth, Germany) and 2% agar (Agar, Carl Roth, Germany) dissolved in aqua destillata at (37 °C) and then cooled for 1 hour at room temperature. This agarose block was then transferred to an embedding cassette and paraffined. The embedding of the lungs in paraffin took place in the Institute of Pathology of the University. Sections with a thickness of 3 µm were made from these paraffin blocks using a Histocrom (Microm, HM 3555, Thermo Fisher Scientific, USA). The slides were dried for 1 day at room temperature and. Afterwards, the sections went through a staining protocol starting with immersing those sections in xylene (Carl Roth, Germany), substituting for 2 x 10 min and rehydrating using descending alcohol series (2 x 5 min 100%, 5 min 96%, 5 min 70% ethanol each, rinsed in aqua distilled water). For the hematoxylin-eosin (HE) staining, the slices were first made in hematoxylin (Carl Roth, Germany) according to Gill III for 5 min, then blue-stained with warm tap water for 5 min, and then rinsed with distilled water. The eosin (Carl Roth, Germany) staining was then performed for 2 min and followed by rinsing with distilled water. The slices went through ascending alcohol series to dehydrate the samples (1 min 96%, 2 x 3 min 100% ethanol and 2 x 10 min xylene substitute). Finally, the samples were covered with Histomount (HX, Merck, KGaA, Germany).

3.3.7. Lung histology analysis

Periodic acid–Schiff (PAS) stained sections were analyzed under the microscope (Olympus, BX51 and Olympus IX81, Japan). The images were randomly selected under the ×10 objective per slide. The numbers of PAS+ mucus-producing goblet cells in the bronchial epithelium were recorded and expressed per millimeter of the basement membrane. Inflammation scores were also identified by the average number of inflammatory cells on an external tangent line between 2 neighboring airway and blood vessel sections.

3.3.8. Fluorescence microscopy

This animal part of this experiment was done by Dr. Hani Harb and the fluorescent microscopy and the analysis were done at the lab of Prof. Dr. Peter Graumann by Dr. Jihad El andari. Here, the *A. lwoffii* cells were stained with DAPI prior to animal inoculation. After collecting the nasopharyngeal sample 4 hours after the *A. lwoffii* application as depicted in PBS into 1.5 ml eppendorf tubes, each tube was poured into 60 µ-Dish, 35 mm high with glass bottom (ibidi) used for microscopy. On the other hand, tissue samples were placed between two glass slides to achieve thinner layers that allow penetration of photons. PBS buffer (20 µl) was added on top of sample to keep the sample hydrated. Images were acquired from the tissue edges and specifically at the thinner layers. All images were acquired here using Zeiss Axio Imager A1 with a TIRF objective having an aperture of 1.45. The microscope is equipped with an EVOLVE EMCCD camera (Photometrics). The operating system used was VisiView (2.1.2). Exposure time set for all images was 500 ms which were subjected to filters DAPI **(Figure 13)**. ImageJ (National Institutes of Health, Bethesda, MD) was employed for processing and analyzing the acquired images.

3.4. *In vitro* experiments

3.4.1. Isolation and *in vitro* stimulation of primary macrophages, myeloid dendritic cells and plasmacytoid dendritic cells

The isolation was done in the BMFZ at Prof. Stefan Bauer lab, Dr. Andreas Kaufmann helps in isolation and differentiation of the cells according to a protocol established in his lab, shortly, for the macrophages the cells were isolated from the bone marrow (BM) of the BALB/c WT mice, erythrocytes are lysed and 5 x 10^6 BM cells were suspended in 10 ml RPMI medium, cells were cultured in 10 cm culture dish (Becton 353003; blue lettering) and M-CSF (Homemade) added to a final concentration of 20 ng/ml. Cells were incubated for 5 days at 37°C; 5% CO2; 100% humidity, at day 3 the M-CSF (Homemade) 20 ng/ml was added again. After day 5 cells were tested by FACS FITC and APC color were used to the analysis the surface marker F4/80 and CD11b, respectively, indicating purity of 48.5 % **(Figure. 1A Suppl)**. For the mDCs, the same protocol for the isolation but we culture the cells with 15% GM-CSF (Homemade) for each 6x10^6 cells/plate or 10% for each 3x10^6. Culture incubate for 7 days in incubator (37°C, 5%CO2), at day 7 the medium was carefully removed and the plate was rinsed

with fresh medium without suspending it, the macrophages are recognizable as white coverings of the panel floor and the floating cell clusters should be visible by microscope are the mDCs, supernatant containing the mDCs was centrifuge and re-suspend then cells were counted for the stimulation, purity was tested by FACS using the CD11c und MHCII antibodies double staining indicating purity of 40.6% **(Figure. 1B Suppl)**. For pDCs, the cells were isolated from the BM, $15x10^6$ cells in 10 ml of the RPMI medium then the FMS-like tyrosine kinase 3 ligand (Flt3L) with end concentration (35 ng/ml) (Homemade) added, cells were incubated for 8 days in incubator (37°C, 5%CO2), at day 8 cells were harvested and tested by FACS using FITC-B220 and APC-CD11c antibodies double staining indicating purity of 37.8 %, pDCs should express both on the surface **(Figure. 1C Suppl)**. After the isolation, those murine cells were washed in 5 ml PBS, centrifuged for 10 minutes at 400× g and then the supernatant was discarded. The cells were re-suspended at a density of 10^6 cells/ml and re-cultured in 24-well plates (Sarstedt, Germany) with medium (RPMI-1640; PAA Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories), L-Glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin (PAA Laboratories). The murine cells were stimulated with microbial stimuli (10^6 CFU freshly live isolated *A. lwoffii*) or LPS (10 ng/mL; from E. coli; Sigma, Germany) as positive control or with medium as negative control **(Figure 14. A)**. After 24 hours the supernatant was harvested and the cytokines were measured using Cytometric Bead Array-CBA (Bio-Plex® 200, Bio-Rad, USA) **(Figure 14. B)**.

3.4.2. Isolation of murine peritoneal macrophages and *in vitro* stimulation

Peritoneal macrophages were isolated from six-week-old female wild-type BALB/c mice. Briefly, mice were injected in the peritoneal cavity with 5-ml syringe (23-G) filled with 1-ml of 3% Brewer thioglycollate medium (Brewers, Sigma-Aldrich, Germany). The inflammatory response was allowed to proceed for 72 hours after the i.p. injection and then peritoneal exudate cells were isolated by washing the peritoneal cavity with 5-7 ml of ice-cold PBS without puncturing the intestine. If the intestine was accidentally punctured, then the mouse was excluded from further processing **(Figure. 15 A)**. The purity of the macrophages was detected using FACS analysis, indicating purity of 85% **(Figure. 2 Suppl)**. Cells were stained with individual mAbs, and the optimal concentrations of antibody indicated by the manufacturer were optimized. 1 µl of Fc-receptor blocking biomaterial such as mouse serum was added to each sample to prevent the nonspecific binding of the mAbs. Then, the samples were incubated on ice

for 15 min. After that, 2 µl of anti-F4/80-APC (APC-anti mouse F4/80 antibody, BioLegend, USA) and anti-CD45-FITC (FITC -anti mouse CD45 antibody, BioLegend, USA) were added to the sample and 2 µl from the isotype APC (APC -anti mouse F4/80 isotype, BioLegend, USA) and FITC (FITC -anti mouse CD45 isotype, BioLegend, USA) were added to the isotype negative control tube. The samples were incubated with the mAbs for 30 min to 1 hour and then samples were centrifuge for 5 to 10 min at 400 × g, 4°C. Afterwards, the supernatants were gently discarded from the tube. Finally, samples were vortex and 150 µl of MACS buffer (PBS + 0.5% BSA) were added to each to be measured using the BD FACS (FACSCanto™ II system, BD, USA).

For the *in vitro* purposes, murine macrophages were centrifuged for 10 minutes at 400 × g and then the supernatant was discarded and the cells were re-cultured in 24-well plates (Sarstedt, Germany) with medium (RPMI-1640; PAA Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories), L-Glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin (PAA Laboratories) at the density of 10^6 cells/ml and stimulated with microbial stimuli (10^6 CFU freshly live isolated *A. lwoffii*), LPS (10 ng/mL; from E. coli; Sigma, Germany) as a positive control, or medium as a negative control. After 24 hours of incubation, supernatants were harvested and the analysis of cytokines was performed using an ELISA specific for IL-6 (R&D Systems, USA), IL-1β, (R&D Systems, USA) and TNF-a (R&D Systems, USA) according to the manufacturer's instructions **(Figure. 15 B)**. These supernatants were used later to stimulate naïve CD4+CD62L T cells isolated from murine spleen.

3.4.3. Isolation of naïve CD4+CD62L+ T cells from mouse spleen

Mouse was killed by cervical dislocation. Then the abdominal cavity was opened, and the spleen was isolated and transferred into the nylon mesh (orange mesh) on the culture dish. Cells were homogenized using syringe and then the cell suspension was transferred into 15 ml Falcon (15 ml Falcon, BD, USA) the dish & the mesh were washed with 3 ml MACS buffer to ensure that all cells were collected. The cells suspension was centrifuged 450x g for 3 minutes at 4 °C. After discarding the supernatant, 1-2 ml of RBCs lysis buffer (Red blood cells, Hypri Max, Sigma, Germany) was added and the cells were incubated for 2 minutes at room temperature. Then the reaction was stopped by adding MACS buffer up to10 ml to each Falcon. The sample was centrifuged again at 450x g for 3 minutes at 4 °C and then the supernatant was

discarded. The cell pellets was re-suspend in 2-5 ml of MACS buffer and then counted by CASY®ton machine (Casy TT; Schaerfe Systems).

3.4.4. Magnetic Cell Sorting-MACS (Miltenyi)

The cell suspension was first filtered through the MACS Pre-seperation filter (Pre-Separation Filters [30 µm] Miltenyi Biotec, Germany). Then the cells were counted using CASY®ton (Casy TT; Schaerfe Systems) and centrifuged 450x g for 5 minutes at 4 °C. After that cells were re-suspended in 400 µL of MACS buffer per 1 x 108 cells and 10 µL of CD4+ T Cell Biotin-Antibody Cocktail per 108 total cells (CD4+T Cell Isolation Kit mouse, Miltenyi Biotec, Germany) were added. This preparation was incubated for 15 minutes at 4 °C in the dark. Then the mixture was further diluted with 300 µL of MACS cold buffer and 200 µL of Anti-Biotin Micro Beads per 108 total cells (CD4+T Cell Isolation Kit mouse, Miltenyi Biotec, Germany). After that, the mixture was incubated for additional 10 minutes in the refrigerator (2−8 °C). Then, after rinsing each column with 3 mL of MACS buffer, the mixture was applied onto the large column (LS Columns, Miltenyi Biotec, Germany) under a magnetic field. The flow-through containing unlabeled cells, representing the enriched CD4+ T cells, was collected and the column was washed with 5 ml buffer. Flow-through was centrifuged at 450x g for 10 minutes at 4 °C and then the supernatant was discarded and the pellet re-suspended in 800 µL of MACS buffer and 200 µL of CD62L (L-selectin) Micro Beads (CD62L MicroBeads, mouse, Miltenyi Biotec, Germany) was added to the mixture. The mixture was mixed gently and incubated for 10 minutes at 4 °C in the dark, and then it was washed with 10 ml MACS buffer and centrifuged at 450x g for 10 minutes at 4 °C. The supernatant was discarded, and the pellet was re-suspended in 500 µL of the buffer. The mixture was applied onto the medium column (MS Columns, Miltenyi Biotec, Germany) under a magnetic field and after rinsing each column with 500 µL of buffer. The columns were washed twice with 500 µL of buffer and then removed from the magnetic separator and placed on a suitable collection tube or 15 ml Falcon and 1 mL of MACS buffer was pipetted onto the column and immediately flush with the magnetically labeled cells by firmly pushing the plunger into the column. The cells were centrifuged at 450x g for 10 minutes at 4 °C and the pellet of naïve CD4+CD62L+ T cells was re-suspended in 1 ml of medium and counted using CASY®ton (Casy TT; Schaerfe Systems).

Cells were maintained in medium (RPMI-1640; PAA Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories), 1% NEAA (PAA Laboratories), 50 µM 2-mercaptoethanol (Roth), L-Glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin (PAA Laboratories) at the density of $2x10^6$ cells/ml. The purity of the cells was tested using FACS analysis, indicating purity of 74% **(Figure. 3 Suppl)**; two Abs were used, APC anti-mouse CD4 Antibody and PE anti-mouse CD62L Antibody) (both BioLegend, USA). 2 µl of each mAbs were used for the samples and 2 µl of the isotype PE (PE-anti mouse CD62L+ isotype, BioLegend, USA) and APC (APC-anti mouse CD+ isotype, BioLegend, USA) was added to the isotype negative control tube. Subsequently, cells were primed with αCD3 (0.5 µg/ml; clone 145-2C11, BioLegend, USA) in pre-coated 48 well-plate and soluble αCD28 mAb (1 µg/ml; clone 37.5, BioLegend, USA) was also added. The naïve T cells were cultured in the presence of the supernatant of macrophages exposed to *A. lwoffii* (50% of macrophage derived supernatant and 50% fresh medium), recombinant murine IL-6 (end concentration of 50 ng/ml, PeproTech, USA), or in medium as a negative control. After seventy-two hours, the medium was replaced in the presence of recombinant murine IL-2 (end concentration of 50 U/ml, PeproTech, USA). Then, the cells were incubated for further 48 hours. Afterwards, the medium was changed again and the cells were re-stimulated with αCD3 (5 µg/ml; clone 145-2C11, BioLegend, USA) in pre-coated 48-plate and after 24 hours the supernatant was collected for cytokine measurement using Cytometric Bead Array-CBA (Bio-Plex® 200, Bio-Rad, USA) as it is showed in **(Figure. 16 B)**

3.4.5. Extracellular staining (FACS)

Extracellular staining is a method used to identify or differentiate the cells based on the specific protein or proteins expressed on their surface. Cells were washed with 2 ml MACS buffer and then centrifuged at 450x g for 3-5 minutes at 4°C. The supernatant was then discarded, and the pellet re-suspended in 1ml MACS buffer. After that, antibodies or isotype mixtures were added for the surface staining. Then, the sample was incubated for 30-45 minutes and washed again with 5 ml MACS buffer and centrifuged at 450x g for 3-5 minutes. The supernatant was discarded, and the pellet re-suspended in 120-200 µl MACS buffer and then measured using FACS machine.

3.4.6. TNF alpha, IL-1β, IL-6, IgE, IgG2a, IgG1 ELISA (enzyme-linked immunosorbent assay)

ELISA is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and cytokines. To measure TNFa, IL-1β, IL-6 and serum immunoglobulins, the ELISA plate was coated with the corresponding coating antibody (50 µL/well) and incubated overnight at 4 °C. Then, the plate was washed four times with the wash buffer (WB; PBS + 0.1 % Tween 20). Afterwards, 100 µL of blocking buffer was applied per each well to block the unspecific binding. The plate was incubated for 2 hours at room temperature on a shaker. Thereafter the plate was washed again four times with the wash buffer (WB; PBS + 0.1 % Tween 20) and 50 µL of the second anti-body was added per well. The plate was incubated for another 2 hours at room temperature and then washed again four times. Then, 25 µL of Streptavidin HRP were added to each well and incubated again for 20-30 minutes in the dark. The plate was washed again eight times and, after that, 100 µL of substrate solution (peroxidase, POD) per well were added, and then, the plate was incubated for 20 minutes. Afterwards, the reaction was stopped by adding 50 µL of 2 M sulfuric acid to each well, changing their color to yellow. The plate was then measured at 450 nm with the Tecan Reader and analyzed with the Magellan 3 software.

3.4.7. Cytometric Bead Array (CBA)

CBA is a new and innovative technology that enables to quantitatively determine the concentration of the cytokines or chemokines from a small amount of sample material (50 µL). This method uses Luminex magnetic beads for the quantification of over 450 biologically relevant targets. Assays specific for inflammation, disease, cancer, cell signaling and growth, apoptosis, toxicity, and more are available. Like ELISA, the assays are based on a capture sandwich immunoassay format. Briefly, the capture antibody-coupled beads are first incubated with samples for a specific time. The plate is then washed, which is followed by an incubation with biotinylated detection antibodies. After another washing step for the unbound biotinylated antibodies, a reporter streptavidin-phycoerythrin conjugate (SA-PE) was added and incubated with the samples. Another washing to remove the unnecessary SA followed, and then, the beads were passed through the array reader measuring the fluorescence of the bound SA-PE **(Figure 9.)**. Mouse cytokine flex sets used by Bio-Rad was able to measure IFN-g, IL-4, IL-5, IL-6, IL-9, IL-10, IL17A, IL-2, TNFa and IL-12p and other cytokines.

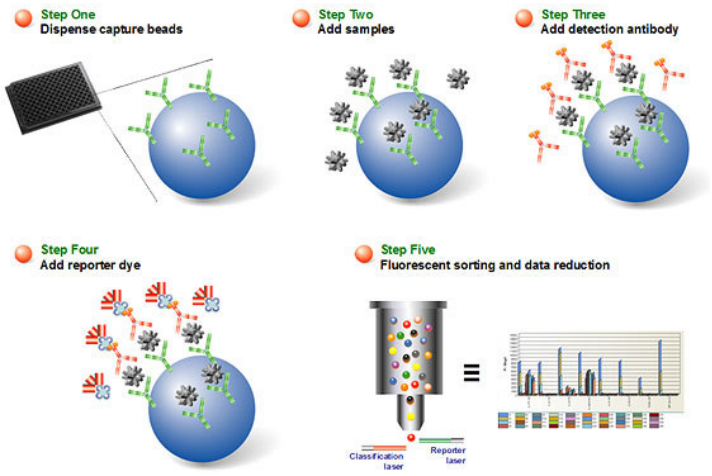


Figure 9. The workflow of the luminex magnetic beads assay (CBA) (graph was taked from Bio-Rad company website)

3.4.8. Statistical Analysis

The graphical representation of the data is conducted using software GraphPadPrism® (version 6, company: Graph Pad Software). For the BAL cytology, BAL cytokine, serum immunoglobulins and histology parameter (Goblet cells and inflammatory score) the two-sided T-test was used to calculate the significance differences between the comparison groups. Bars represent means ± SEMs (n = 6-8 per group), *P < .05, **P < .01, and ***P < .001.

3.5. Microbiome analysis

The samples were collected from the IL-6 KO and WT littermate animals and then transferred to Prof. Martin Blaser lab in USA for the microbiome analysis.

The methods were used at Blaser lab are clarified in the paragraphs, 3.5.2, 3.5.3, 3.5.4

3.5.1. Faecal samples collections

The contents of cecum were collected from the IL-6 KO and the WT littermate mice in cryopreservative tubes then the tubes were snap freezed in liquid nitrogen, then stored at -80 °C for further analysis. The samples were collected at the end of the experiment on day 67 i.e. after the mice pretreated w/o *A. lwoffii* (17 time every second day) and subjected or not subjected to the OVA model of asthma.

3.5.2. Microbial DNA isolation

DNA was extracted using the DNeasy PowerSoil HTP 96 Kit from Qiagen, which can be used for up to 0.25 g of cecum content to each sample. The square well mat from a PowerBead plate was removed then the samples were added to the well plate. After that, 750 µl of PowerBead solution was added to the each well followed by 60 µl of solution C1; the plate was secured tightly and placed on the plate's shaker at speed 20 Hz for 10 minutes. The plate then centrifuge at room temperature for 6 minutes at 4500 x g, and the supernatant moved to a new clean 1 ml collection plate. To the collection plate, 250 µl of solution C2 was added then the plate was vortexed gently and centrifuged at room temperature for 6 minutes at 4500 x g.

In the next step, the supernatant was transferred carefully to a new clean 1 ml collection plate then 200 µl of solution C3 was added, and again the plate was vortexed and centrifuged for 6 minutes at 4500 x g.

For the DNA isolation, only 650 µl of supernatant moved to a new 2 ml collection plate and 650 µl of solution C4 was added then the samples pipet up and down to mix. 650 µl of the mixture was loaded to each well of the spin plate, the spin plate was centrifuged at room temperature for 3 minutes at 4500 x g, the supernatant was discarded then the second 650 µl of the supernatant loaded to the spin plate and we repeated the centrifugation step. After that, the supernatant was discarded, and 500 µl of solution C5-D was added to the spin plate. The plate was centrifuged at room temperature for 3 minutes at 4500 x g, the supernatant was discarded and the plate centrifuged again for 5 minutes at 4500 x g. The supernatant was discarded again and the spin plate allowed to air dry for 10 minutes at room temperature.

In the last step, 100 µl of solution C6 was added to the center of each well, and then we centrifuged at room temperature for 3 minutes at 4500 x g. The DNA was isolated and ready in the bottom of the spin plate and it was collected and frozen at –80°C to be used for downstream applications.

3.5.3. DNA sequencing and OTUs table

After the DNA was extracted using the MoBio PowerSoil DNA Extraction Kit, the microbial 16S rRNA gene was amplified with barcoded fusion primers, targeting either the V1-2 (Fierer *et al.*, 2008) or the V4 (Caporaso *et al.*, 2012) region of the ribosomal DNA. MiSeq platforms protocol from illumine company was applied to sequence the cecum extracted DNA, the average number of the reads after sequencing was 50.949 with minimum number of reads 33.279 and maximal number of reads 65.240.

The QIIME 2 pipeline (Caporaso *et al.*, 2010) was used for quality filtering of DNA sequences, demultiplexing (sorting out the barcodes for each sample), and taxonomic assignment in order to create the OTU table.

OTU table contains the number of sequences that are observed for each taxonomic unit (OTUs) in each sample. Columns usually represent samples and rows represent genera or species taxonomic units (OTUs). OTU file was uploaded and processed by QIIME 2 program in order to create LEfSe (Linear discriminant analysis effect size) graphs for different comparisons to determine the differences in the enriched taxa under the influence of four conditions, first, the influence of *A. lwoffii* pretreatment alone, second, the influence of OVA sensitization alone, third, the influence of *A. lwoffii* pretreatment in OVA-sensitized mice and fourth, the influence of the genotype, in

presence/absence of both *A. lwoffii* and OVA sensitization, or in presence of *A. lwoffii* pretreatment alone, or in presence of OVA alone.

3.5.4. Taxonomic analysis

The LEfSe (Linear discriminant analysis effect size) tool was used to compare relative taxa abundance between two groups. LEfSe results showing significant differences between two groups are plotted as a graph that has bars which represent the effect size (LDA) for a particular taxa in a certain group. The length of the bar represents a log10 transformed LDA score for the phylogenetic sequence data, in simple words, the LDA represent the number of the reads per organism in the sample. The colors represent which group that taxa are found to be more abundant compared to the other group, this code color was clarified in the legend of each graph.

(https://twbattaglia.gitbooks.io/introduction-to-qiime/content/lefse.html)

4. Results

4.1. Protective effects induced by *A. lwoffii* against asthma in mouse model are slightly different between the lyophilized or live bacteria

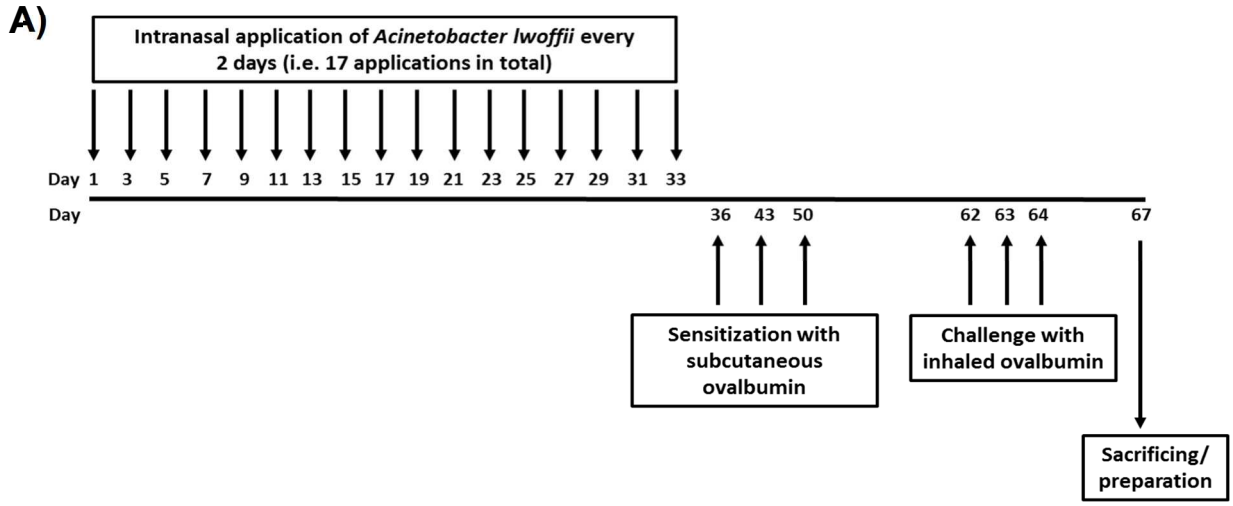


Figure 10. A) Comparison between live and lyophilized *A. lwoffii* on the protective effect against asthma development. *In vivo* experiment layout in which live or lyophilized *A. lwoffii* was applied 17 times every second day. After the last application, mice were three times sensitized with OVA once per week, and then, challenged for three days with 1% OVA through a nebulizer, once per day. The analysis was conducted 48 hours from the last challenge at day 67.

B)

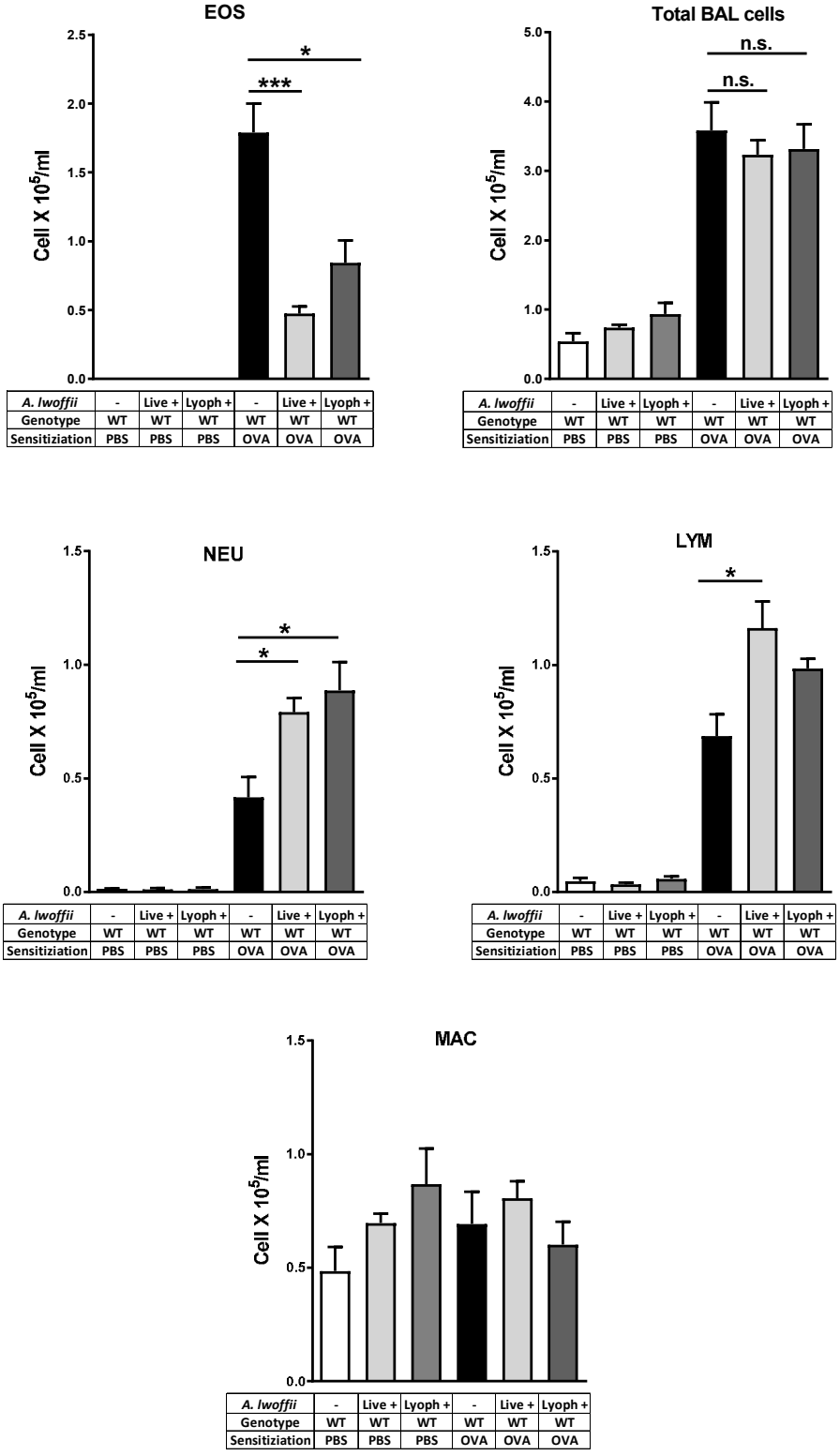
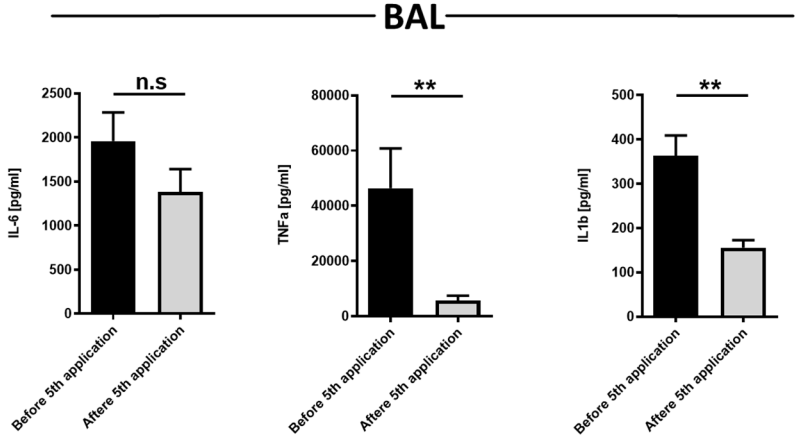


Figure 10. B) Comparison between live and lyophilized *A. lwoffii* on the protective effect against asthma development. BAL cell analysis for OVA and PBS groups treated with live or lyophzlized *A. lwoffii*; PBS used as control; Bars represent means ± SEMs (n = 6 per group), *P < .05, **P < .01, and ***P < .001.

In this experiment, we aimed to determine if the protection effect against asthma induced by *A. lwoffii* is stronger or weaker when applying live or lyophilized bacteria intranasally to the mice subjected later to OVA-experimental asthma model. Every second day, the mice were repeatedly internasally treated with 10^8 CFU of living or dead *A. lwoffii* in a final volume of 50 µl PBS; in total, 17 intranasal application were performed. The control group was treated with PBS as a negative control. The treatment was stopped one day before the starting with three subcutaneous (s.c) ovalbumin (OVA) sensitization steps performed once per week, on the days 36, 43 and 50. The sensitization was followed by an inhaled challenge with aerosolized OVA (1% wt/vol diluted in PBS) for 20 minutes, performed once daily on the days 62, 63 and 64 **(Figure 10. A)**. Forty-eight hours from the last challenge, the mice were sacrificed and the bronchoalveolar lavage was collected for differentiation analysis under the confocal microscope. The evaluation of the data from the BAL cytology clearly indicated that live *A. lwoffiii* has a stronger effect against eosinophilic asthma than the lyophilized one, as the reduction in eosinophils was much higher in the group treated with live *A. lwoffi* compared with the reduction in the group treated with lyophilized *A. lwoffiii*. On the other hand, neutrophils and lymphocytes were induced in both live and lyophilized *A. lwoffii* groups but no changes on the levels of the macrophages were observed **(Figure 10. B).**

4.2. Intranasal exposure to *A. lwoffii* indicates a strong increase of interleukin 6 (IL-6) locally and systemically, with no tolerance developed

A)



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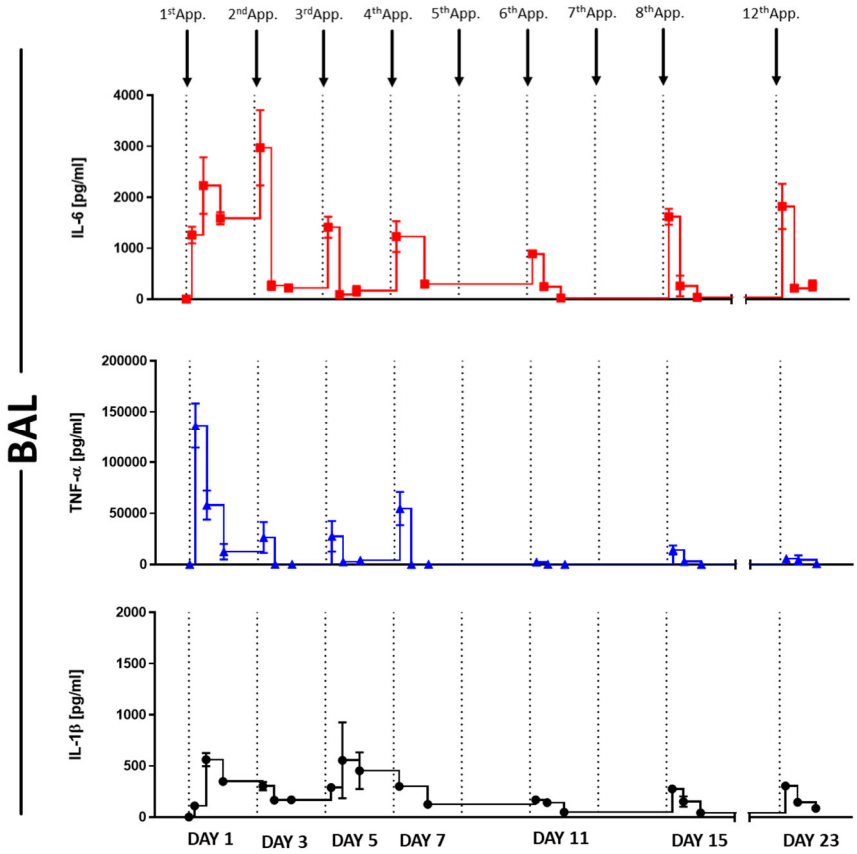


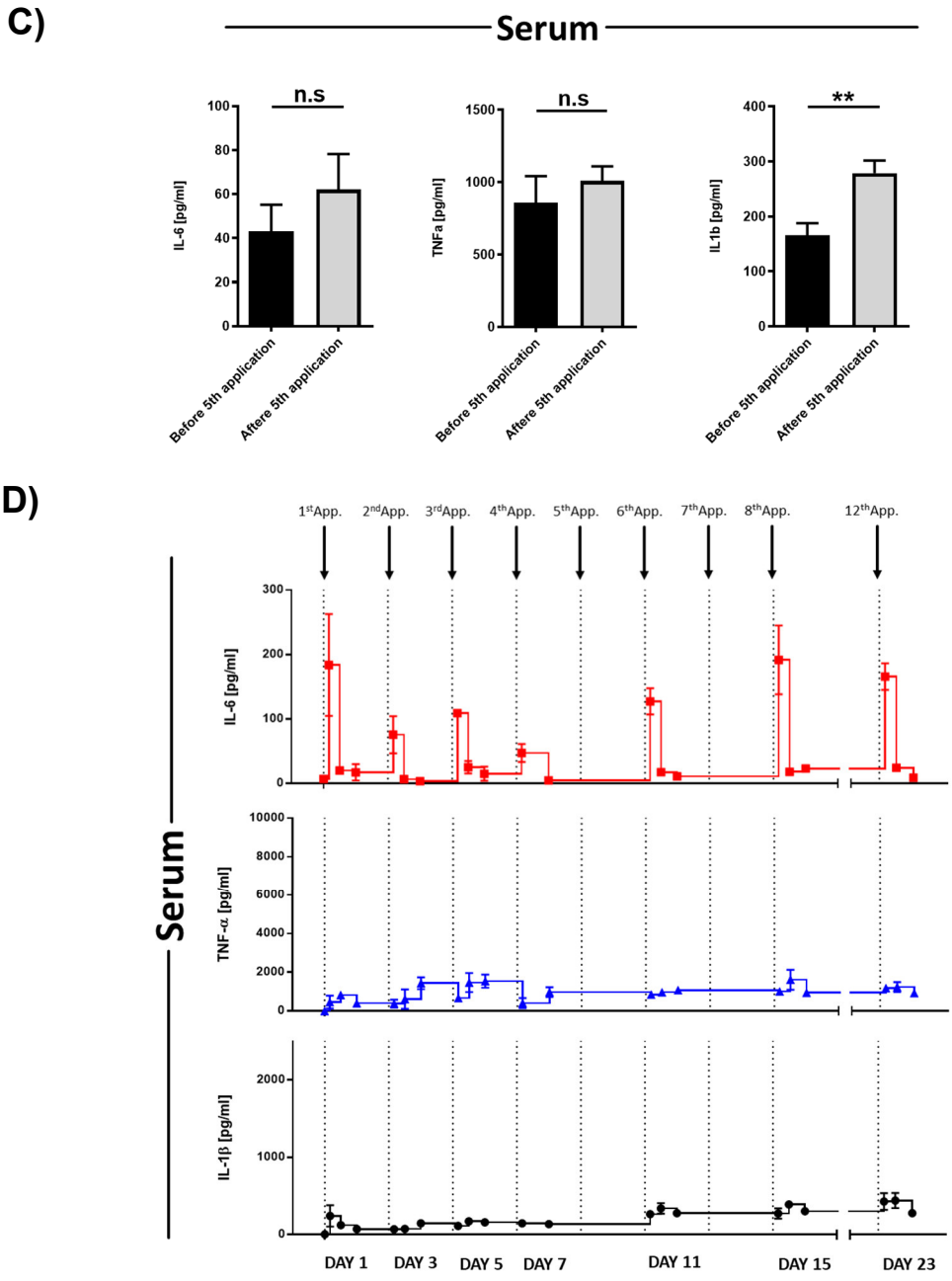


Figure 11. Chronic intranasal exposure to *A. lwoffii* influnces an innate immune response, IL-6 was mostly recognized. WT Mice were treated with *A. lwoffii* every second day from day 1 until day 23, comprising 12 different applications. On days 1, 3, 5, 7, 11, 15 and 23, blood and BAL have been collected from 2-3 mice at different time points (8, 12 and 24 hours) for the analysis of the pro-inflammatory cytokines IL-6, TNFa and IL-1b . **(A**). BAL cytokines IL-1b, IL-6 and TNF-a were measured using R&D ELISA Kit. **(B).** The sum of the BAL cytokine peaks-concentrations (8 hours) before and after the 5th application of *A. lwoffii*; no tolerance was detected for IL-6 levels, while it was observed for IL-1b and TNF-a levels. **(C).** Serum cytokines IL-1b, IL-6 and TNF-a were measured using R&D ELISA Kit. **(D).** The sum of the serum cytokine peaks-concentrations (8 hours) before and after the 5th application of *A. lwoffii*; no tolerance was detected for IL-6 levels, while a further induction was observed for IL-1b and same levels for TNF-a .

In order to investigate the pattern in which *A. lwoffii* stimulates the innate immune system, mice were repeatedly intranasally treated with 10^8 CFU of living *A. lwoffii* in a final volume of 50 µl PBS for each application. The *A. lwoffii* was applied for 23 days, every second day. Blood and BAL were collected from the scarified mice (2-3 mice) at different time points (8, 12 and 24 hours) after each application for 12 applications. Pro-inflammatory cytokines (IL-6, TNFa and IL-1b) in serum and BAL were measured using R&D ELISA Kit.

The data indicates a decrease in the response of TNF-a and IL-1b in the BAL after 5th application of the *A. lwoffii* **(Figure 11. A& B)**. Both cytokines achieved a tolerance locally in the lungs (BAL) after the 5th application of the *A. lwoffii* as it is presented in **(Figure 11. B).** On the other hand, the serum IL-1b levels were increased after the 5th application of *A. lwoffii* **(Figure 11. C)** but the serum TNFa levels stay similar before and after or the 5th application of *A. lwoffii* **(Figure 11. C).**

Interestingly, a very pronounced, fast and transient but repeated inflammatory response was observed for the IL-6 locally in lungs (BAL); IL-6 was increased to the peak 8 hours after the application and then transiently decreased till 24 hours. The effects pattern observed for the IL-6 levels in the lungs were similar to those in serum.

The pattern of innate immune responses assessed locally and systemically after an intranasal application of *A. lwoffii* shed some light on the important role of IL-6 in mediating the effects of *A. lwoffii*.

4.3. *IL-6* knockout abolishes the protective effect of *A. lwoffii* against eosinophilic asthma in a mouse model

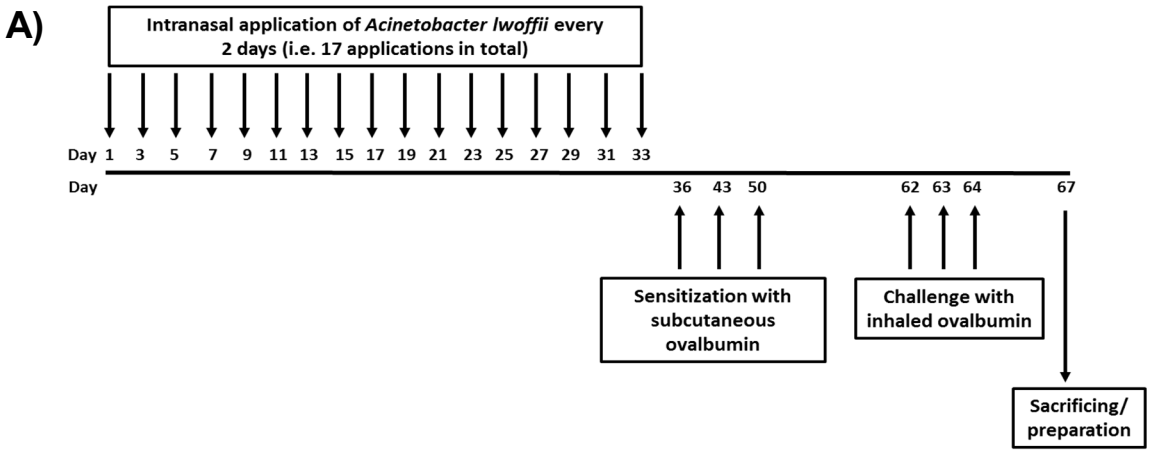


Figure 12. A) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-6 KO mice. Layout of the *in vivo* experiment in which *A. lwoffii* was applied 17 times every second day. After the last application, mice were three times sensitized with OVA once per week, andthen, challenged once per day with 1% OVA through a nebulizer for three days. The analyses were conducted 48 hours from the last challenge at day 67.

B)

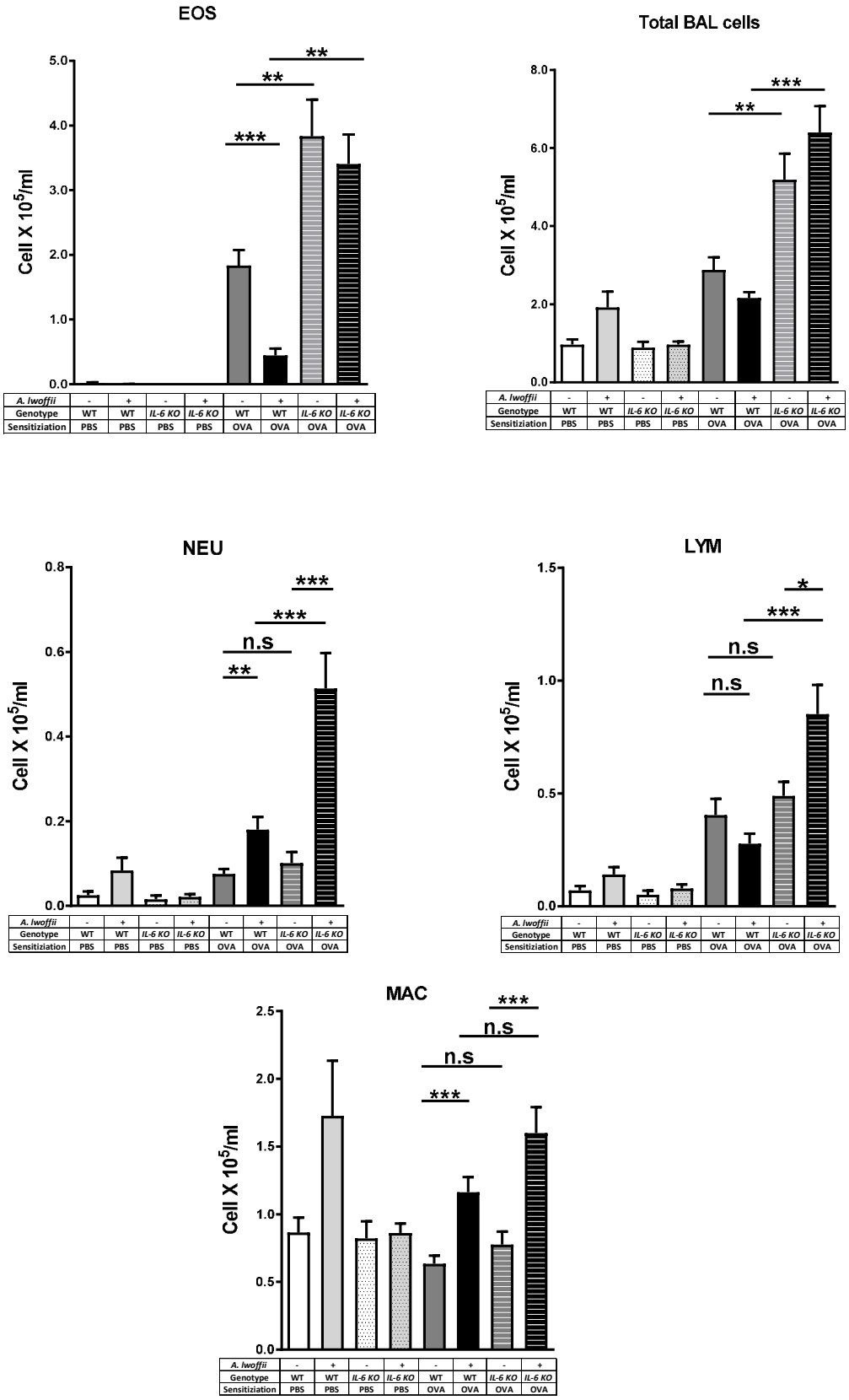


Figure 12. B) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-6 KO mice. BAL cell analysis for OVA and PBS groups treated w/o *A. lwoffii*. Bars represent means ± SEMs (n = 8 per group), *P < .05, **P < .01, and ***P < .001.

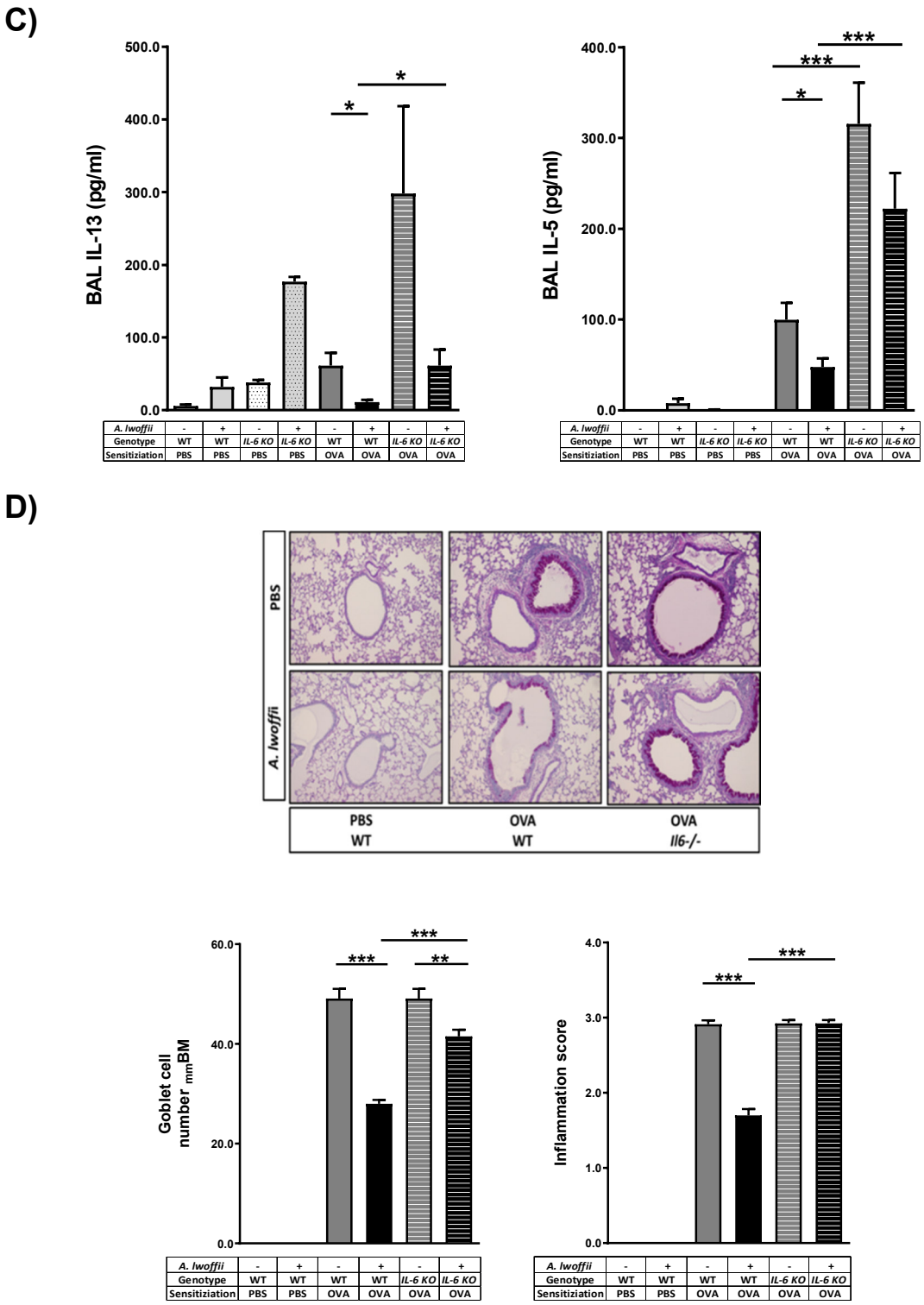


Figure 12. C&D) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-6 KO mice. (C). BAL cytokines IL-5 and IL-13 measurement for OVA and PBS groups treated w/o *A. lwoffii*. **(D).** Representative microphoto-graphs of airways from mice after PAS staining of goblet cell and the calculations of the inflammation score and Goblet cell score for OVA and PBS groups treated w/o *A. lwoffii*, NOT enough biomaterials were available from the IL-6 KO PBS group treated w/o *A. lwoffii* to conduct lung histology . Bars represent means ± SEMs (n = 8 per group), *P < .05, **P < .01, and ***P < .001.

E)

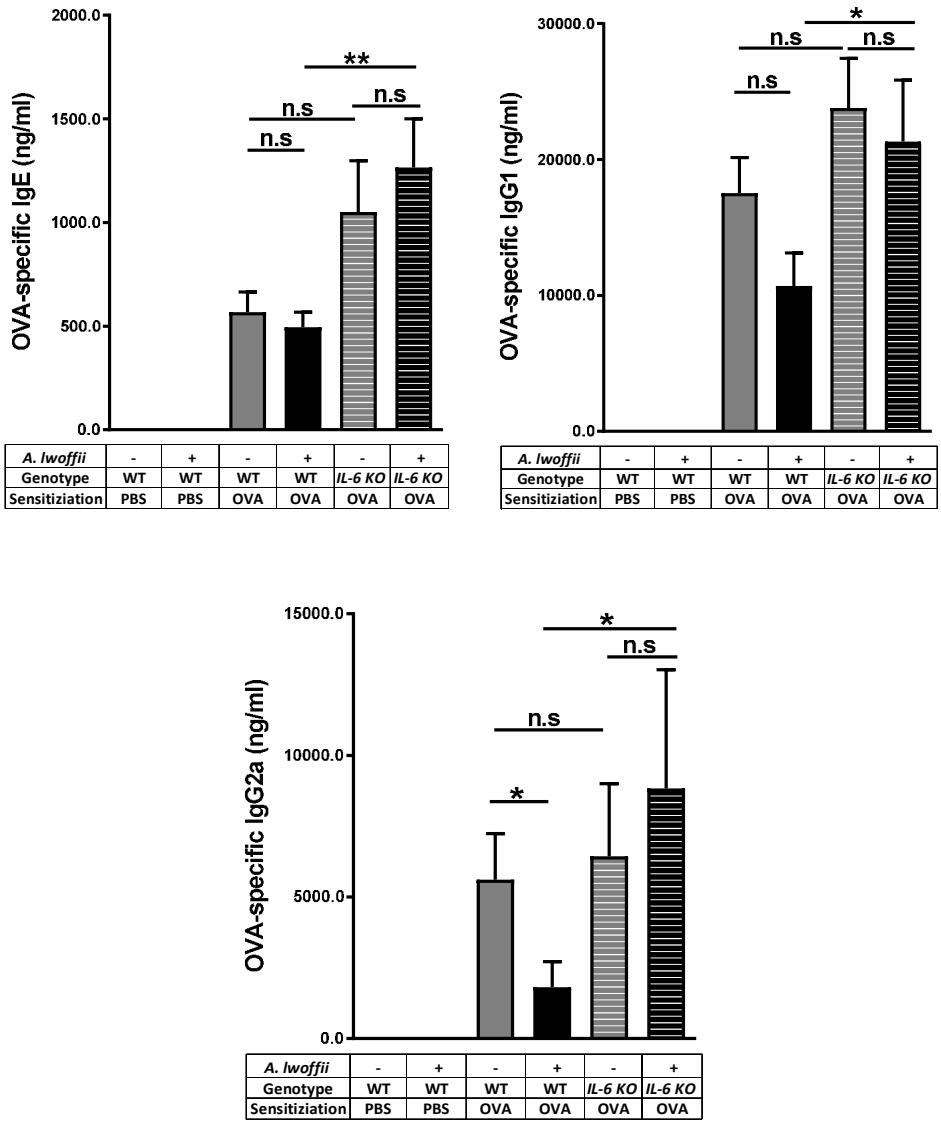


Figure 12. E) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-6 KO mice. OVA-specific serum antibody levels for OVA and PBS groups treated w/o *A. lwoffii*, the serum from the IL-6 KO PBS group treated w/o *A. lwoffii* were not enough to measure the immunoglobulins. Bars represent means ± SEMs (n = 8 per group), *P < .05, **P < .01, and ***P < .001.

In 2009, Melanie Conrad and her colleagues (Conrad *et al*., 2009), first observed increased levels of IL-6 in BAL obtained from mice treated with *A. lwoffii*. Those results together with own observation of the pronounced pattern of increased IL-6 levels after each of multiple applications of *A. lwoffii* suggested a mechanistic role of this pro-inflammatory cytokine in the protective effects of *A. lwoffii* against asthma.

Based on own observations and the previous data, it seems that IL-6 is potentially induced by intranasal application of *A. lwoffii* in WT (wild-type) mice, with no tolerance and it might be crucial for the protective effects against asthma development. Therefore, we hypothesized that IL-6 functionally contributes to the protective effects induced by *A. lwoffii* against allergic airway inflammation. To test own hypothesis, mice with WT and IL-6 KO (IL-6 knockout) background were intranasally treated with 10^8 CFU of living *A. lwoffii* in a final volume of 50 µl PBS, or with PBS as a negative control, 17 times, every second day. Then, the mice were subjected to OVA-Th-2 experimental asthma model. The treatment with *A. lwoffii* stopped one day prior to (s.c.) ovalbumin (OVA) sensitization, performed three times, on the days 36, 43 and 50. Afterwards, at days 62, 63 and 64, the mice were exposed to aerosolized OVA (1% wt/vol diluted in PBS) for 20 minutes **(Figure 12. A)**

Forty-eight hours after the last inhaled challenge with OVA, the mice were sacrificed and the bronchoalveolar lavage (BAL) fluid was collected for the cell differentiation analysis conducted with a confocal microscope as it is described in detail in the methodological section.

The protective effects were observed only in the WT animal pre-treated with *A. lwoffii* and subsequently subjected to OVA-Th-2 experimental asthma model but not in mice subjected to OVA-Th-2 experimental asthma but first pre-treated with PBS only. Attractively, these protective effects were abolished in IL-6 KO mice pre-treated with *A. lwoffii*.

The data demonstrated a significant decrease in the number of eosinophils, a hallmark of eosinophilic asthma, in the BAL fluid from the WT mice subjected to OVA-Th-2 experimental asthma model and pre-treated with *A. lwoffii* compared to the OVA-sensitized WT mice but not treated with *A. lwofii.* The reduction in eosinophil counts was, however, absent in the IL-6 KO mice subjected to OVA model of asthma, regardless of *A. lwoffii* pretreatment **(Figure 12. B)**.

Interestingly, it was also noticed that the asthmatic phenotype was much stronger in OVA-sensitized IL-6 KO mice compared with the asthmatic phenotype in OVA-sensitized WT mice. The eosinophil numbers were much higher in both IL-6 KO mice groups (pre-treated or not pre-treated with *A. lwoffii* and subjected to OVA-Th-2 experimental asthma model) compared with the WT mice not treated with *A. lwoffii* although subjected to OVA-Th-2 experimental asthma model.

In contrast, the numbers of neutrophils and macrophages were significantly induced in OVA-sensitized WT and IL-10 KO mice after pretreatment with *A. lwoffii* compared with the not treated mice. In addition, neutrophils, macrophages, and lymphocytes were induced in the PBS sensitized WT mice after the treatment with *A. lwoffii*.

The number of lymphocytes was significantly increased in the OVA-sensitized IL-6 KO mice after the treatment with *A. lwoffii*, but it was not changed in the OVA-sensitized WT mice, after the treatment with *A. lwoffii* **(Figure 12. B)**.

The cytokine measurements performed in the BAL demonstrated a reduction in the IL-5 and IL-13 levels in the OVA-sensitized WT animals pretreated with *A. lwoffii* compared with the not treated. This reduction in the IL-13 and IL-5 was not observed in the OVA-sensitized IL-6 KO mice pretreated with *A. lwoffii* **(Figure 12. C)**, additionally, the IL-13 was induced in the non-OVA-sensitized IL-6 KO and WT mice after the treatment with *A. lwoffii* compared to PBS control.

The lung histology revealed a reduction in peri-bronchial and peri-vascular inflammatory cell infiltration (inflammatory score) in the OVA-sensitized WT mice after the treatment with *A. lwoffii* compared with the OVA-sensitized WT but not treated with *A. lwoffii.* This reduction was completely absent in OVA-sensitized IL-6 KO mice, regardless of *A. lwoffii* pretreatment.

On the other hand, *A. lwoffii* treatment almost completely hindered the development of mucus-producing goblet cells in OVA-sensitized WT mice after the completion of *A. lwoffii* treatment, here, we observed the same effect for the OVA-sensitized IL-6 KO mice **(Figure 12. D)**. The microphoto-graphs of airways from mice after PAS staining were comparable with the lung histology calculation.

Here, NOT enough biomaterials (lungs) from the IL-6 KO PBS group treated w/o *A. lwoffii* were available to conduct lung histology **(Figure 12. D)**.

Furthermore, IgE, IgG2a, and IgG1 were significantly higher in the OVA-sensitized IL-6 KO mice treated with *A. lwoffii* compared with the OVA-sensitized WT mice treated with *A. lwoffii*. The protective effects of *A. lwoffii* in WT mice was only noticed for the IgG2a which is significantly decreased in the OVA-sensitized WT mice pre-treated with *A. lwoffii* compared with OVA-sensitized WT mice not treated with *A. lwoffii*. The rest of comparisons in OVA-sensitized animals did not reach statistical significance.

Here, the serum from the IL-6 KO PBS group treated w/o *A. lwoffii* were not enough to measure the immunoglobulins **(Figure 12. E)**.

4.4. *In vivo A. lwoffii* first contact cells

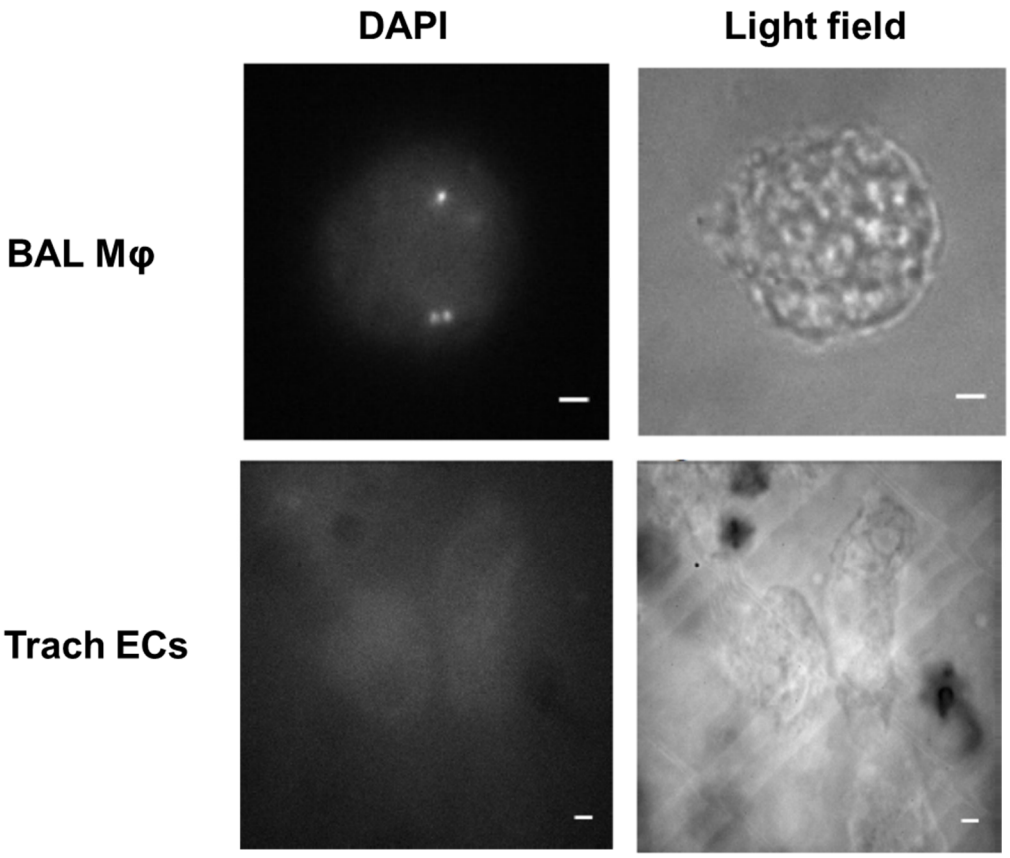


Figure 13. DAPI-pre-stained, intranasally applied *A. lwoffii*. The mouse was killed 4 hours from the *A. lwoffii* application. The data showed that the *A. lwoffii* was taken up by BAL macrophages but not tracheal epithelial cells.

To identify the first contact cells for *A. lwoffii*, the tracheal epithelial cells and alveolar macrophages was studied. To this experiment the animal part was done by Dr. Hani Harb and the fluorescent microscopy and the analysis were done at the lab of Prof. Dr. Peter Graumann by Dr. Jihad El andari. The *A. lwoffii* bacterium was stained with DAPI prior to animal inoculation. DAPI (4,6-diamidino-2-phenylindole), a DNA-specific fluorochrome, was used to detect the presence of the bacteria in the host cells. DAPI specifically binds to double-stranded DNA, emitting a blue fluorescence when excited by 365-nm UV light. After the bacteria stained with DAPI, it was introduced intranasally to the mice and, after 4 hours from the treatment, the mice were sacrificed, and the nasopharyngeal and bronchial epithelial cells were collected for further fluorescent microscopy analysis. The procedure of tissue fluorescent microscopy was conducted as it is clarified in detail in the methodological section. The samples were analyzed using Zeiss Axio Imager A1 with a TIRF objective having an aperture of 1.45. The microscope is equipped with an EVOLVE- EMCCD camera (Photometrics). The operating system used was VisiView (2.1.2). The DAPI-stained *A. lwoffii* bacterium was detected in the alveolar macrophages but not in the epithelial tracheal cells as it is clearly visible in the figure **(Figure 13.)**.

The data demonstrated that the macrophages play a key role as the front line of host contact with *A. lwoffii*. Here, it seems that the macrophages can engulf and digest of *A. lwoffii*, which might be ended by presenting of the *A. lwoffii* antigens to CD4+ T cells. On the other hand, the stimulation the macrophages with *A. lwoffii* bacteria could polarize the macrophages to secret of different cytokines once the crosstalk is started.

In contrast, we were not able to detect DAPI-stained *A. lwoffii* in the tracheal respiratory epithelial cells. Thus, tracheal respiratory epithelial cells seem not to be the point of the initial contact between *A. lwoffii* and host cells. The finding of a crosstalk between the *A. lwoffii* and macrophages was further investigated in *in vitro* model.

4.5. *In vitro* stimulation of primary macrophages, plasmacytoid dendritic cells and myeloid dendritic cells with *A. lwoffii*

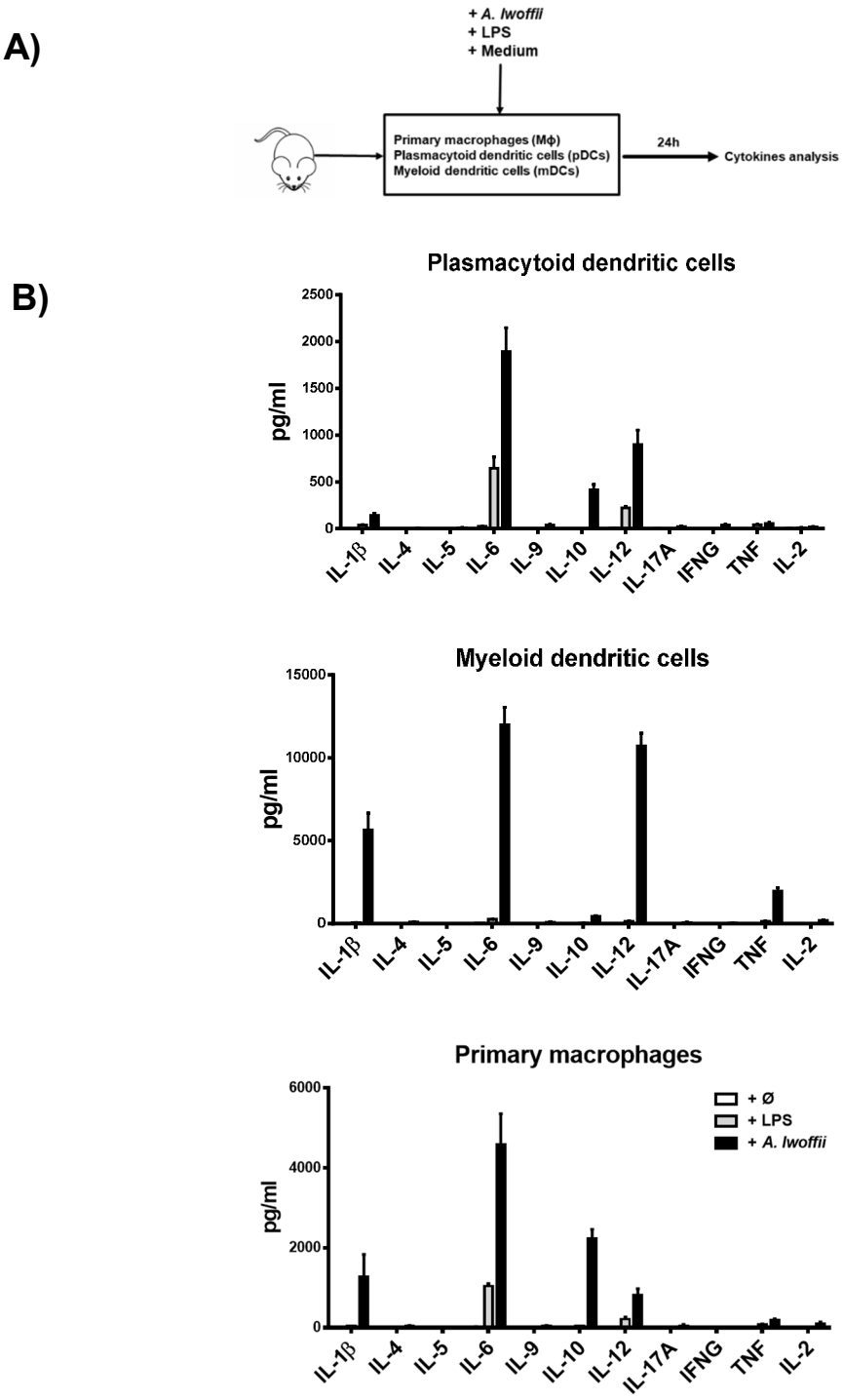


Figure 14. *In vitro* stimualtion of innate immune cells with *A. lwofii* (A). Primary macrophages (Mϕ), plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs) were isolated, and then, stimulated for 24 hours with either LPS (10 ng/ml), $1X10^6$ CFU of *A. lwoffii* or medium as a negative control, as it is described in detail in the methodological section. **(B).** the supernatant was collected and cytokines were measured using CBA-Bio-Plex® 200, Bio-Rad, USA. *A. lwoffii* was able to stimulate Mϕ, pDCs and mDCs to secrete huge amounts of IL-6, the data generated from 3 experiment in triplicate.

The aim of this experimental part was to analyze the innate immune response generated by primary macrophages, plasmacytoid dendritic cells and myeloid dendritic cells. Cells were isolated and differentiated by Dr. Andreas Kaufmann in the BMFZ, Marburg, then tested by FACS as described in the methodology and in **(Figure. 1 Suppl)**.

The cells were stimulated with 10^6 CFU freshly live isolated *A. lwoffii*, LPS (10 ng/ml) as a positive control, or medium as a negative control. After 24 hours of incubation, the supernatant was harvested. The cytokine profile was measured using Cytometric Bead Array-CBA (Bio-Plex® 200, Bio-Rad, USA), as it is clarified in the **(Figure 14. A)**.

Primary macrophages and plasmacytoid and myeloid dendritic cells cultured *in vitro* in the presence of *A. lwoffii* secreted huge amounts of IL-6 and generally smaller amounts of several other classical cytokines of professional non-B-cell antigen-presenting cells such as IL-12, IL-10 and IL-1b. Our investigations *in vitro* demonstrated IL-6 to be the major first line, innate immunity cytokine secreted in response to the contact with *A. lwoffii*, which is at the same in line with the crucial role of IL-6 in mediating the effects of *A. lwoffii* against allergic asthma **(Figure 14. B)**.

4.6. *In vitro* isolation of murine peritoneal macrophages and their stimulation with *A. lwoffii*

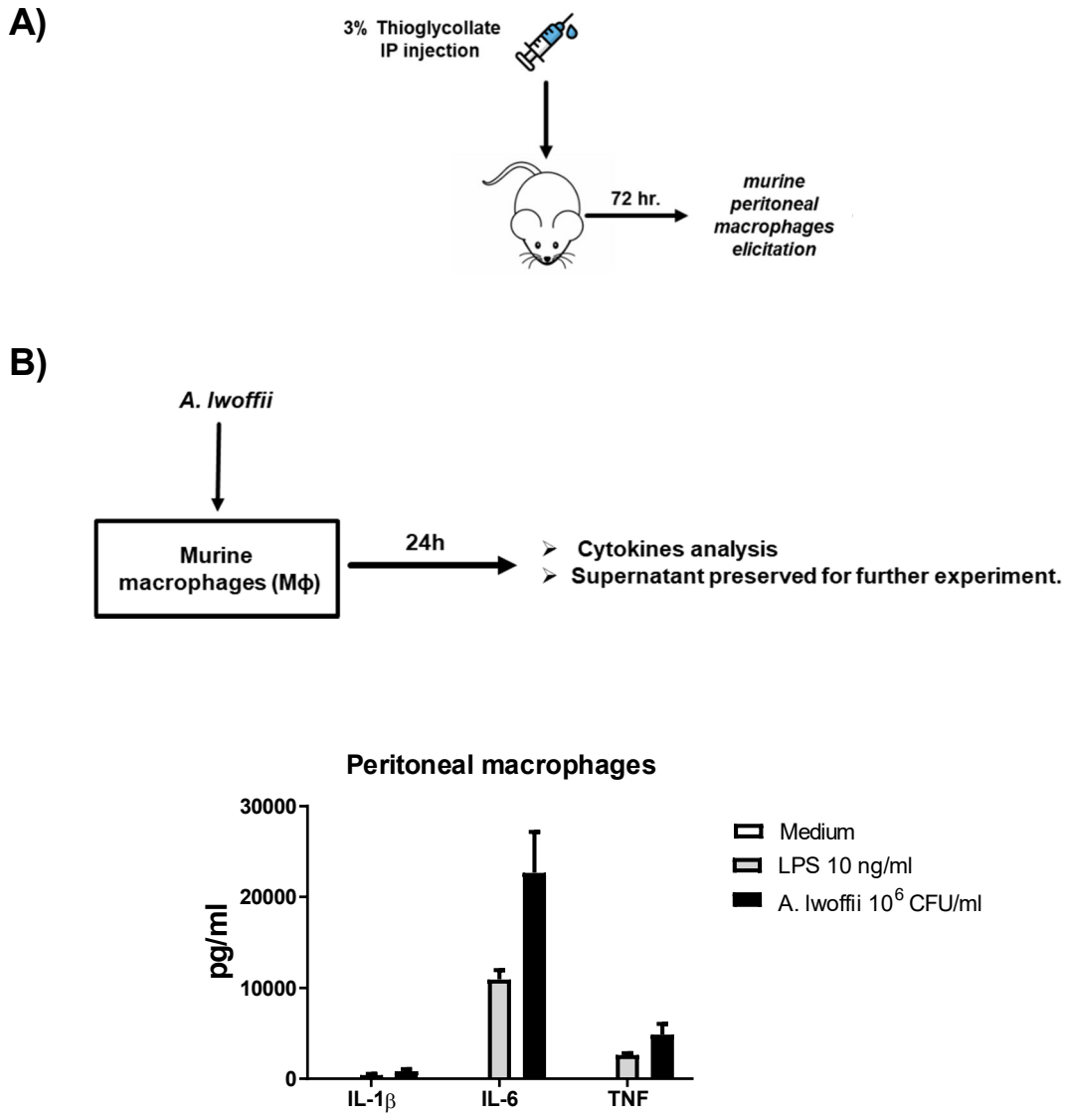


Figure 15. ***In vitro A.lwoffi*-stimulation of the murine peritoneal macrophages. (A).** Murine peritoneal macrophages were isolated from the peritoneal cavity of WT mice after 72 hours of stimulation using peritoneal injection of the 3% Brewer thioglycollate; FACS was used to determine the purity of isolation **(Figure. 2 Suppl). (B).** Murine peritoneal macrophages were stimulated with *A. lwoffii* for 24 hours; the supernatant was collected for further experiments and pro-inflammatory IL-6, IL1b and TNFa cytokines were measured using R&D Kit, data for the cytokines are generated from two independent experiments in triplicate.

Murine peritoneal macrophages were isolated from the peritoneal cavity of (5-6) WT mice after 72 hours of intraperitoneal injection with 3% Brewer thioglycollate **(Figure 15. A)**. The purity of macrophages was tested with FACS machine using anti-F4/80-APC (APC-anti mouse F4/80 anti-body, BioLegend, USA) and anti-CD45-FITC (FITC -anti mouse CD45 anti-body, BioLegend, USA) **(Figure. 2 Suppl)**.

The murine macrophages were cultured in the presence of 10^6 CFU live *A. lwoffii*, LPS (10 ng/ml) as a positive control, or medium as a negative control. After 24 hours of incubation, the supernatant was collected and pro-inflammatory IL-6, IL1b and TNFa cytokines were measured, huge amounts of IL-6 was produced from the *A. lwoffii* stimulated with peritoneal macrophages compared with the LPS stimulated macrophages, less amounts from TNFa were observed **(Figure 15. B)**. Moreover, the supernatant from macrophages exposed *A. lwoffii* or to medium were used for further stimulation of naïve mouse T cells.

4.7. *In vitro* stimulation of naïve CD4+T cells with the supernatant from *A. lwoffii*-exposed macrophages or recombinant IL-6

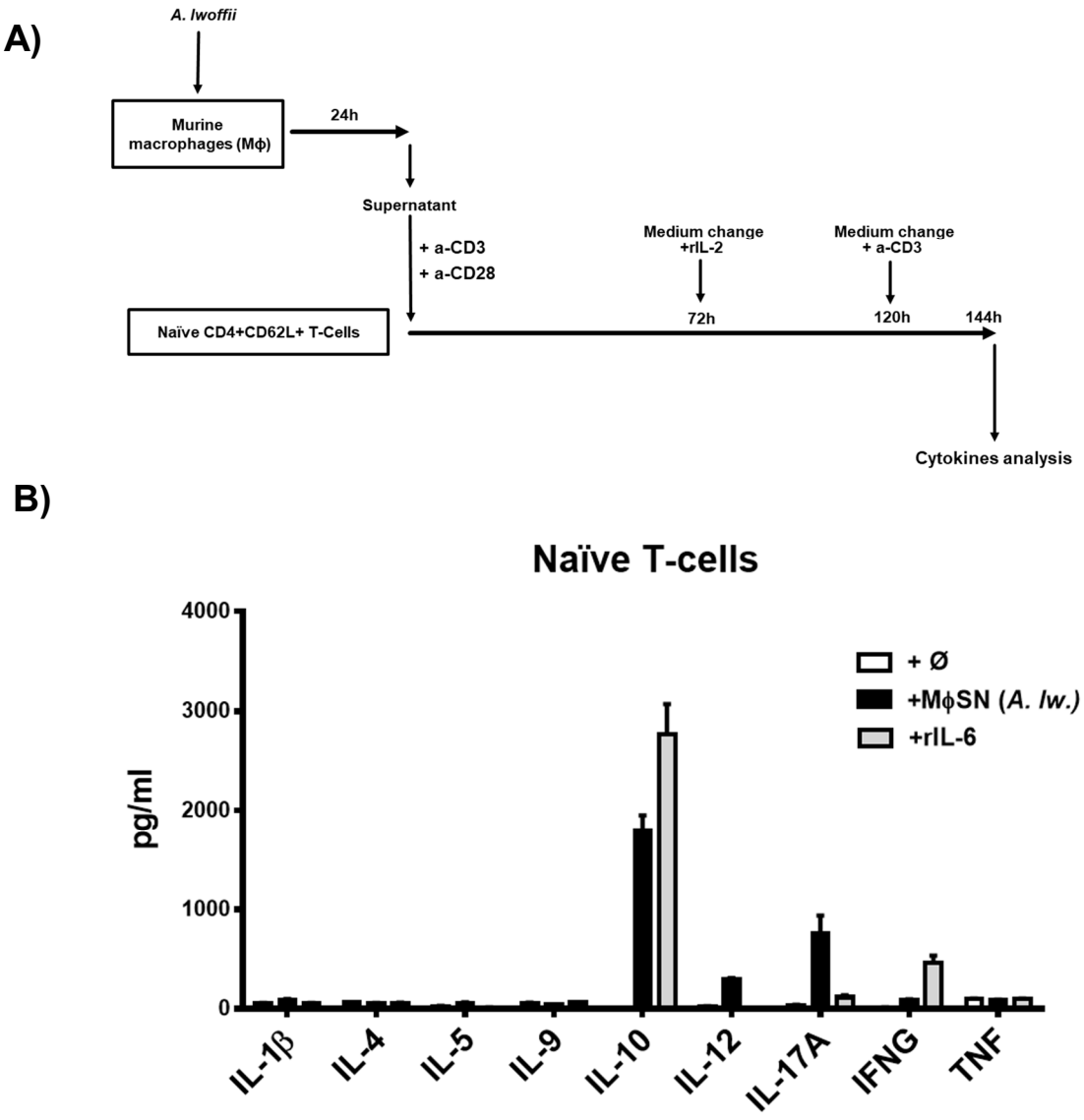


Figure 16. *In vitro* stimulation of naïve CD4+T cells with the supernatant from *A. lwoffii*-exposed macrophages or recombinant IL-6. (A). Layout of the *in vitro* naïve T cell stimulation; the naïve CD4+CD62L+ Tcells isolated from the spleen of wide type mice were cultured in an a-CD3 (0.5 µg/ml) 48-coated plate plus a-CD28 (1 µg/ml). The supernatant from primary macrophages (Mϕ) exposed to *A. lwoffii*, recombinant IL-6 (50 µg/ml), or medium as a negative control were added to naïve T cells. **(B).** The cytokine profile of T cells was measured using Bio-Plex® 200 system. Supernatant from macrophages exposed to *A. lwoffii* was able to stimulate IL-10 secretion from T cells, and the recombinant IL-6 mimicked the same effect.

CD4+CD62L + T cells were isolated from the spleen of WT mice as it is described in the methodological section. The purity of the CD4+T cells was tested with FACS machine, indicating a purity of 76%, (APC anti-mouse CD4 Antibody, BioLegend, USA) and (PE anti-mouse CD62L Antibody, BioLegend, USA) were used **(Figure. 3 Suppl)**.

The naïve T cells were cultured in the presence of the supernatant from the macrophages exposed to *A. lwoffii* (50% of macrophage-derived supernatant and 50% medium), recombinant murine IL-6 (end concentration of 50 ng/ml, PeproTech, USA), or in medium used as negative control. After seventy-two hours, the medium was replaced in the presence of recombinant murine IL-2 (end concentration of 50 U/ml, PeproTech, USA). The cells were then incubated for further 48 hours. Afterwards, the medium was changed again, and the cells were re-stimulated with αCD3 (5 μg/ml; clone 145-2C11, BioLegend, USA) in a pre-coated 48-plate. After 24 hours the supernatant was collected for cytokine measurement **(Figure 16. A& B).**

The data demonstrated that culturing naïve mouse T cells with supernatant from *A. lwoffii*-exposed macrophages or rIL-6 led to production of high amounts IL-10 and IL-17A or only IL-10, respectively. Both, supernatant from *A. lwoffii*-exposed macrophages and rIL-6, induced production of IL-10.

4.8. *IL17* knock out plays no role in the protective effect of *A. lwoffii* against eosinophilic asthma in a mouse model

A)

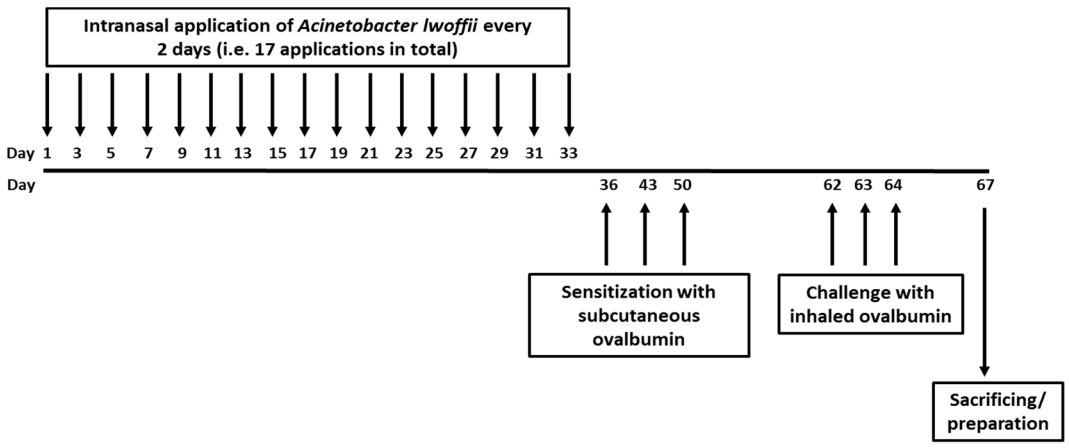


Figure 17. A) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-17 KO mice. Layout of the *in vivo* experiment in which *A. lwoffii* was applied 17 times every second day. After the last application, mice were sensitized with OVA once per week for three times, and then, challenged once per day with 1% OVA through a nebulizer for three days. The analyses were conducted 48 hours from the last challenge at day 67.

B)

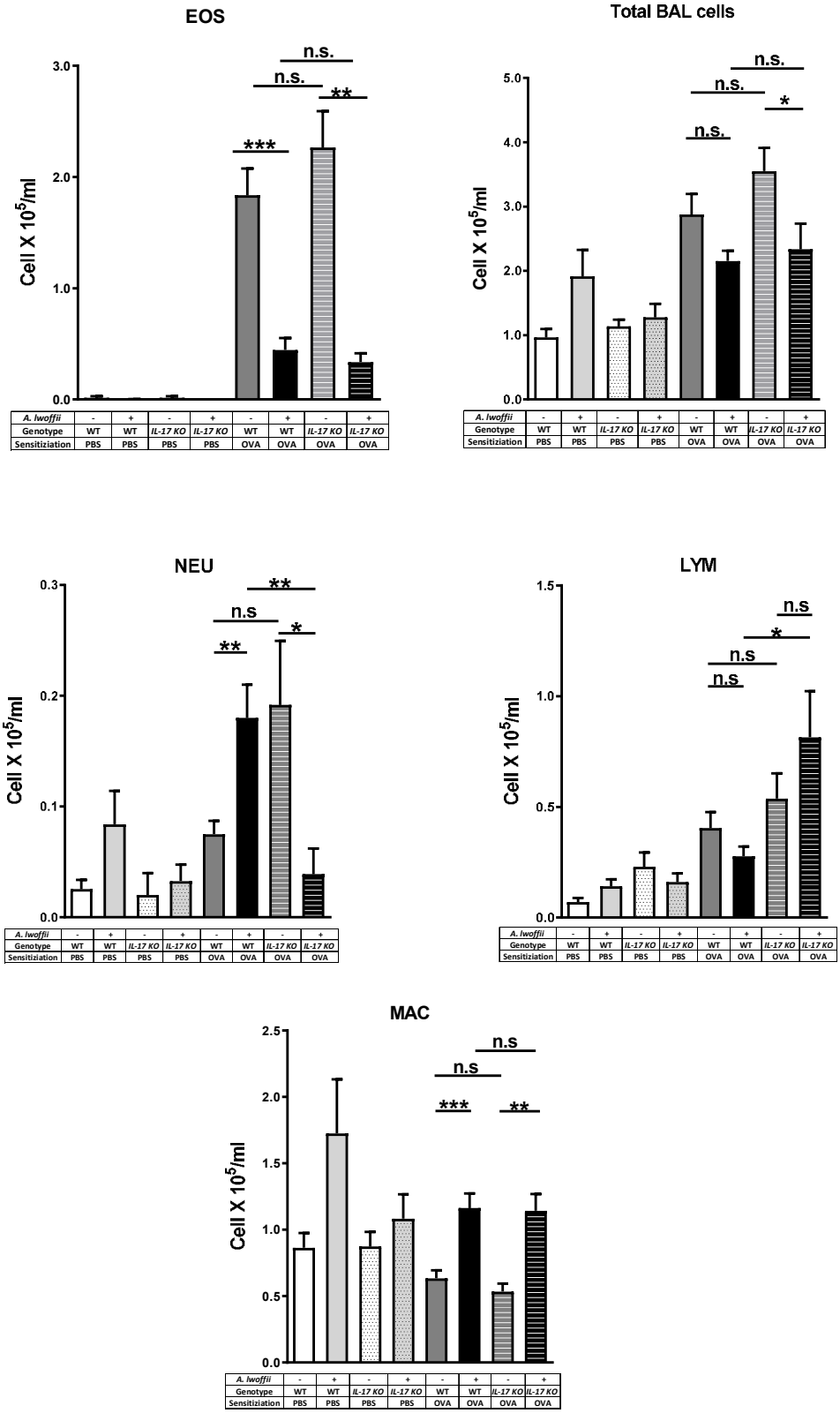
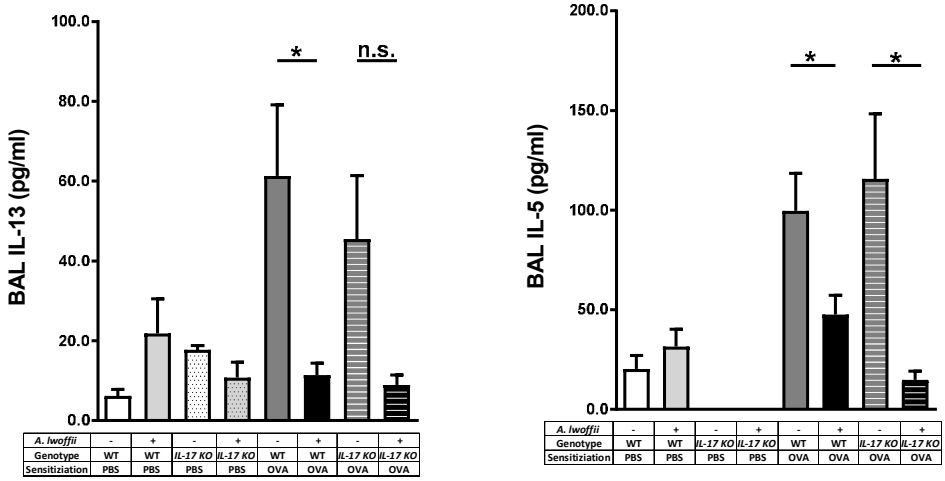


Figure 17. B) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-17 KO mice. BAL cell analysis for OVA and PBS groups treated w/o *A. lwoffii*. Bars represent means ± SEMs (n = 8 per group), *P < .05, **P < .01, and ***P < .001.

C)



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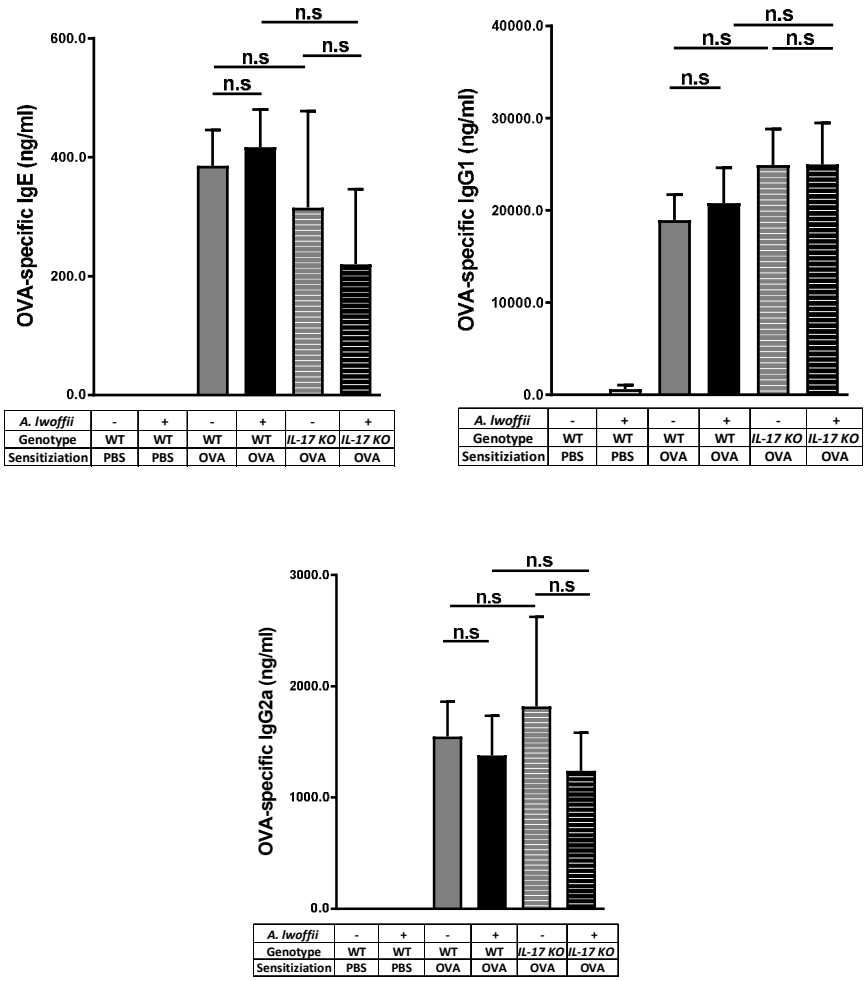


Figure 17. C& D) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-17 KO mice. (C). BAL cytokines IL-5 and IL-13 measurement for OVA and PBS groups treated w/o *A. lwoffii*. **(D).** OVA-specific serum antibody levels for OVA and PBS groups treated w/o *A. lwoffii*, the serum from the IL-17 KO PBS group treated w/o *A. lwoffii* was not enough to measure the immunoglobulins. Bars represent means ± SEMs (n = 8 per group), *P < .05, **P < .01, and ***P < .001.

A crucial role of IL-6 as the first response to the *A. lwoffii* application was shown in mice. Moreover, the IL-6 might play an important role in modulating of the adaptive immune system towards non-/anti-allergic direction. On the top of that, our investigations demonstrated that IL-17 production is thought to arise from naïve T cells primed with IL-6. Therefore, we sought to get further insights into the underlying mechanism downstream of IL-6 production and to understand the role of IL-17 in the protective effects against asthma induced by *A. lwoffii*.

WT (wild-type) and IL-17 KO (IL-17 knockout) mice received intranasally 10^8 CFU of living *A. lwoffii* in a final volume of 50 µl PBS or PBS as a negative control 17 times every second day. Then the mice were subjected to OVA-Th-2 experimental asthma model; the *A. lwoffii* pre-treatment stopped one day prior to the first ovalbumin (OVA) sensitization, performed subcutaneously three times on the days 36, 43 and 50. On days 62, 63 and 64, the mice were exposed to aerosolized OVA (1% wt/vol diluted in PBS), always for 20 minutes **(Figure 17. A).**

Forty-eight hours from the last inhaled challenge with OVA, the mice were sacrificed and the bronchoalveolar lavage (BAL) fluid as collected for the cell differentiation analysis conducted using the confocal microscope, as it is described in detail in the methodological section.

In OVA-sensitized WT and IL-17 KO mice, the pretreatment with *A. lwoffii* was able to reduce the counts of eosinophils in the BAL compared with the OVA-sensitized WT and IL-17 KO mice which were not treated with *A. lwoffii*. The reductions in eosinophil counts in both WT and IL17 KO animals, demonstrating that IL17 plays no role in the protective effects induced by *A. lwoffii* **(Figure 17. B)**.

In contrast, the numbers of BAL macrophages were influenced in the WT and IL-17 KO mice after the treatment with *A. lwoffii* compared with those were not treated with *A. lwoffii,* regardless of the OVA or non-OVA-sensitization.

Besides, in the WT mice, the neutrophils were induced due to the treatment with *A. lwoffii* compared with the *A. lwoffii* not-treated mice, regardless of the OVA or non-OVA-sensitization. But in the OVA-sensitized IL-17 KO mice it was observed that the treatment with *A. lwoffii* reduced the number of the neutrophils compared with the not-treated OVA-sensitized IL-17 KO.

On the other hand, in the non-OVA sensitized IL-17 KO mice, no change was observed on the number of the neutrophils if the mice treated w/o *A. lwoffii*.

For the lymphocytes, not much were observed for many comparisons in the WT or in the IL-17 KO regardless of the *A. lwoffii* pretreatment or OVA sensitization **(Figure 17. B)**.

The cytokine measurements performed in the BAL demonstrated the same reduction in the IL-5 of the OVA-sensitized WT or IL-17 KO mice pretreated with *A. lwoffii* compared with the OVA sensitized WT or IL-17 KO mice not treated with *A. lwoffii*. IL-13 levels were significantly reduced in OVA-sensitized WT animals pretreated with *A. lwoffii* compared with the not treated mice. This effect was weaker or not significant in OVA-sensitized IL-17 KO animals pretreated with *A. lwoffii* compared with the not treated mice **(Figure 17. C)**. The IL-13 and IL-5 levels were induced in the non-OVA sensitized WT mice after the treatment with *A. lwoffii*.

The differences in concentrations of the OVA-specific serum antibodies (IgE, IgG2a, and IgG1) did not reach statistical significance, with the immunoglobulin concentrations being similar within and between the OVA-sensitized WT and IL-17 KO mice regardless of *A. lwoffii* pretreatment **(Figure 17. D)**.

Here, the serum from the IL-17 KO PBS group treated w/o *A. lwoffii* was not enough to measure the immunoglobulins, and the lung histology was not conducted while the lungs were preserved in RNA later -80 C for future microbiome analysis.

4.9. *IL10* knock out abolishes the protective effects of *A. lwoffii* against eosinophilic asthma in a mouse model

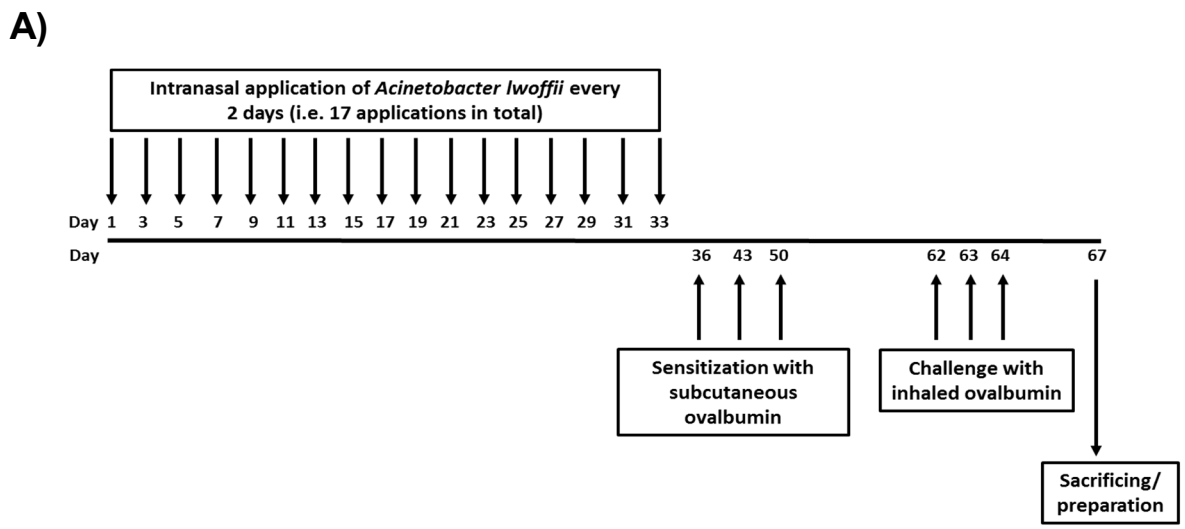


Figure 18. A) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-10 KO mice. Layout of the *in vivo* experiment in which *A. lwoffii* was applied 17 times every second day. After the last application, mice were sensitized with OVA once per week for three times, and then, challenged once per day with 1% OVA through a nebulizer for three days. The analyses were conducted 48 hours from the last challenge at day 67.

B)

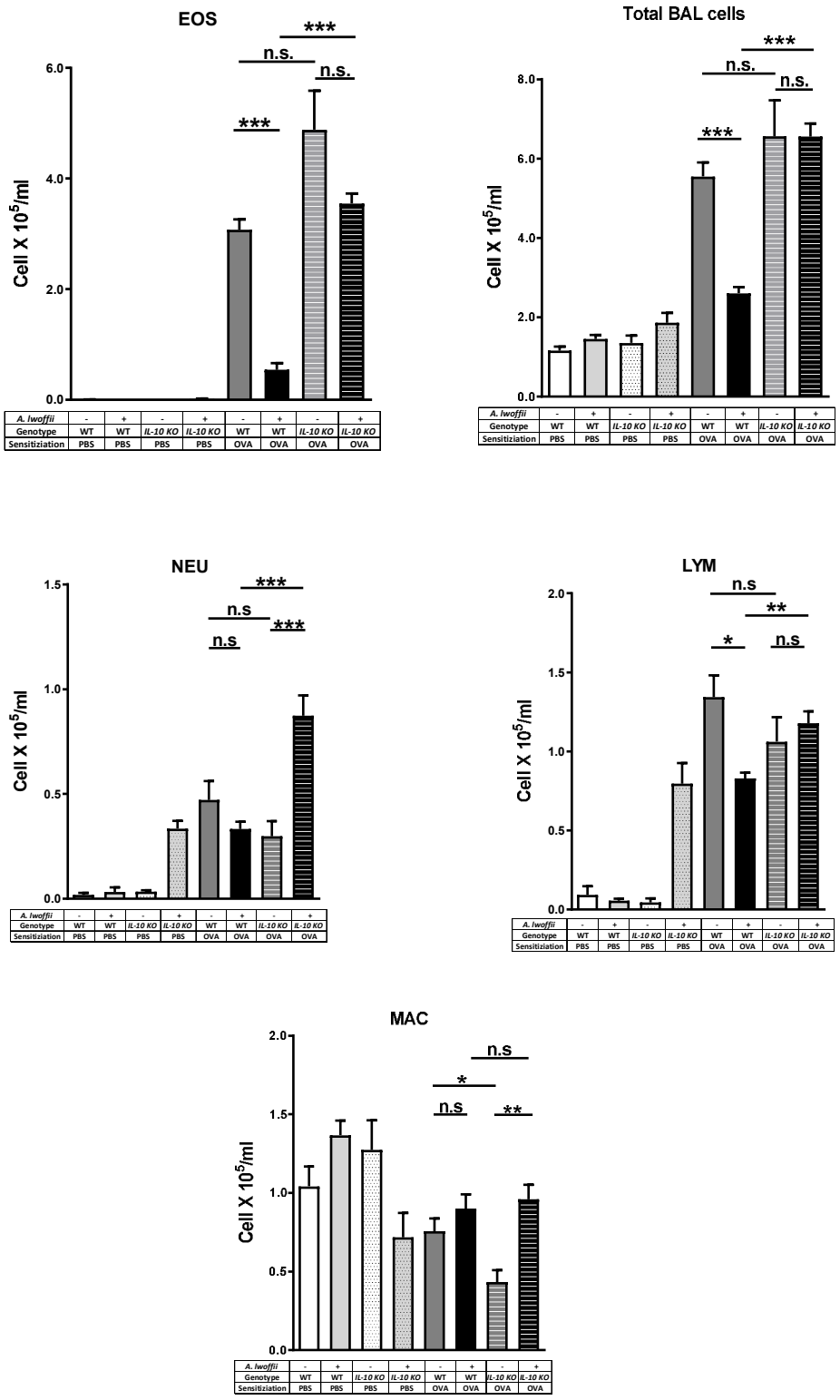


Figure 18. B) The protective effects of *A. lwoffii* against asthma development in WT compared with IL-10 KO mice. BAL cell analysis for OVA and PBS groups treated w/o *A. lwoffii*. Bars represent means ± SEMs (n = 6 per group), *P < .05, **P < .01, and ***P < .001.

Current *in vitro* data showed a huge production of IL-10 from the naïve T cells primed with supernatant from macrophages exposed to *A. lwoffii*. To analyze whether IL-10 functionally contributes to the protective effects of *A. lwoffii* against allergic asthma development, WT (wild-type) and IL-10 KO (IL-10 knockout) mice received intranasally 10^8 CFU of living *A. lwoffii* in a final volume of 50 µl PBS or PBS as a negative control, 17 times, every second day. Then, the mice were subjected to OVA-Th-2 experimental asthma model. The pre-treatment with *A. lwoffii* stopped one day prior to the first ovalbumin (OVA) sensitization, performed subcutaneously three times on the days 36, 43 and 50. Then, the mice were exposed to aerosolized OVA (1% wt/vol diluted in PBS) for 20 minutes at days 62, 63 and 64 **(Figure 18. A)**. Forty-eight hours after the last inhaled challenge with OVA, the mice were sacrificed and the bronchoalveolar lavage (BAL) fluid was collected for the cell's differentiation analysis conducted using the confocal microscope, as described in detail in the methodological section.

The protective effects of *A. lwoffii* against asthma development were observed only in the OVA-sensitized WT mice pre-treated with *A. lwoffii* compared with the not treated mice. The protective effects were abolished in the OVA-sensitized IL-10 KO mice if the mice treated or not treated with *A. lwoffii*.

The data demonstrated a significant decrease in the number of eosinophils in the BAL fluid from the OVA-sensitized WT mice pre-treated with *A. lwoffii* compared with the OVA-sensitized WT not-treated with *A. lwoffii*, but the reduction in eosinophils counts was disappeared in the OVA-sensitized IL-10 KO mice pre-treated with *A. lwoffii* compared with the not treated **(Figure 18. B)**.

Besides, it was noticed that the asthmatic phenotype was much stronger in the OVA-sensitized IL-10 KO mice (regardless of *A. lwoffii* pretreatment) compared with the asthmatic phenotype in OVA-sensitized WT mice not treated with *A. lwoffii* .

Eosinophil counts were much higher in both OVA-sensitized IL-10 KO mice groups (pre-treated or not pre-treated with *A. lwoffii*) compared with the WT animals not treated with *A. lwoffii* although subjected to OVA-Th-2 experimental asthma model.

On the other hand, the numbers of lymphocytes were decreased in OVA-sensitized WT mice pretreated with *A. lwoffii* compared with the not-treated mice.

In the OVA-sensitized IL-10 KO group, the number of the lymphocytes was not different if the mice were treated w/o *A. lwoffii*, but in absence of OVA-sensitization the lymphocytes were highly induced after the treatment with *A. lwoffii* **(Figure 18. B)**.

In absence of OVA sensitization macrophages were increased in the WT mice but decreed in the IL-17 KO mice after the pretreatment with *A. lwoffii*. In presence of OVA the macrophages were not changes after the treatment with *A. lwoffii* but in the IL-17 KO mice they were significantly increased under the influence of *A. lwoffii* treatment. Besides, the macrophages in the PBS control groups were higher than the macrophages in the OVA control groups of the WT or IL-10 KO mice. The neutrophils significantly increased in the IL-10 KO mice after the treatment with *A. lwoffii*, regardless of the OVA sensitization **(Figure 18. B)**.

The difference was not significant in presence/absence of OVA of the WT mice if we compared the *A. lwoffii* treated mice with the not treated mice. For this experiment the lungs and serum were preserved for future microbiome analysis.

4.10. Genotype effect on the relative abundance of taxa in cecum microbiota in absence of both *A. lwoffii* pre-treatment and OVA-sensitization (IL-6 KO versus WT)

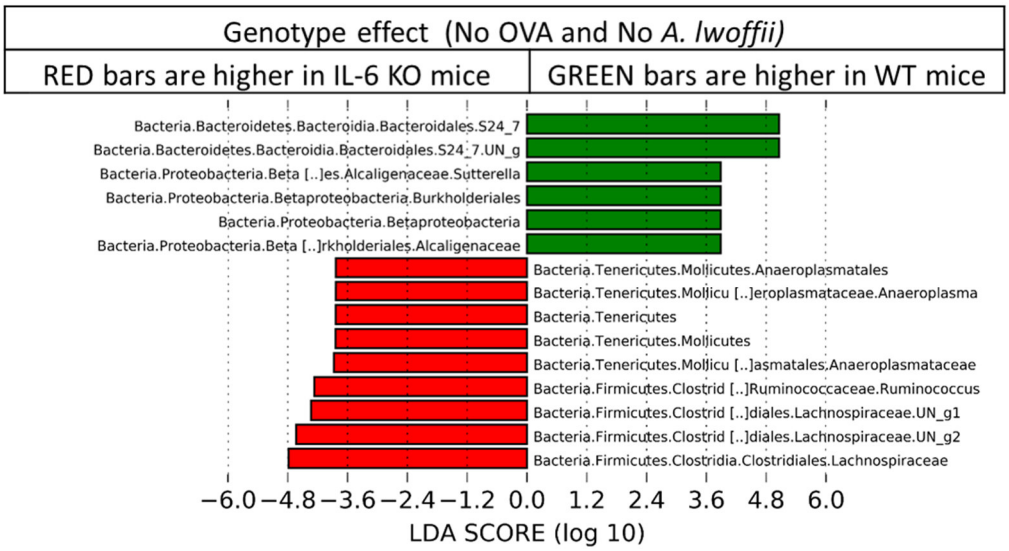


Figure 19. Genotype effect on the taxa abundance of the cecum microbiota, in absence of both *A. lwoffii* pre-treatment and OVA (IL-6 KO versus WT). LEfSe (Linear discriminant analysis effect size) was used to compare relative abundance of taxa between the two groups. LEfSe are plotted as a graph that has bars which represent the effect size (LDA) for a particular taxa in a certain group. The length of the bar represents a log10 transformed LDA score. The colors represent which group that taxa was found to be more abundant compared to the other group. GREEN bars, significant higher in WT mice, RED bars, significant higher in IL-6 KO mice. All mice were not treated with *A. lwoffii* and not subjected to OVA model of asthma, (UN_g2) unclassied genus.

Microbiome analysis conducted on the cecum contents of the samples which were collected from WT and IL-6 KO mice on day 67 **(Figure 12. A)**. The animals were neither pretreated with *A. lwoffii* nor subjected to OVA sensitization. Moreover, the differences in phylum-, class-, order-, family- and genus-level relative abundance between IL-KO and WT littermate were determined using LEfSe and plotted as a bars graph. The length of the bar represents a log10 transformed LDA score **(Figure 19)**.

In the WT littermate the relative abundance of *S24_7.UN_g* and *Sutterella* were significantly higher (GREEN bars) compared with IL-6 KO mice. And the relative abundance of *Lachnospiraceae.UN_g1*, *Lachnospiraceae.UN_g2*, *Ruminococcus* and *Anaeroplasma* were significantly higher in the IL-6 KO mice (RED bars) compared with WT littermate, as it is described in **(Figure 19)**.

4.11. Genotype effect on the relative abundance of taxa in cecum microbiota in presence of *A. lwoffii* pre-treatment but no OVA-sensitization (IL-6 KO versus WT)

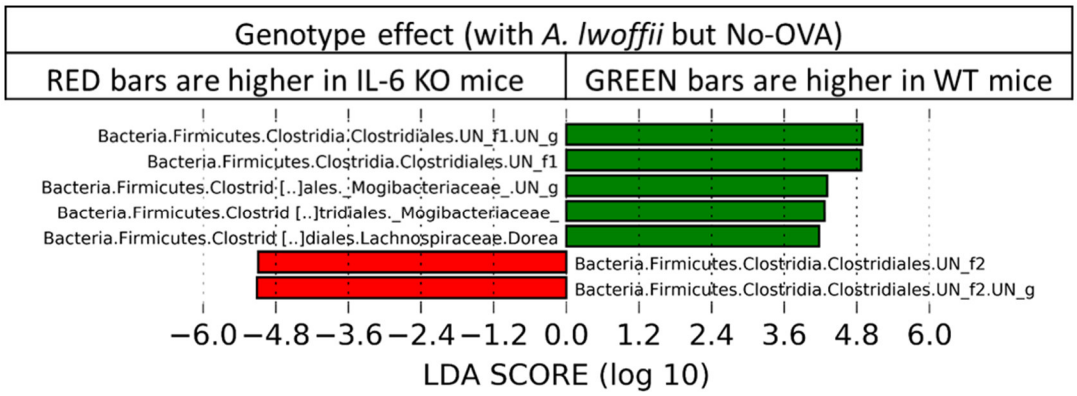


Figure 20. Genotype effect on the taxa abundance of the cecum microbiota, in presence of *A. lwoffii* pretreatment but No OVA (IL-6 KO versus WT). LEfSe (Linear discriminant analysis effect size) was used to compare relative abundance of taxa between the two groups. LEfSe are plotted as a graph that has bars which represent the effect size (LDA) for a particular taxa in a certain group. The length of the bar represents a log10 transformed LDA score. The colors represent which group that taxa was found to be more abundant compared to the other group. GREEN bars, significant higher in WT mice, RED bars, significant higher in IL-6 KO mice. All mice were pretreated with *A. lwoffii* and not subjected to OVA model of asthma, (UN_f) unclassied family, (UN_g) unclassied genus.

Microbiome analysis conducted on the cecum contents of the samples which were collected from WT and IL-6 KO mice on day 67, i.e. after the completion of the intranasal treatment with *A. lwoffii* comprising 17 applications performed every second day, the animals were not subjected to OVA model of asthma **(Figure 12. A)**.

The differences in phylum-, class-, order-, family- and genus-level relative abundance between IL-KO and WT littermate were determined using LEfSe and plotted as a bars graph. The length of the bar represents a log10 transformed LDA score **(Figure 20)**.

In the WT littermate mice, the relative abundance of *Mogibacteriaceae.UN_g, Clostridiales.UN_f1.UN_g* and *Dorea* were significantly higher (GREEN bars) compared with the IL-6 KO mice. And the relative abundance of *Clostridiales.UN_f2.UN_g* was significantly higher in the IL-6 KO mice (RED bars) compared with the WT littermate, as it is described in **(Figure 20)**.

4.12. Genotype effect on the relative abundance of taxa in cecum microbiota in absence of *A. lwoffii* pre-treatment but with OVA-sensitization (IL-6 KO versus WT)

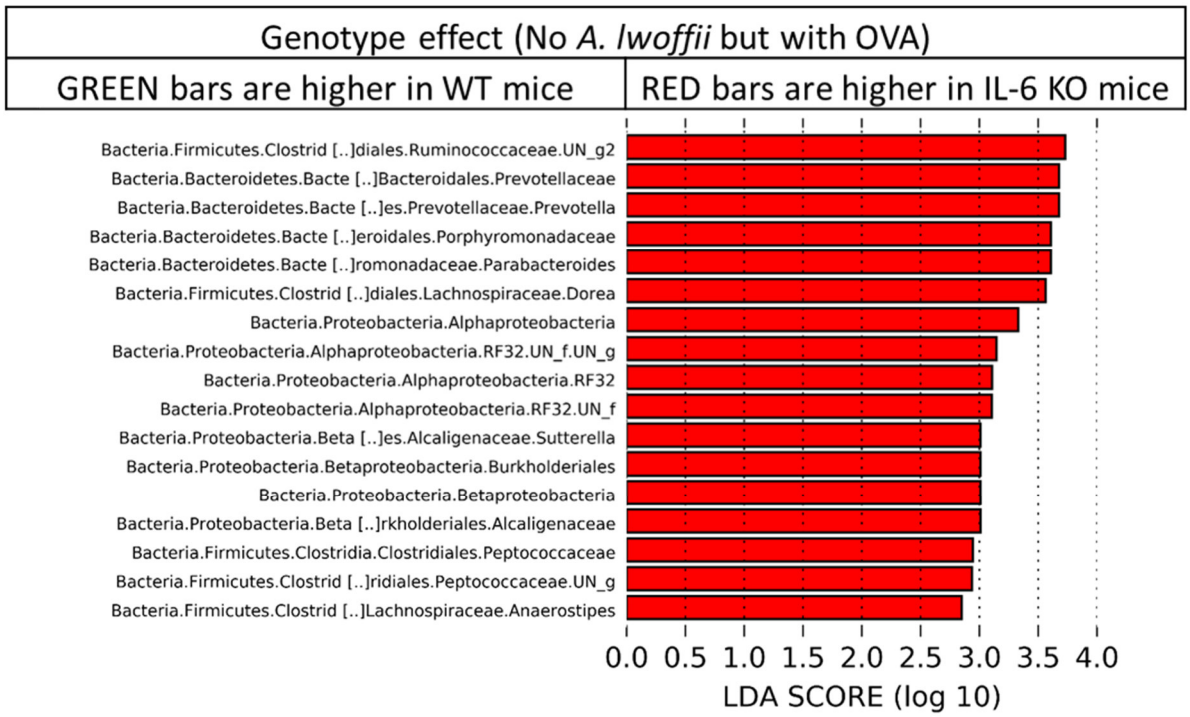


Figure 21. Genotype effect on the taxa abundance of the cecum microbiota, in absence of *A. lwoffii* pretreatment but with OVA (IL-6 KO versus WT). LEfSe (Linear discriminant analysis effect size) was used to compare relative abundance of taxa between the two groups. LEfSe are plotted as a graph that has bars which represent the effect size (LDA) for a particular taxa in a certain group. The length of the bar represents a log10 transformed LDA score. The colors represent which group that taxa was found to be more abundant compared to the other group. GREEN bars, significant higher in WT mice (no taxa were detected to be higher in the WT compared with IL-6 KO), RED bars, significant higher in IL-6 KO mice. All mice were subjected to OVA model of asthma and not treated with *A. lwoffi,i* (UN_f) unclassied family, (UN_g) unclassied genus.

Microbiome analysis conducted on the cecum contents of the samples which were collected from WT and IL-6 KO mice on day 67 **(Figure 12. A)**, the animals were subjected to OVA model of asthma and not pre-treated with *A. lwoffii*. The differences in phylum-, class-, order-, family- and genus-level relative abundance between IL-6 KO and WT littermate mice were determined using LEfSe and plotted as a bars graph.

The length of the bar represents a log10 transformed LDA score, the absence of the GREEN bars is indicating that no taxa were detected to be higher in the WT compared to IL-6 KO **(Figure 21)**.

In the WT littermate, no taxa were detected to be higher compared with the IL-6 KO (GREEN bars are absence) but in the IL-6 KO mice the relative abundance of *Ruminococcaceae.UN_g2,Prevotella,Sutterella,Anaerostipes,Dorea,Parabacteroides, Peptococcaceae.UN_g, and RF32.UN_f.UN_g* were higher (RED bars) compared with WT littermate **(Figure 21)**.

4.13. Genotype effect on the relative abundance of taxa in cecum microbiota in presence of both *A. lwoffii* pre-treatment and OVA-sensitization (IL-6 KO versus WT)

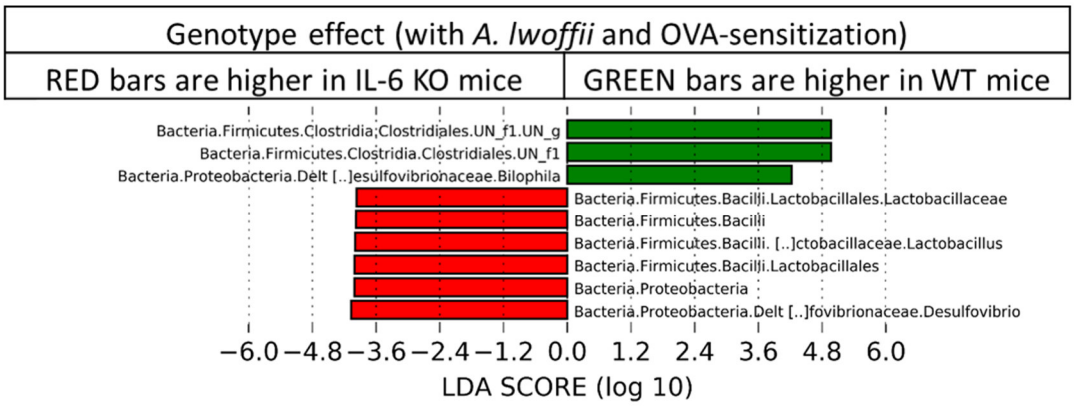


Figure 22. Genotype effect on the taxa abundance of the cecum microbiota in presence of *A. lwoffii* pretreatment and OVA sensitization (IL-6 KO versus WT). LEfSe (Linear discriminant analysis effect size) was used to compare relative abundance of taxa between the two groups. LEfSe are plotted as a graph that has bars which represent the effect size (LDA) for a particular taxa in a certain group. The length of the bar represents a log10 transformed LDA score. The colors represent which group that taxa was found to be more abundant compared to the other group. GREEN bars, significant higher in WT mice, RED bars, significant higher in IL-6 KO mice. All mice were pretreated with *A. lwoffii* and subjected to OVA model of asthma, (UN_g2) unclassied genus.

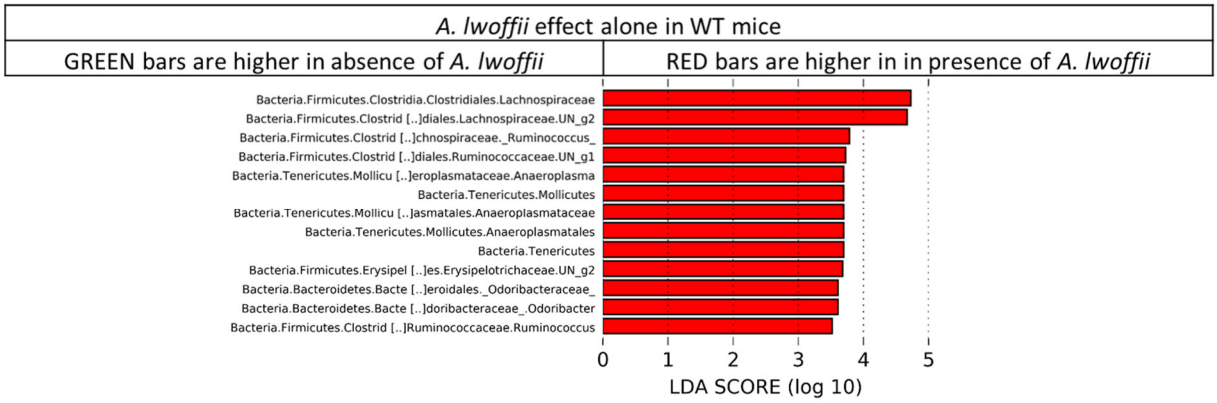
Microbiome analysis conducted on the cecum contents of the samples which were collected from WT and IL-6 KO mice on day 67, i.e. after the completion of the intranasal treatment with *A. lwoffii* comprising 17 applications performed every second day and subjecting the mice to OVA model of asthma **(Figure 12. A)**.

The differences in phylum-, class-, order-, family- and genus-level relative abundance between IL-KO and WT littermate were determined using LEfSe and plotted as a bars graph. The length of the bar represents a log10 transformed LDA score **(Figure 22)**.

In the WT littermate mice, the relative abundance of *Clostridiales.UN_f1.UN_g* and *Bilophila* were significantly higher (GREEN bars) compared with the IL-6 KO mice. And the relative abundance of *Lactobacillus* and *Desulfovibrio* were significantly higher in the IL-6 KO mice compared with WT littermate (RED bars), as it is described in **(Figure 22)**.

4.14. Effect *A. lwoffii* pre-treatment (alone) on the relative abundance of taxa in cecum microbiota of the WT and IL-6 KO mice (no *A. lwoffii* versus *A. lwoffii*)

A)



B)

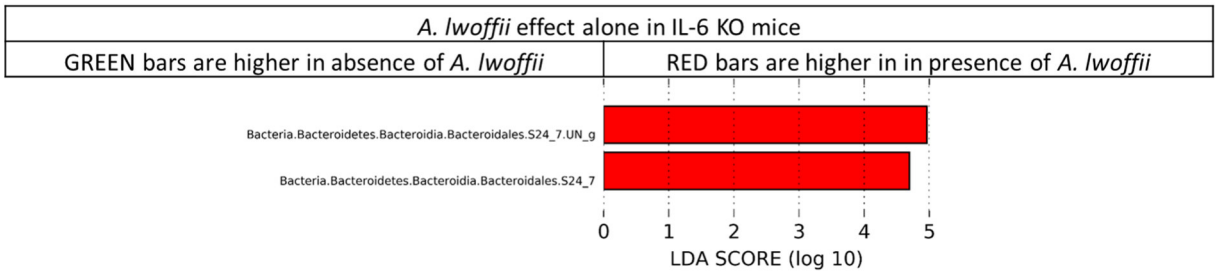


Figure 23. Effect of *A .lwoffii* pretreatment alone on the relative abundance of taxa in cecum microbiota of the WT and IL-6 KO mice (No *A. lwoffii* versus *A. lwofii*). LEfSe (Linear discriminant analysis effect size) was used to compare relative abundance of taxa in presence/absence *A. lwoffii* in **(A)**. WT mice and **(B)**. IL-6 KO mice. LEfSe are plotted as a graph that has bars which represent the effect size (LDA) for a particular taxa in a certain group. The length of the bar represents a log10 transformed LDA score. The colors represent which group that taxa was found to be more abundant compared to the other group. GREEN bars, significant higher in absence of *A. lwoffii* (no taxa were detected to be higher in the absence of *A. lwoffii* pretreatment in both the WT and the IL-6 KO mice), RED bars, significant higher in presence of *A. lwoffii,* (UN_f) unclassied family, (UN_g) unclassied genus.

Microbiome analysis conducted on the cecum contents of the samples which were collected from WT and IL-6 KO mice on day 67 **(Figure 12. A)**, here we compared the presence of *A. lwoffii* pretreatment with the absence of the *A. lwoffii* pretreatment, first in the WT **(Figure 23. A)** and second, in the IL-6 KO mice **(Figure 23. B),** all animals were NOT subjected to OVA model of asthma.

The differences in phylum-, class-, order-, family- and genus-level of the relative abundance in presence or absence of *A. lwoffii* pretreatment were determined in IL-6 KO and WT mice using LEfSe and plotted as a bars graph. The length of the bar represents a log10 transformed LDA score.

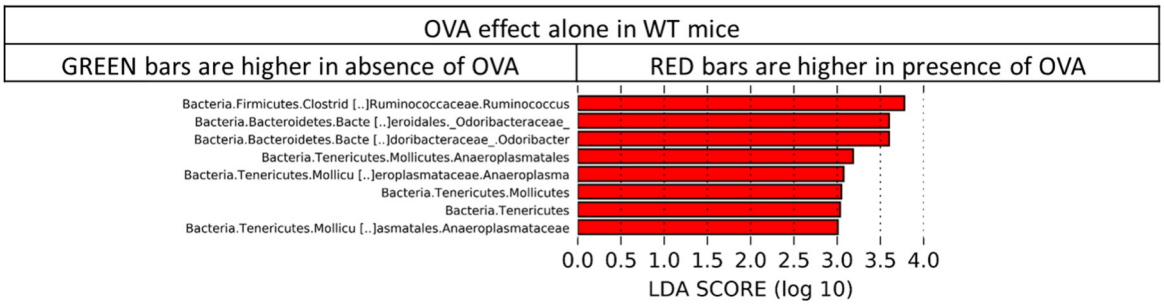
The absence of the GREEN bars is indicating that no taxa were detected to be higher in the absence of *A. lwoffii* pretreatment in both the WT and the IL-6 KO mice, compared with the presence of *A. lwoffii* pretreatment **(Figure 23. A& B)**.

In the WT littermate, pretreatment with *A. lwoffii* results in higher of the relative abundance of *Odoribacter, Ruminococcus, Erysipelotrichaceae.UN_g2, Anaeroplasma*, *Ruminococcaceae.UN_g1* and *Lachnospiraceae.UN_g2* (RED bars), compared with the absence of *A. lwoffii* (**Figure 23. A)**.

On the other hand, in the IL-6 KO mice the pretreatment with *A .lwoffii* results in higher of the relative abundance of *S24_7.UN_g* (RED bars) compared with the absence of *A. lwoffii* **(Figure 23. B)**.

4.15. Effect of OVA sensitization (alone) on the relative abundance of taxa in cecum microbiota of the WT and IL-6 KO mice (no OVA versus OVA)

A)



B)

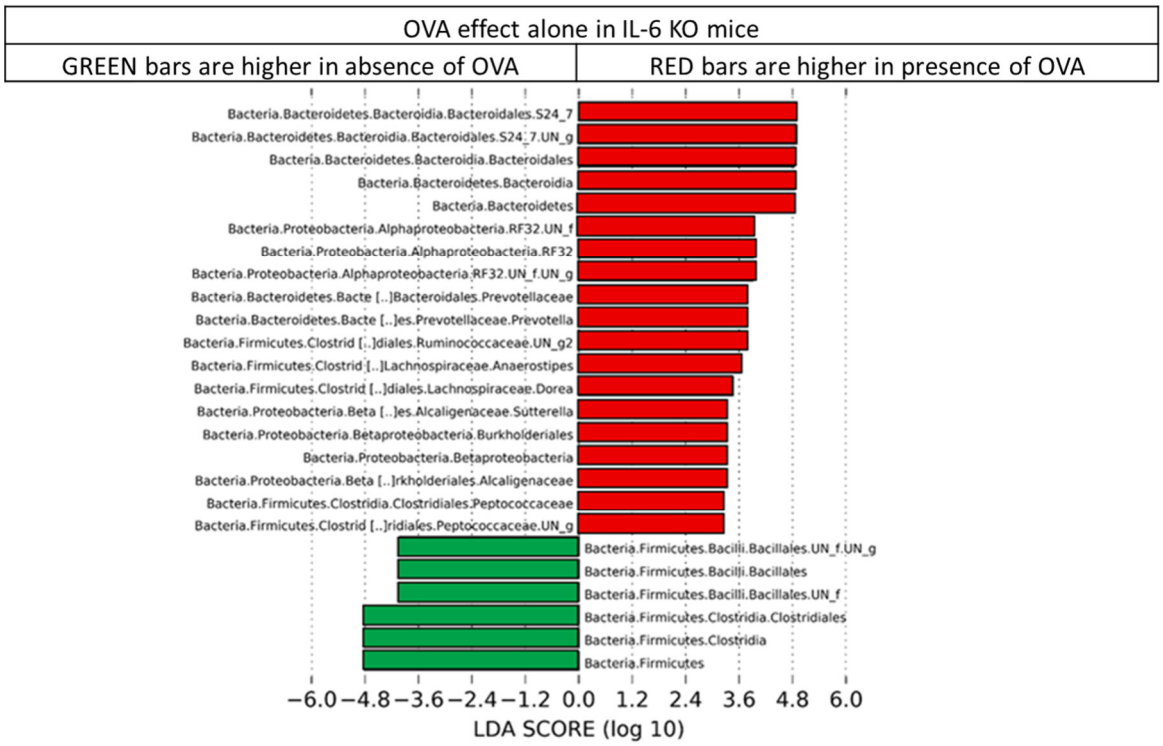


Figure 24. Effect of OVA sensitization alone on the relative abundance of taxa in cecum microbiota of the WT and IL-6 KO mice (No OVA versus OVA). LEfSe (Linear discriminant analysis effect size) was used to compare relative abundance of taxa in presence/absence of OVA sensitization in **(A)**. WT mice and **(B)**. IL-6 KO mice. LEfSe are plotted as a graph that has bars which represent the effect size (LDA) for a particular taxa in a certain group. The length of the bar represents a log10 transformed LDA score. The colors represent which group that taxa was found to be more abundant compared to the other group. GREEN bars, significant higher in absence of OVA sensitization (no taxa were detected to be higher in absence of OVA-sensitization in the WT mice), RED bars, significant higher in presence of OVA sensitization, (UN_f) unclassied family, (UN_g) unclassied genus.

Microbiome analysis conducted on the cecum contents of the samples which were collected from WT and IL-6 KO mice on day 67 **(Figure 12. A)**, here we compared the effect of presence of absence of the OVA sensitization. First in the WT **(Figure 24. A)** and second, in the IL-6 KO mice **(Figure 24. B),** all animals were NOT pretreated with *A. Iwoffii*.

The differences in phylum-, class-, order-, family- and genus-level of the relative abundance in presence or absence of OVA sensitization were determined in IL-6 KO and WT mice using LEfSe and plotted as a bars graph. The length of the bar represents a log10 transformed LDA score **(Figure 24. A& B)**.

The absence of the GREEN bars in the WT mice is indicating that no taxa were detected to be higher in absence of OVA-sensitization in the WT mice **(Figure 24. A)**.

In the WT littermate, OVA sensitization results in higher of the relative abundance of *Odoribacter, Ruminococcus* and *Anaeroplasma* (RED bars) compared with the non-OVA sensitized WT mice **(Figure 24. A)**.

In the IL-6 KO, first, the OVA sensitization results in higher of the relative abundance of *Prevotella*, *S24_7.UN_g, Anaerostipes, Peptococcaceae.UN_g, Dorea, Ruminococcaceae.UN_g2, Sutterella* and *RF32.UN_f.UN_g* (RED bars) compared with the non-OVA sensitized IL-6 KO mice, second, the absence of OVA sensitization results in higher of the relative abundance of *Bacillales.UN_f.UN_g* and *Clostridiales* (GREEN bars) compared with the non-OVA sensitized IL-6 KO mice **(Figure 24. B)**.

4.16. Effect of *A. lwoffii* pre-treatment on the relative abundance of taxa in cecum microbiota of OVA-sensitized WT and IL-6 KO mice (no *A. lwoffii* versus *A. lwoffii*)

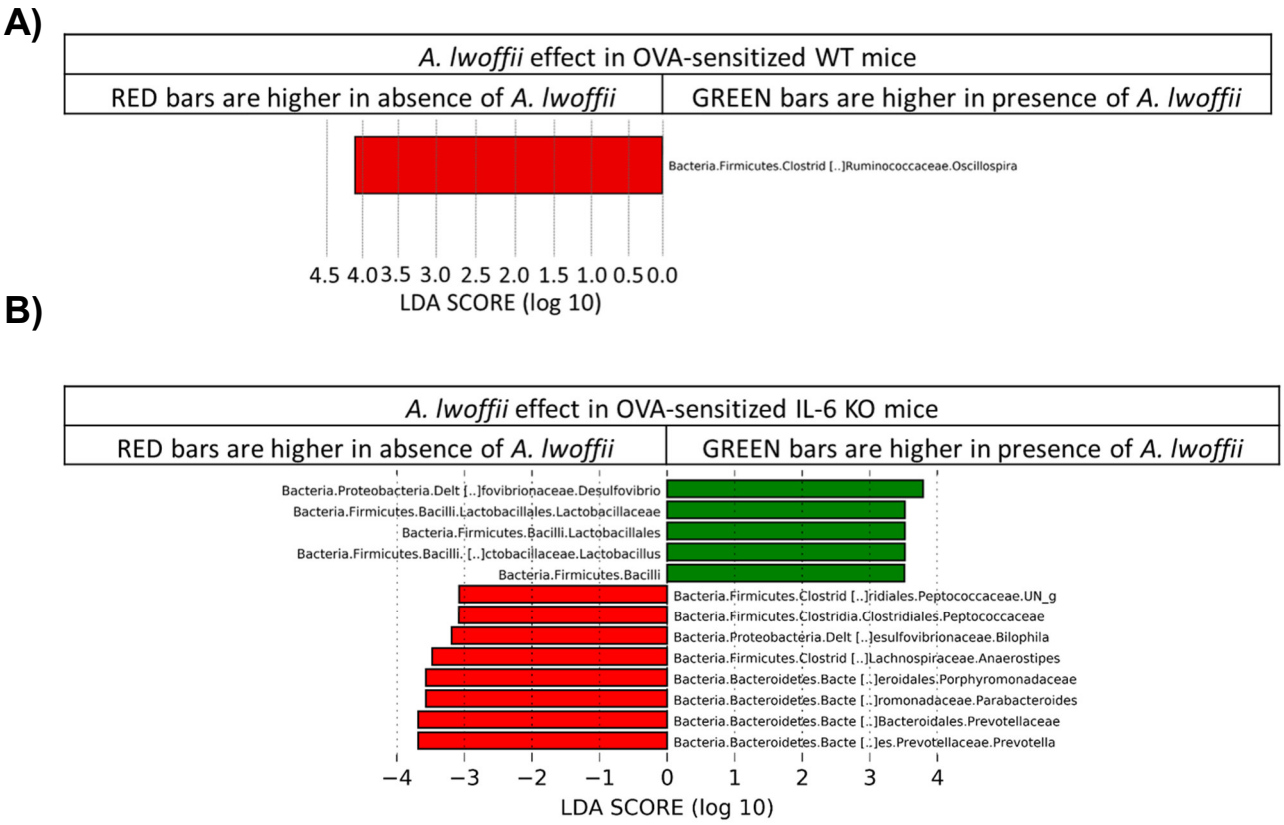


Figure 25. Effect of *A .lwoffii* on the relative abundance of taxa in cecum microbiota of OVA-sensitized WT and IL-6 KO mice (No *A. lwoffii* versus *A. lwoffii*). LEfSe (Linear discriminant analysis effect size) was used to compare relative abundance of the taxa in presence/absence *A. lwoffii* pretreatment of the **(A).** OVA-sensitized WT mice and **(B).** the OVA-sensitized IL-6 KO mice. LEfSe are plotted as a graph that has bars which represent the effect size (LDA) for a particular taxa in a certain group. The length of the bar represents a log10 transformed LDA score. The colors represent which group that taxa was found to be more abundant compared to the other group. GREEN bars, significant higher in presence of *A. lwoffii* pretreatment (no taxa were detected to be higher in presence of *A. lwoffii* pretreatment of the OVA-sensitized WT mice), RED bars, significant higher in absence of *A. lwoffii* pretreatment. All animals from IL-6 KO or WT mice subjected to OVA model of asthma, (UN_f) unclassied family, (UN_g) unclassied genus.

Microbiome analysis conducted on the cecum contents of the samples which were collected from WT and IL-6 KO mice on day 67 **(Figure 12. A)**, here we compared the effect of presence or absence of *A. lwoffii* pretreatment in OVA-sensitized mice.

First in the OVA-sensitized WT mice **(Figure 25. A)** and second, in the OVA-sensitized IL-6 KO mice **(Figure 25. B).** All animals were subjected to OVA model of asthma.

The differences in phylum-, class-, order-, family- and genus-level of the relative abundance in presence or absence of *A. lwoffii* in OVA-sensitized mice were determined in IL-6 KO and WT mice using LEfSe and plotted as a bars graph. The length of the bar represents a log10 transformed LDA score **(Figure 25. A& B)**.

The absence of the GREEN bars in the OVA-sensitized WT mice is indicating that no taxa were detected to be higher in presence of *A. lwoffii* pretreatment.

In the OVA-sensitized WT littermate and in the absence of *A. lwoffii* pretreatment the relative abundance of *Oscillospira* (RED bars) was higher compared with the *A. lwoffii* pretreated OVA-sensitized WT **(Figure 25. A)**.

In the OVA-sensitized IL-6 KO mice the presence of *A. lwoffii* pretreatment results in higher of the relative abundance of *Desulfovibrio and Lactobacillus* (GREEN bars) compared with the OVA-sensitized IL-6 KO mice not treated with *A. lwoffii*.

In contrast, the absence of *A. lwoffii* pretreatment in the OVA-sensitized IL-6 KO mice results in higher of the relative abundance of *Peptococcaceae.UN_g, Bilopila, Anaerostipes, Prevotella* and *Parabacteroides* (RED bars) compared with the presence of *A. lwoffii* in the OVA-sensitized IL-6 KO mice **(Figure 25. B)**.

5. DISCUSSION

Epidemiologic studies has identified natural microbial exposure as an important environmental exposome factor that provides allergic and asthma protection in a prenatal window of opportunity (Brand *et al.*, 2011) (Braun-Fahrländer *et al.*, 2002). Furthermore, although it has been suggested that early exposure to a farming environment reduces the risk of development of allergic diseases and asthma later in life (Von Mutius, 2016), not much is known on the underlying mechanisms. The key findings among the cohort studies examining both endogenous and exogenous microbial exposures report an inverse correlation between the increase of the microbial exposure and asthma development, which is in line with the basis of the hygiene hypothesis (Von Mutius, 2016). This protective effect against allergic disease and asthma is sustained into adulthood and has been reproduced in numerous studies comparing between the children growing up in farm and nonfarm environment (Von Mutius and Vercelli, 2010) (Radon *et al.*, 2004). It has been previously demonstrated that the bacteria isolated from the cowshed microflora such as *L. lactis* or *A. lwoffii* are able to produce a protective effect against asthma development in mice. *A. lwoffii* is one of the Gram-negative bacteria that have been identified in the Bavarian farms because of their relative abundance in cowshed microflora (Debarry *et al.*, 2007a).

The purpose of this work is to demonstrate the underlying mechanism of the protective effect induced by *A. lwoffii* against the development of asthma in the mouse model of Th2-eosinophilic asthma, to highlight the role of innate and adaptive immune system responses to *A. lwoffii*, and also to figure out how cytokine environment produced by innate immune system upon contact with *A. lwoffii* is shaping the adaptive immune system responses towards the protective effect against asthma development. Furthermore, the goal is to get further insights into the role of the cecum microbiome in mediating this protective effect and to determine the changes occurring in the gut microbiota after chronic intranasal exposure of *A. lwoffii*.

Our findings in mice demonstrated that chronic exposure to *A. lwoffii* stimulates local and systemic innate immunity as reflected by increased levels of interleukin-6 in lungs and serum. Further investigations in mice showed that wild-type but not IL-6 knockout mice pretreated with *A. lwoffii* develop much less severe allergic airway inflammation in the ovalbumin (OVA) model, as shown by no reduction in the number of the eosinophils, peribronchial and perivascular inflammatory cell infiltration, thus

demonstrating the cruel role of IL-6 to the protective effect of *A. lwoffii*. The data also demonstrated that the *in vitro* macrophages exposed to *A. lwoffii* secrete huge amount of IL-6, which in turn stimulates synthesis of IL-10 by T cells after culturing the naïve T cells with supernatant from *A. lwoffii*-exposed macrophages. Further assessments in mice revealed that IL-10 but not IL-17 KO mice pretreated with *A. lwoffii* have failed to show protective effects against allergic airway inflammation in the ovalbumin (OVA) model, no evidence for reduction in the eosinophilia of the lung; therefore, IL-10 seems to play an important role in downstream the protective effect of *A. lwoffii* on adaptive immunity. On the top of that, the microbiota might also contribute to the protective effect induced by *A. lwoffii* via possible interaction between IL-6 or IL-10, the changes in the relative abundance of taxa in the cecum microbiome abundance were studied in the IL-6 KO and WT littermate mice to figure how the cecum microbiome might contribute to the protective effect of *A. lwoffii* through determining of specific taxa. Our investigations demonstrated that *Ruminococcaceae* family maybe underlying the immunological/anti-allergic phenotypes in the IL-6 KO and *A. lwoffii* is having its immunological effects through the inhibition of these organisms.

Due to differences in the properties, activity and survival rate between the live and lyophilized bacteria (Conde-Islas *et al.*, 2019), it was aimed to determine if the protective effect differs between live and lyophilized *A. lwoffii* in mouse model of Th2-eosinophilic asthma. The mice were chronically exposed to 10^8 CFU of both lyophilized and live *A. lwoffii* intranasally which is followed by OVA-model of asthma **(Figure 10. A)**. Two days after the last challenge with OVA, the mice were sacrificed, and cell cytology of the BAL was studied. The results from the bronchoalveolar lavage cytology were demonstrated that the reduction in eosinophilic counts was significantly much higher in the group treated with a live *A. lwoffii* compared with those which were treated with lyophilized *A. lwoffii.* The neutrophils and lymphocytes were induced in both live and lyophilized *A. lwoffii* groups but no changes on the levels of the macrophages were observed **(Figure 10. B).**

Altogether, those findings clearly demonstrated that the protective effect of live *A. lwoffii* was much stronger compared with the lyophilized one. Thus, the decision was made to apply a live *A. lwoffii* for the future *in vivo* or *in vitro* experiments.

Since the innate immune system plays an essential role in the initial immune activation and recognition of *A. lwoffii*, which subsequently modulates the adaptive immune responses, it was decided to investigate the pattern in which *A. lwoffii* stimulates the

innate immune system by measuring three of the major pro-inflammatory cytokines (IL-6, IL1b and TNFa) locally in the lung (BAL) and systemically in the blood (serum) of the mice. The measurements were done at different time points in relation to the intranasal treatment with (10^8 CFU) of live *A. lwoffii*. The mice were received *A. lwoffii* 12 times, every second day. Blood and BAL were collected directly after the first application then every 8, 12 and 24 hours from each application, and the cytokines were measured using R&D ELISA kit. TNF-a and IL-1b were increased to the peak after 8 hours and then decreased after 12 and 24 hours from each application **(Figure 11. B& D)**. This pattern was reproduced till the fifth *A. lwoffii* application, and then, both cytokines (TNF-a and IL-1b) achieved a tolerance locally in the lung **(Figure 11. A)** but not systemically in the serum, in which the levels IL-1b was increased and TNF-a was the same **(Figure 11. C)**. In contrast, IL-6 has a different pattern from TNF-a and IL-1b; the levels of IL-6 increased to the peak after 8 hours from *A. lwoffii* application and decreased after 12 and 24 hours from each application locally and systemically **(Figure 11. B& D)**. Interestingly, the fast and transient inflammatory response of IL-6 to each of the single-doses of bacteria had the same pattern after each application of *A. lwoffii*, so no tolerance developed **(Figure 11. A &C)**. It was thus speculated that an endotoxin tolerance or LPS desensitization might develop after multiple applications of the gram-negative *A. lwoffii* bacteria, and the two of the earliest cytokines secreted upon *A. lwoffii* stimulation, TNF-a and IL-1b, were able to set up an LPS-like tolerant state in mice (Morrison and Ryan, 1987) (Epstein and Parrillo, 1993) (van der Poll and van Deventer, 1999). Many studies reported in the past were able to demonstrate that giving IL-1b to the mice in different concentration was able to produce a tolerance to LPS and, besides, a low production of TNF-a was observed in these mice(Alves-Rosa *et al.*, 2002). Additionally, the mice which were treated with TNF-a alone were not able to develop endotoxin tolerance, but this effect was completely abolished when TNF-a and IL-1b were administered simultaneously. The chronic exposure to *A. lwoffii* might be able to educate the innate immune cells by priming innate immune cells, which appeared to limit the amount of active TNF-a and IL-1b produced by tissue macrophages in response to a bacterial challenge **(Figure 11. A)** but not systemically in the serum, while the TNF-a and IL-1 b levels were significantly increased **(Figure 11. C)** (Netea, Quintin and Van Der Meer, 2011) (Gardiner and Mills, 2016).

The exhibited unusual response of IL-6 was very pronounced locally and systemically and, besides, the mice did not show any signs of systemic inflammation such as weight

loss or lethargy throughout the experiment. This findings indicate that the IL-6 response to *A. lwoffii* application seems to be at least partially crucial for the protective effect against asthma development induced by *A.lwoffii* application. Therefore, it was further sought to investigate the role of IL-6 to the protective effect against asthma induced by chronic exposure of intranasal *A. lwoffii*.

To elaborate the role of IL-6, the IL-6 KO (IL-6 knockout) and WT (wild-type) mice received intranasally 10^8 CFU of living *A. lwoffii* or PBS as control every second day for which followed by OVA model of asthma as it is described in **(Figure 12. A)**. BAL was collected and centrifuged; the cytokines IL-5 and IL-13 were measured in the supernatant and the cell pellet was re-suspended in PBS for cytology analysis. Lung was also collected for lung histology analysis, here the IL-6 KO PBS control mice treated w/o *A. lwoffii* were missed. The serum was also collected for serum immunoglobulins measurements (IgE, IgG1 and IgG2a), we also missed the IL-6 KO PBS control treated w/o *A. lwoffi*. and serum. The chronic exposure to *A. lwoffii* was able to induce a very weak inflammatory response to OVA in WT mice, as shown by a significant decreases in the number of eosinophils in the BAL fluid but this effects was absent in the OVA-sensitized IL-6 KO mice, regardless of the *A. lwoffii* pretreatment. Besides, the asthmatic phenotype was much stronger in IL-6 KO mice compared with the asthmatic phenotype in WT mice **(Figure 12. B)**. The IL-5 and IL-13 cytokine measurements from the BAL are also indicated pronounced protective effects of *A. lwoffii*, as the treatment with *A. lwoffii* was able to reduce the levels of IL-5 and IL-13 measured in the BAL fluid of OVA-sensitized WT animals, as shown in **(Figure 12. C)**. These protective effect was abolished in the OVA-sensitized IL-6 KO mice treated w/o *A. lwoffii*.

In addition, lung histology revealed an effect similar to that observed in the cytospine analysis of the OVA-sensitized WT mice pretreated with *A. lwoffii*; a reduction was seen in the inflammatory cell infiltration in WT mice after the completion of *A. lwoffii* treatment followed by OVA model. This reduction was completely absent in OVA-sensitized IL-6 KO mice, independently of the fact if the mice were treated or not treated with *A. lwoffii*. Consistently, *A. lwoffii* treatment almost completely hindered the development of mucus-producing goblet cells in WT mice after the completion of *A. lwoffii* treatment followed by OVA model in OVA-sensitized WT and IL-6 KO mice. The microphoto-graphs of airways from mice after PAS staining were comparable with the lung histology calculation **(Figure 12. D).**

Furthermore, IgE, IgG2a, and IgG1 were significantly higher in the OVA-sensitized IL-6 KO mice treated with *A. lwoffii* compared with the OVA-sensitized WT mice treated with *A. lwoffii*. The protective effects of *A. lwoffii* in WT mice was only noticed for the IgG2a which is significantly decreased in the OVA-sensitized WT mice pre-treated with *A. lwoffii* compared with OVA-sensitized WT mice not treated with *A. lwoffii*. The rest of comparisons in OVA-sensitized animals did not reach statistical significance. Here, the serum from the IL-6 KO PBS group treated w/o *A. lwoffii* were not enough to measure the immunoglobulins **(Figure 12. E)**.

Several possible reasons may explain the finding of the serum immunoglobulins in OVA-sensitized WT mice pretreated with *A. lwoffii*. It might be that the protective effect of *A. lwoffii* are not strong enough to override the high concentrations of serum immunoglobulins and the protective effect was located in the lung and do not expand systemically. One might also speculate that the protective effect of *A. lwoffii* occurs in a B cell–independent manner, in other words, *A. lwoffii* chronic exposure was not able to reduce the sensitization but rather decrease the inflammatory responses in the airways.

Taken together, the data are supporting the concept that IL-6 is crucial for the development of the protective effect against asthma induced by *A. lwoffii* treatment.

According to our current knowledge on the innate immune system, it constitutes the first line of host defense against infectious or non-infectious pathogens, and therefore, it plays a crucial role in the early recognition and subsequent triggering of a pro-inflammatory response to bacteria (Mogensen, 2009).

Epithelial cells are providing a mucosal barrier against infectious or not infectious bacteria and contribute to the mucociliary clearance function. There is growing evidence that epithelial cells are of particular relevance as initial sensors of danger through phagocytosis used by epithelial cells to maintain homeostasis in lung (Günther and Seyfert, 2018). It has been also demonstrated that the epithelial cells are also able to recruit inflammatory cells such as DCs, which otherwise lie above and below the basement membrane in a resting or immature state. These DCs derived from blood are able to uptake the antigens of the bacteria and present them to the adaptive immune cells on their surface (Allard, Panariti and Martin, 2018).

Inoculation of the DAPI-stained *A. lwoffii* into the lung of the mice and the assessment of the nasopharyngeal and bronchial epithelial cells after four hours from *A. lwoffii* application were revealed that *A. lwoffii* was detectable inside the macrophages,

demonstrating them to be the front line of the host contact with *A. lwoffii*. At the same time, *A. lwoffii* was not detectable in the tracheal respiratory epithelial cells **(Figure 13.)**. These findings suggested an important role of macrophages in the recognition and phagocytose of *A. lwoffii* upon the intranasal application to the animal, resulting subsequently in a polarization of the macrophages to produce cytokines once the contact occurred.

Innate immunity is maintained in part by antigen presenting cells (APCs) including dendritic cells and macrophages, as listed before. To better understand of the pattern of the innate immune response to *A. lwoffii*, the *in vivo* experiment was followed by an *in vitro* model in which, three major types of innate immune cells or antigen presenting cells which were targeted. The bone marrow cells were isolated from the BALB/c mice and differentiated into primary macrophages (PM), plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (moDCs) by Dr. Andreas Kaufmann. The cells were subsequently incubated for 24 hours with 10^6 CFU freshly prepared live *A. lwoffii*, LPS (10 ng/ml) as a positive control, or medium as negative control **(Figure 14. A)**. Afterwards, the supernatants were collected, and cytokines were measured using Cytometric Bead Array-CBA (Bio-Plex® 200, Bio-Rad, USA) **(Figure 14. B)**. Consistently with our previous *in vivo* experiment, the primary macrophages, plasmacytoid and myeloid dendritic cells cultured *in vitro* in the presence of *A. lwoffii* secreted huge amounts of IL-6 and generally smaller amounts of several other classical cytokines of professional non-B-cell antigen-presenting cells such as IL-12, IL-10 and IL-1b **(Figure 14. B)**. Taken together, the data strongly supported the original hypothesis suggesting the crucial role of IL-6 as the first player from the innate immune system possibly modulating subsequent responses of the adaptive immune system, resulting in the protective effect against asthma development induced in mice by *A. lwoffii* exposure.

Further experiment were done to figure out the role on the innate immune system in shaping the adaptive immune responses, and to determine the crosstalk between the innate and adaptive immune system upon the *A. lwoffii* treatment. It has been demonstrated that the crosstalk between APCs and T cells shapes the innate and adaptive immune responses to pathogenic or non-pathogenic bacteria. On their surface, APCs can present the LPS of the bacteria to the T cells and then directly influence T cell activation and differentiation (Gaudino and Kumar, 2019) (Jain and Pasare, 2017).

For better understanding of the downstream effects of *A. lwoffii* on adaptive immunity, an *in vitro* experiment was conducted, in which the murine peritoneal macrophages were isolated from the peritoneal cavity of BALB/c WT mice after stimulation with 3% Brewer thioglycolate to increase the yield of elucidated macrophages. Then, these macrophages were cultured in the presence of *A. lwoffii* for 24 hours to, LPS (10 ng/ml) as a positive control, or medium as negative control, i.e. the same conditions as in previous *in vitro* experiment **(Figure 15. B)**.

The supernatants from *A. lwoffii*- exposed macrophages were used for subsequent stimulation of the CD4+CD62L+ T cells (naïve T helper cells) isolated from the spleen of the WT mice. The naïve CD4+ T cells were cultured on anti-CD3 (0.5 µg/ml) coated plate together with of anti-CD28 (1µg/ml). Afterwards, the supernatant from *A. lwoffii*-exposed macrophages, recombinant IL-6 (rIL-6), or pure medium as negative control were added to the cells. The plate was incubated for 72 hours at 37°C, and then, the medium was replaced, and recombinant IL-2 was added to further expand T cells. Cells were incubated for another 48 hours. Thereafter, they were washed and cultured again on anti-CD3 coated plate for 24 hours. Finally, the supernatants were collected for cytokines measurements performed using Cytometric Bead Array-CBA (Bio-Plex® 200, Bio-Rad, USA) **(Figure 16. A& B)**. It was observed that naïve T cells produced huge amounts of IL-10 under influence of either the supernatant from *A. lwoffii*-exposed macrophages or recombinant IL-6 (rIL-6) **(Figure 16. B)**. Moreover, the supernatant from *A. lwoffii*-exposed macrophages was able to stimulate the secretion of IL-17 from naïve T cells. These findings suggested an important role of IL-10 and IL-17 in mediating the protective effect against asthma induced by *A. lwoffii* downstream of IL-6. The positive correlation between *Acinetobacter* species and IL-10 production has been studied in human PBMCs of healthy or atopic subjects; the data indicated that *Acinetobacter* species influenced most strongly the production of IL-10 in healthy subjects but not in atopic individuals (Fyhrquist *et al.*, 2014).

Our data from the *in vitro* stimulation of the naïve T cells with supernatant from *A. lwoffii*-exposed macrophages point toward a potential role of the IL-10 and IL-17 in the protective effects against asthma development induced by *A. lwoffii*. Therefore, it was hypothesized that IL-17 and/or IL-10 are involved in this protective effect downstream of IL-6.To test the hypothesis, two independent *in vivo* experiments were

conducted using either IL-17 KO or IL-10 KO mice. The experimental layout was described in the **(Figure 17. A) (Figure 18. A)**.

In the IL-17 KO versus littermate WT *in vivo* experiment, the chronic exposure to *A. lwoffii* was able to reduce an inflammatory response induced by OVA independently of the genotype, as evidenced by significant decrease in the numbers of eosinophils in the BAL fluid in both WT and IL-17 KO mice pre-exposed to *A. lwoffii* and subsequently subjected to the OVA-Th-2-eisonoplilic asthma model compared with the not treated mice **(Figure 17. B)**.

Furthermore, the IL-5 was indicated a pronounced protective effect of *A. lwoffii* regardless of the genotype, IL-5 levels in the BAL was decreased after the treatment with *A. lwoffii* in OVA-sensitized WT and IL-17 KO mice compared with the not treated mice **(Figure 17. C)**. The IL-13 was significantly decreased in OVA-sensitized WT mice pretreated with *A. lwoffii* and has a tendency to decrease in the OVA-sensitized IL-17 KO mice under the effect of *A. lwoffii* **(Figure 17. C)**.

The IgE, IgG2a, and IgG1 immunoglobulin measurements in the serum of OVA-sensitized WT or IL-17 KO pre-treated w/o *A. lwoffii* demonstrated no significant differences between the groups but here, the biomaterials were not enough to measure the serum immunoglobulins in IL-17 KO PBS mice treated w/o *A. lwoffii* **(Figure 17. D)**. In the OVA-sensitized WT mice littermate to IL-10 KO mice, the chronic exposure to *A. lwoffii* was able to strongly reduce the inflammatory response induced by OVA **(Figure 18. B)** in a manner similar to that observed in “IL-6 KO experiment” **(Figure 12. B)** as shown by significant decreases in the numbers of eosinophils in the BAL fluid while in contrast, the protective effect was completely abolished in the IL-10 KO mice with no reduction in the number of the eosinophils in OVA-sensitized IL-10 KO mice after the treatment with *A. lwoffii* **(Figure 18. B)**. Besides, the allergic phenotype was much stronger in terms of lung eosinophilia in the IL-10 KO mice compared with the WT, independently if the mice were pre-exposed to *A. lwoffii* or not The data from both genotypes clearly proved the essential role of IL-10 but not IL-17 in mediating the protective effect against asthma development induced by *A. lwoffii*.

The bacterial biomass of the lung is considered relatively less than the bacterial biomass of the gastrointestinal trac (Arumugam *et al.*, 2011). Generally, the composition of the bacteria in the lung is determined through the elimination and immigration mechanisms via the epithelium and mucociliary clearance of the lung. The dysbiosis or the disturbance of this physiological system can influence the shape and responses of both the innate and adaptive immune system, which in turn contribute to the development of respiratory disease such as allergy and asthma (Abrahamsson *et al.*, 2012) (Inagaki *et al.*, 1996).

Different conditions may influence the bacterial proliferation in the lung or cecum such as oxygen tension, pH, temperature, effector inflammatory cell disposition, and epithelial cell but, most importantly, the environmental exposure such as microbes. *A. lwoffii* is one among those microbes that could influence the lung or gut microbiota (Martin *et al.*, 2015) (Wilson and Hamilos, 2014).

Growing evidence suggests that the gut commensal microbiota is an important regulator of the innate immune system which seems to be crucial in early period during life where intestinal microbiome development is important for the regulation of an appropriate immune response in the lung through the gut-lung axis (Clarke, 2014) (Sassone-Corsi and Raffatellu, 2015). The chronic inflammatory disease of the lung such as asthma seems to be influenced via the shifts in the composition or diversity of the gut microbiome especially in the cecum where most of the bacterial fermentation processes take place (Den Besten *et al.*, 2013). Based on all the facts listed above, it was speculated that the chronic exposure to *A. lwoffii* may induces changes in the gut microbiota which is might partially contributed to the *A. lwoffii*-induced protective effect of the cytokines against asthma development.

Therefore, the efforts were made to get further insights into the interaction between IL-6 and microbiome changes in the cecum and its role in mediating the protective effects of *A. lwoffii*. It was speculated that the changes of the lung microbiota due to *A. lwoffii* application might be somehow translocated into the gut thus influencing the relative abundance of some taxa of the gut microbiota. The indirect regulation of the gut microbiome by the changes in the lung microbiome would be referred to so-called a lung-gut axis. The goal of the preliminary investigations reported here was to assess (1) the influence of chronic *A. lwoffii* application on the relative abundance of the taxa in the gut microbiota of the WT and IL-6 KO mice, (2) the influence of OVA-sensitization on the relative abundance of the taxa in gut microbiota of the WT and IL-6 KO mice,

(3) how the *A. lwoffii* pretreatment may change the abundance of specific taxa in the gut of the OVA-sensitized WT and IL-6 KO mice (4). the influence of the genotype on the taxa of the cecum in IL-6 KO compared with WT littermate mice in presence or absence of both *A. lwoffii* pretreatment and OVA sensitization, or in presence of *A. lwoffii* alone or in presence of OVA-sensitization alone.
The samples for the analysis of the microbiome were obtained from the IL-6 KO and WT littermate (IL-6 experiment).The biomaterials for the microbiome analysis were collected on day 67, after the end of the experiment. In order to obtain the microbiome sequencing data of the cecum content, the samples were transferred to an external partner in USA, Prof. Martin Blaser lab. Prof. Blaser performed next-generation sequencing and bioinformatics, which made it possible to generate a graphical representation of the taxa which is sent back to Marburg for the interpretation and analysis. The cecum content samples were used to generate the taxa comparisons due to the importance of the cecum in the bacterial fermentation and the high diversity of the bacteria which is observed for the cecum.

By comparing the IL-6 KO with WT littermate without *A. lwoffii* pretreatment or OVA sensitization, the *Lachnospiraceae.UN_g1*/*g2* and *Ruminococcus* were higher in the IL-6 KO mice **(Figure 19)** this might indicating their susceptibility to immune control. The families *Lachnospiraceae* and *Ruminococcaceae* have been described to be able to modulate the adaptive immune responses by increasing *Foxp3* and IL-10 producing T cells and maintaining the balance of Treg/Th-17 in mesenteric lymph node (MLN) cells (Lindenberg et al. 2019) (Atarashi *et al.*, 2013) (Atarashi *et al.*, 2011) (Norbäck *et al.*, 2018) (Sun *et al.*, 2015). On the other hand, the *S24_7.UN_g* and *Sutterella* indicate less abundance in the IL-6 KO mice, either that they need the immune milieu, or they are out-competed by organisms that had been suppressed by the cytokines (e.g. *Ruminococcus*).

It could be speculated that the increase in the *Lachnospiraceae.UN_g1*/*g2* and *Ruminococcus* maybe underlying the immunological/anti-allergic phenotypes in the IL-6 KO and *A. lwoffii* is having its immunological effects through the inhibition of the*Ruminococcaceae* family. *Ruminococcaceae* was not only higher in the IL-6 KO mice but also in the OVA-sensitized IL-6 KO mice **(Figure 21)**. Additionally, in the absence of *A. lwoffii Ruminococcaceae*> *Oscillospira* were increased in the OVA-sensitized WT mice (not protected group) compared with the *A. lwoffii* pre-treated WT mice where it was less abundance due to *A. lwoffii* pretreatment **(Figure 25. A)**, further

assessment is required in germ free mice by inoculate 10 microbe consortium versus 8 microbes and then study the phenotypes.

Moreover, in the combined model of *A. lwoffii* pretreatment and OVA-sensitization, *Lactobacillus* and *Desulfovibrio* were higher in the IL-6 KO mice compared with WT **(Figure 22)**. The relative abundance *Desulfovibrio* and *Lactobacillus* were also higher in the OVA-sensitized IL-6 KO mice pretreated with *A. lwoffii* **(Figure 25. B)**.Studies on mice showed that some genus of the *Lactobacillus* can induced the proliferation/expansion of Treg cells in the circulation, spleen, lung and lymph nodes, and reduced inflammatory parameters induced by OVA challenge in sensitized mice (Karimi *et al.*, 2009), our investigations indicate an higher abundance of the *Lactobacillus* in the not protected IL-6 KO mice and decrees in the WT protected mice **(Figure 22)**.However, not much was observed by comparing the IL-6 KO with WT littermate after pretreatment with *A. lwoffii* alone*. Mogibacteriaceae_.UN_g, Dorea and Clostridiales.UN_f1.UN_g* were higher in WT and lower in the IL-6 KO **(Figure 20)**. Here, it seems that *A. lwoffii* pretretament override the genotype effect.

In the WT pretreated with *A. lwoffii* the relative abundance of *Odoribacter, Ruminococcus, Erysipelotrichaceae.UN-g2, Anaeroplasma*, *Ruminococcaceae.UN-g1* and *Lachnospiraceae.UN-g2* were higher compared with the absence of *A. lwoffii* **(Figure 23. A)**. Not much was observed in the IL-6 KO mice where only *S24_7.UN_g* was increased in presence of *A. lwoffii* **(Figure 23. B)**.

In addition, the effects of OVA sensitization on the relative abundance of cecum taxa were studied in WT and IL-6 KO mice by comparing the non-OVA-sensitized with the OVA-sensitized mice. The OVA sensitization in WT mice enhances the relative abundance of *Odoribacter, Ruminococcus* and *Anaeroplasma* **(Figure 24. A)**.

More changes were determined in the IL-6 KO mice. The OVA sensitization of the IL-6 KO mice results in higher abundance of the *Prevotella*, *S24_7.UN_g, Anaerostipes, Peptococcaceae.UN_g, Dorea, Ruminococcaceae.UN_g2, Sutterella* and *RF32.UN_f.UN_g.* On the other hand, the absence of OVA sensitization results in higher abundance of the *Bacillales.UN_f.UN_g* and *Clostridiales* **(Figure 24. B)**.

In the OVA-sensitized WT mice, the absence of *A. lwoffii* pretreatment (not protected group) results in higher abundance of *Oscillospira* **(Figure 25. A)** which is decreased in presence of *A. lwoffii* (protected group), human studies demonstrate the opposite

where the *Oscillospira* have been inversely associated with atopy and asthma (Fujimura *et al.*, 2016) (Arrieta *et al.*, 2015) (Savage *et al.*, 2018).

In the OVA-sensitized IL-6 KO mice, the presence of *A. lwoffii* pretreatment results in increase of the relative abundance of *Desulfovibrio* and *Lactobacillus* and decrees of the relative abundance of *Peptococcaceae.UN_g, Bilopila, Anaerostipes, Prevotella* and *Parabacteroides* **(Figure 25. B)**. Several previously published studies demonstrated that the abundance of *Prevotella* was reduced in the lung of the asthmatic patients. Hence, many speculations can be made as to the role of *Prevotella* in the gut microbiota of healthy individuals but the homeostatic role of *Prevotella* remains largely unknown in asthma (Yadava *et al.*, 2016) (Neff *et al.*, 2016).

As a conclusion, the protective effects of *A. lwoffii* against the development of eosinophilic airway inflammation seem to be mediated by IL-6. Thus IL-6 might be an important modulator of adaptive immune system towards non-/anti-allergic direction. Since IL-17 knockout mice showed same results as in wild-type (WT), therefore, IL-17 is not important and play no role in this protective effect.

On the other hand, the protective effect is abolished in IL-10 knockout mice, which indicate a fundamental role of IL-10 in the protective effect of *A. lwoffii* against asthma development. On the top of that, the higher abundance of the *Ruminococcaceae* family is indicating their susceptibility to immune control which is maybe underlying the immunological/anti-allergic phenotypes in the IL-6 KO mice, the *A. lwoffii* is having its immunological effects through the inhibition of the *Ruminococcaceae* family. Further investigations in germ free mice are required to address the role *Ruminococcaceae* in immune control and additional analysis would be also necessary to determine the microbiota changes in the cecum of the IL-10 KO and WT littermate mice, which is ongoing.

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Potaczek, D. P. *et al.* (2017b) ‘Epigenetics and allergy: from basic mechanisms to clinical applications’, *Epigenomics*, 9(4), pp. 539–571. doi: 10.2217/epi-2016-0162.

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7. Supplementary data.

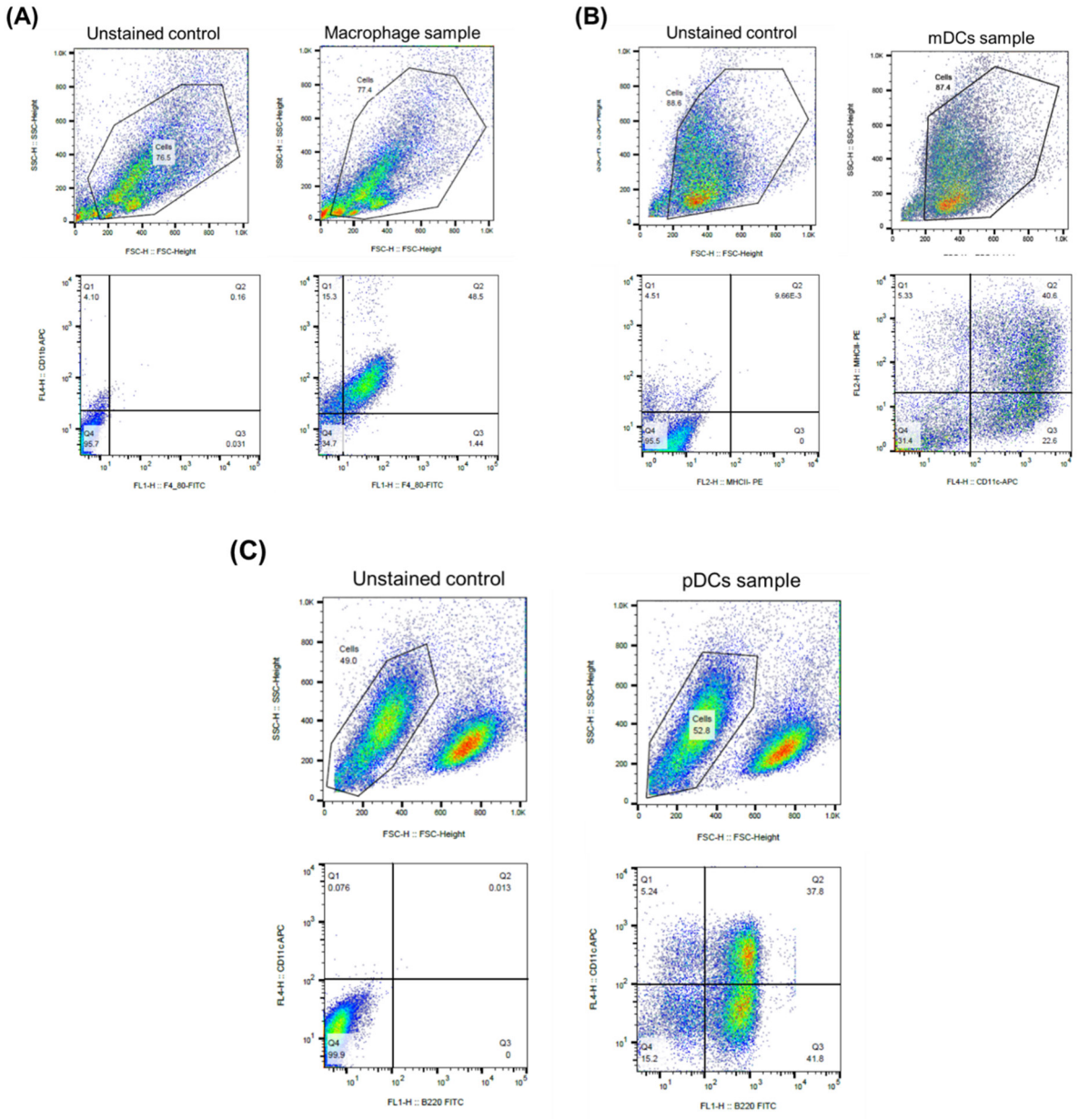

Figure. 1 Suppl. FACS analysis of the murin differentiated PM, mDCs and pDCs. Cells were isolated from (BM) then diffrentaied within indicated time into PM, mDCs or pDCs. FACA analysis was done to asses the cells differentiation **(A).** differentiated macrophages using double staing with FITC-F4/80 and APC-CD11b antibidies, indicating prurity of 48.5%. **(B).** differentiated mDCs using douple staining with APC-CD11b and PE-MHCII, indicating prurity of 40.6%. **(C).** differentiated pDCs using douple staining with FITC-B220 and APC-CD11c, indicating prurity of 37.8%. Unsatined cells were used as negative control in all experiments **A**, **B** and **C**.

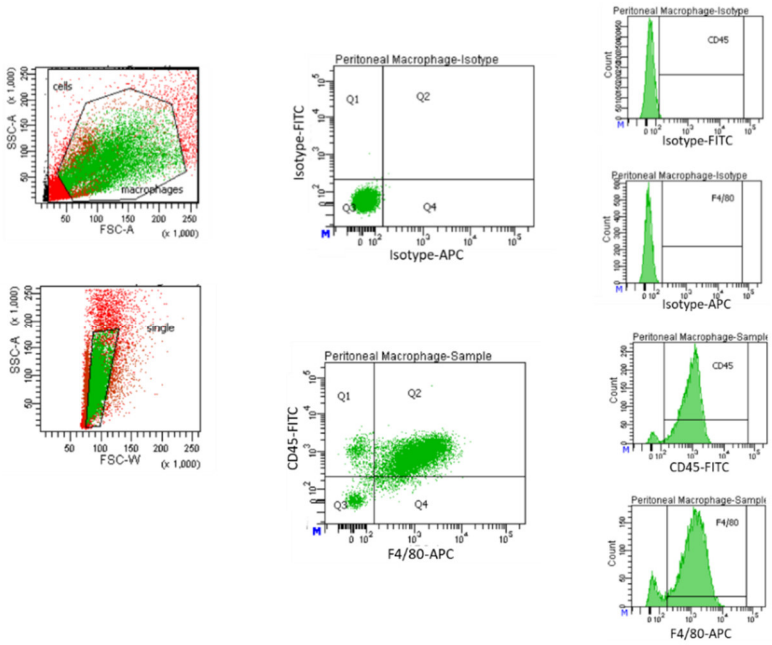

Figure. 2 Suppl. Purity of the murine peritoneal macrophages. Murine peritoneal macrophages isolated from the peritoneal cavity of WT mice after 72 hours of intraperitoneal injection with 3% Brewer thioglycollate. The purity of macrophages was tested with FACS machine using anti-F4/80-APC (APC-anti mouse F4/80 anti-body, BioLegend, USA) and anti-CD45-FITC (FITC -anti mouse CD45 anti-body, BioLegend, USA), indicating purity of 85%.

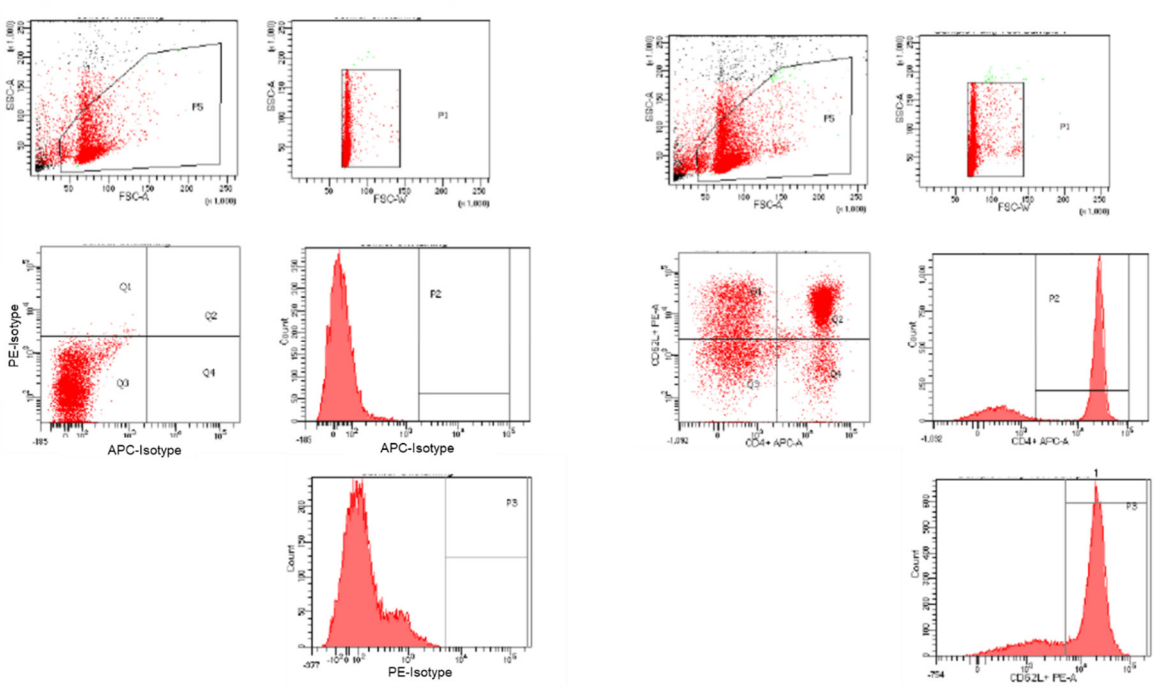

Figure. 3 Suppl. Purity of the naïve CD4 T cell by FACS analysis. APC anti-mouse CD4 Antibody and PE anti-mouse CD62L Antibody (both BioLegend, USA). 2 µl of each mAbs were used for the samples and 2 µl of the isotype PE (PE-anti mouse CD62L+ isotype, BioLegend, USA) and APC (APC-anti mouse CD+ isotype, BioLegend, USA), indicating purity of 74%..

8. Figures and tables

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9. SUMMARY

BACKGROUND: Early childhood or even prenatal exposures to farm bacteria such as *Acinetobacter lwoffii* have been demonstrated an association with a decreased incidence of allergy and asthma later in life. Studies in mouse models demonstrated that intranasal application of *A. lwoffii* stimulates local and systemic innate immunity as reflected by increased the levels of the pro-inflammatory cytokines, especially interleukin-6 (IL-6) levels in lungs and serum.

AIMS AND HYPOTHESIS: The key concept of this study was to understand different mechanistic pathways of the *A. lwoffii* protective effect against asthma. We hypothesized that interleukin-6 (IL-6) might contributes to the protective effect of *A. lwoffii* against asthma/allergy development. We further supposed that treatment with *A. lwoffii* stimulates the innate immune system in which it can skew the function of the adaptive immune system towards mechanisms know to favor lower asthma and/or allergy susceptibility. Furthermore, we expand our hypothesis to highlight the role of the cecum microbiota changes in mediating the protective effects of the *A. lwoffii* against asthma in the WT and IL-6 KO mice.

METHOD AND RESULTS: We first investigate the innate immune response up on contact with *A. lwoffii*, the mouse was chronically treated with *A. lwoffii* and then the blood and bronchial lavage were collected for pro-inflammatory cytokines measurement, TNFa and IL-1b developed tolerance after the 5th application but the IL-6 exhibit unusual response with no tolerance during the repeated treatment. Further assessments in Wild-type and IL-6 knockout mice pre-exposed to *A. lwoffii* and subjected to the OVA model of airway inflammation demonstrate a pivotal role of IL-6 in mediating the protective effects of *A. lwoffii*. This data was supported with the *in vitro* data where the mouse primary macrophages (PM), plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs) exposed to *A. lwoffii* secreted huge amounts of IL-6, as measured in the culture supernatants. Culturing of naïve murine T cells with supernatant from *A. lwoffii*-exposed macrophages or recombinant IL-6 induce the secretion of high levels of IL-10 and less extend of IL-17. Subsequent animal experiment revealed that IL-10 but not IL-17 deficient mice pretreated with *A. lwoffii* failed to develop a protection against airway inflammation, suggesting that IL-10 but not IL-17 plays an important role in mediating the effects of *A. lwoffii* downstream of IL-6.

On the top of that, the microbial DNA from the cecum content of the IL-6 KO and WT littermate mice were isolated and sequenced to investigate the changes in the relative abundance of taxa in the cecum microbiome. The genotype effect, the *A. lwoffii* effect, the OVA sensitization effect and the *A. lwoffii* effect in OVA sensitized mice were studied in IL-6 KO and WT littermate animal. The data demonstrated an important role of *Ruminococcaceae* family which is indicating their susceptibility to immune control which is maybe underlying the immunological/anti-allergic phenotypes in the IL-6 KO mice, the *A. lwoffii* is having its immunological effects through the inhibition of the *Ruminococcaceae* family.

CONCLUSION: Protective effects of *A. lwoffii* against asthma development seem to be at least partly mediated by IL-6. Thus IL-6 might be an important modulator of adaptive immune system towards non-/anti-allergic direction. The *in vitro* macrophages exposed to *A. lwoffii* secrete huge amount of IL-6, which in turn stimulates synthesis of IL-10 and IL-17 by T cells after culturing the naïve T cells with supernatant from *A. lwoffii*-exposed macrophages. Subsequent experiment in mouse model demonstrates that IL-10 but not IL-17 play an important role in downstream the protective effects of *A. lwoffii* on adaptive immunity. Furthermore, the microbiome analysis in the IL-6 KO and WT littermate indicates a possible immune regulation via *Ruminococcaceae* family which is maybe underlying the immunological/anti-allergic phenotypes in the IL-6 KO mice, the A*. lwoffii* is having its immunological effects through the inhibition of the *Ruminococcaceae* family. Further investigations in germ free mice are required to address the role *Ruminococcaceae,* and additional in-depth investigations, including those on the possible interaction with IL-10, are ongoing.

10. CURRICULUM VITAE

Personal information:

Name: Bilal Alashkar Alhamwe

Date of birth: 03.09.1987

Place of birth: Damascus, Syria

Adress: Sankt-Martin Str. 21, 35039 Marburg

Telefon number: 0176/25933474

Nationality: Syrian

E-Mail: dbilaliust@gmail.com ; alashkab@staff.uni-marburg.de

Work Experience:

04/2015–today Ph.D. student in clinical and molecular immunology.
Faculty of Medicine, Philipps Universität Marburg
„Microbiome changes may contribute to the protective effect of *Acinetobacter lwoffii* against asthma development mediated by interleukins 6 and 10“

02/2014–today Member of the Board of Directors at Lamset hanan Charitable organization.

01/2012 –12/2014 Medical Advisor
Boehringer Ingelheim Pharmaceutical company,
Damascus-Syria

201/2012–12/2014 Medical Advisor
Alpha Pharmaceutical industries,
Damascus-Syria

01/2011 –12/2014 Part Time-Hospital pharmacist
Alasndalus university hospital.
Damascus-Syria

01/2010 –12/2012 Medical Rep
Alpha Pharmaceutical industries,
Damascus-Syria

Education:

04/2015–today Ph.D. student in clinical and molecular immunology.
Faculty of Medicine, Philipps Universität Marburg

01/2015 –01/2016 Diploma, Political science and Goverment
Department of Politics and Public Administration,
University of Konstanz, Konstanz, Germany.

10/2010 –04/2014 Master of Business Administration (MBA)
Syrian Virtual University (SVU), Damascus, Syria
Graduaion grade: 73.65% (Good)

10/2005–05/2010 Bachelor of Pharmacy (B.Sc.)
International University of Science and Technology (IUST),
Daraa, Syria
GPA: 3.10/4 (Very Good)

Awards and prizes:

- Travel grant (Allergie im Fokus-DGAKI-Frankfurt-November. 2019).
- Best Poster together with PD. Dr. Daniel Potaczek
  (EAACI Congress-May 2018 Munich)
- European Respiratory Society (ERS) International Congress, together with
  PD. Dr. Daniel Potaczek, Research highlights (held in Paris in 2018)
- Best poster and travel grant (Eosinophile Granulozyten-DGAKI-Kassel-June. 2017).
- DAAD Scholarship (Leadership for Syria Program); funding period 04.2014-09.2019.

Membership:

Since 2017 Junior Member in the European Academy of Allergy and Clinical Immunology (EAACI).

Since 2016 Junior Member in Deutsche Gesellschaft für Allergologie und klinische Immunologie e.V. (DGAKI)

Since 2015 Memebership in the DAAD German Academic Exchange Service Alumi, Germany

Since 2015 Memebership in *inVIVO* Planetary Health group.

Marburg, 2020.

11. PUBLICATIONS AND CONGRESS PAPERS:

11.1. PUBLICATION OF THE PHD PROJECT:

Microbiome alterations may contribute to the cytokine protective effect of *Acinetobacter lwoffii* against asthma development

B. Alashkar Alhamwe[1,2], H. Harb[1,3], Z. Gao[4], E. Kilic-Niebergall[1], F. Alhamdan[1], F. Busato[5], S. Miethe[1], J. El Andari[4], A. Kaufmann[5], P. L. Graumann[4], D. Kesper[1], M. Blaser[4], J. Tost[5], H. Garn[1], D. P. Potaczek[1]*, H. Renz[1]*.

[1]Institute of Laboratory Medicine, member of the German Center for Lung Research (DZL) and the Universities of Giessen and Marburg Lung Center (UGMLC), Philipps-University Marburg, Marburg, Germany.

[2]College of Pharmacy, International University for Science and Technology (IUST), Daraa, Syria

[3]Division of Immunology, Boston Children's Hospital, Boston, MA, USA.

[4]Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, NJ.

[5]The Laboratory for Epigenetics and Environment, Centre National de Recherche en Genomique Humaine, CEA–Institut de Biologie Francois Jacob, Paris, France

[4]LOEWE SYNMIKRO, LOEWE Center for Synthetic Microbiology and Department of Chemistry, Philipps-University Marburg, Marburg, Germany

[5]Institute for Immunology, Philipps-University Marburg, Marburg, Germany

***Equal senior author contribution**

(Manuscript in preparation…)

11.2. ADDITIONAL PUBLICATIONS

RESERCH ARTICLES

1. Mingjing Hu*****, MD; **Bilal Alashkar Alhamwe, BSc***; Brigitte Santner-Nanan; Sarah Miethe, MSc; Hani Harb, PhD; Harald Renz, MD; Daniel P. potaczek, MD†; Ralph K. Nanan, MD, PhD†, *Short chain fatty acids augment differentiation and function of human iTreg cells .*
   *****Authors contributed equally.
   (in preparation).
2. Hani Harb*****, **Bilal Alashkar Alhamwe***, Nathalie Acevedo, Paolo Frumento, Catharina Johansson, Lisa Eick, Nikos Papadogiannakis, Johan Alm, Harald Renz, Daniel P. Potaczek and Annika Scheynius. (2019)***. Epigenetic Modifications in Placenta Are Associated with the Child's Sensitization to Allergens*. Hindawi BioMed Research International Volume.19, p.1-11.
   *****Authors contributed equally. **(IF: 2.58)**
3. Suzanne Abbring, Johanna Wolf, Veronica Ayechu-Muruzabal, Mara A.P. Diks, **Bilal Alashkar Alhamwe**, Fahd Alhamdan, Hani Harb, Harald Renz, Holger Garn, Johan Garssen, Daniel P. Potaczek and Betty C.A.M. van Esch. (2019). *Raw Cow's Milk Reduces Allergic Symptoms in a Murine Model for Food Allergy—A Potential Role For Epigenetic Modifications*." Nutrients Vol.11. P. 1-15. **(IF: 4.19)**
4. Acevedo, Nathalie, Paolo Frumento, Hani Harb, **Bilal Alashkar Alhamwe**, Catharina Johansson, Lisa Eick, Johan Alm, Harald Renz, Annika Scheynius, and Daniel P. Potaczek. (2019). *Histone Acetylation of Immune Regulatory Genes in Human Placenta in Association with Materal Intake of Olive Oil and Fish Consumption*. International Journal of Molecular Sciences Vol. 20, p.1-10; **(IF: 4.18)**
5. Daniel P.Potaczek, Sebastian D. Unger, NanZhang, Styliani Taka, Sven Michelß, Nesibe Akdağ, Feng Lan, Markus Helfer,Christoph Hudemann, Markus Eickmann, Chrysanthi Skevaki, Spyridon Megremis,AnneSadewasser,**Bilal Alashkar Alhamwe**,FahdAlhamdan, Mübeccel Akdis, Michael R.Edwards, Sebastian L. Johnston, Cezmi A. Akdis, Stephan Becker, Claus Bachert, Nikolaos G. Papadopoulos, Holger Garn, HaraldRenz. (2019). *Development and*

characterization of DNAzyme candidates demonstrating significant efficiency against human rhinoviruses. Journal of Allergy and Clinical Immunology. Volume 143, P. 1403-1415. **(IF: 14.11)**

6. Chrysanthi Skevaki, Christoph Hudemann, Mikhail Matrosovich, Christian Möbs, Sinu Paul, Andreas Wachtendorfa, **Bilal Alashkar Alhamwe**, Daniel P. Potaczek, Stefanie Hagner, Diethard Gemsa, Holger Garn, Alessandro Sette, Harald Renz. (2018). *Influenza-derived peptides cross-react withallergens and provide asthma protection.* Journal of Allergy and Clinical Immunology Volume 142. p. 804–814. **(IF: 14.11)**

REVIEW ARTICLES

1. **Alashkar Alhamwe, Bilal**, Fahd Alhamdan, Andreas Ruhl, Daniel P Potaczek, and Harald Renz. (2020). *The Role of Epigenetics in Allergy and Asthma Development*. Current Opinion in Allergy and Clinical Immunology Vol. 20. p. 48–55. **(IF:3.46)**
2. **Bilal Alaskhar Alhamwe**, Razi Khalaila, Johanna Wolf, Verena von Bülow, Hani Harb, Fahd Alhamdan, Charles S, Susan L. Prescott, Antonio Ferrante, Harald Renz, Holger Garn and Daniel P. Potaczek. (2018). *Histone Modifications and Their Role in Epigenetics of Atopy and Allergic Diseases*. Allergy, Asthma, and Clinical Immunology. Vol. 14, p.1-16. **(IF: 2.66)**
3. Potaczek, Daniel P.; Harb, Hani; Michel, Sven; **Alashkar Alhamwe, Bilal**; Renz, Harald; Tost, Jörg (2017). *Epigenetics and allergy: from basic mechanisms to clinical applications*. Epigenomics Vol. 9, p. 539-571 **(IF: 4.40)**
4. Harb, Hani; **Alashkar Alhamwe, Bilal**; Garn, Holger; Renz, Harald; Potaczek, Daniel P. (2016). *Recent developments in epigenetics of pediatric asthma.* Current Opinion in Pediatrics. Vol. 28, p. 754–763**. (IF: 2.67)**

11.3. CONGRESS PAPERS/ABSTRACTS:

ACCEPTED ABSTRACTS:	
The DZL Annual Meeting, January 23 - 24, 2020, Travemünde, Lübeck.	**B. Alashkar Alhamwe,** H. Harb, F. Alhamdan, S. Miethe, J. Tost, H. Garn, M. Blaser, D. P. Potaczek*, H. Renz*. ***Microbiome changes may contribute to the protective effect of Acinetobacter lwoffii against asthma development mediated by interleukins 6 and 10.***
32. Mainzer Allergie-Workshop 21-23 March, 2020, DGAKI, Mainz.	**B. Alashkar Alhamwe,** H. Harb, F. Alhamdan, J. Tost, H. Garn, Martin Blaser, D. P. Potaczek1*, H. Renz1*. ***The protective effect of Acinetobacter lwoffii against asthma development is mediated by interleukins 6 and 10.***
31. Mainzer Allergie-Workshop 21-23 March, 2019, DGAKI, Mainz.	**B. Alashkar Alhamwe**, H. Garn*, D. P. Potaczek*, H. Renz*. ***Anti-allergic/anti-asthmatic effects of A. lwoffii are at least partly underlain by macrophage-secreted il-6 further and resulting production of il-10 by T cells.***
ERS International Congress, 15–19 September, 2018, Paris, France.	**B. Alashkar Alhamwe*,** H. Harb*, E. Kilic-Niebergall, S. Miethe, F. Alhamdan, J. El Andari, A. Kaufmann, S. Hagner-Benes, D. Kesper, P. L. Graumann, H. Garn†, D. P. Potaczek†, H. Renz†. ***Interleukin-6 plays an important role in the development of Acinetobacter lwoffii-mediated protection against allergic asthma.***
The Allergie im Fokus | Eosinophile Granulozyten Workshop DGAKI June, 2018. Kassel, Bad Wilhelmshöhe.	**B. Alashkar Alhamwe**, H. Harb, E. Kilic-Niebergall, D. Kesper, H. Garn, D. P. Potaczek, H. Renz. ***Interleukin-6 plays an important role in the development of acinetobacter lwoffii-mediated protection against allergic asthma.***

International DZL Symposium, April 13 - 14, 2018 in Munich, Germany. Munich Lung Conference 2018,	**B. Alashkar Alhamwe**, H. Harb, E. Kilic-Niebergall, D. Kesper, H. Garn, D. P. Potaczek, H. Renz. ***Interleukin-6 plays an important role in the development of Acinetobacter lwoffii-mediated protection against allergic asthma.***

12. Verzeichnis der akademischen Lehrer

Philipps Marburg Universität

Meine akademischen Lehrer waren die Damen und Herren

Harald Renz, Daniel P. Potaczek, Reinhard Geßner, Holgar Garn, Michael Lohoff, Magdalena Huber, Till Adhikary, Hani Harb, Elke Pogge von Strandmann.

13. ACKNOWLEDGEMENTS

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Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel: „*Mechanistic studies on the allergy protective effect of Acinetobacter lwoffii in the mouse*“ im Institut für Laboratoriumsmedizin unter Leitung von Prof. Dr. H. Renz mit Unterstützung durch PD Dr. Daniel P. Potaczek ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt. Ich versichere, dass ich sämtliche wörtlichen oder sinngemäßen Übernahmen und Zitate kenntlich gemacht habe. Mit dem Einsatz von Software zur Erkennung von Plagiaten bin ich einverstanden.

Marburg 20./04./2020 Bilal Alashkar Alhamwe

„Die Hinweise zur Erkennung von Plagiaten habe ich zur Kenntnis genommen.“

Marburg 20./4./20.. Prof. Dr. Harald Renz

Universitätsklinikum
Gießen und Marburg GmbH
Institut für Laboratoriumsmedizin und Pathobiochemie
Molekulare Diagnostik
- Standort Marburg -
Direktor: Prof. Dr. Harald Renz
Baldingerstr., 35043 Marburg
Telefon (0 64 21) 58-6 62 34/-5
Telefax (0 64 21) 58-6 55 94