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„The influence of genetics on the response to different
allergens in mouse models of allergic asthma“

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ABSTRACT

Allergic asthma is a chronic inflammatory disease of the lungs characterized by chronic allergic airway and lung inflammation, airway hyperresponsiveness to usually innocuous stimuli, particularly specific allergens, in addition to excessive mucus production of the airway epithelium and the occurrence of allergen specific IgE antibodies. Although in many cases, asthma can be treated effectively with inhaled corticosteroids and sympathomimetic drugs as the most common therapies, it still represents a major burden on patients' lives and has a tremendous impact on health budgets worldwide. A complex interplay of individual genetic differences as well as environmental factors influence the development, maintenance and outcome of the disease. Mouse models of allergic asthma can be utilized to study these mechanisms to ultimately aid in the development of new compounds to prevent and treat allergic asthma.

In previous experiments conducted in our lab, we observed an eosinophil-dominated immunological response of C57BL/6 mice to intranasally administered low fat powdered milk (milk) but not to Ovalbumin from chicken egg (OVA). BALB/c mice did not show such a differential response to milk and OVA. Our aim was to confirm these results in a new set of experiments to demonstrate the influence of genetic differences in our mouse model of asthma. Furthermore, we wanted to explore the capabilities of lipopolysaccharide (LPS) with added doses of LPS to milk ranging from $10^{-4}\mu\text{g}$ to $1\mu\text{g}$ as an immunological modulator in asthmatic, milk sensitized BALB/c mice. Lastly, we wanted to reproduce the eosinophil-dominated immunological reaction of C57BL/6 mice to milk using LPS-depleted OVA with added doses of LPS ranging from $10^{-5}\mu\text{g}$ to $10^{-2}\mu\text{g}$.

In this work, an acute model of allergic asthma based on exclusive respiratory delivery of allergens was used. Experimental asthma was monitored via differential cell counts of the bronchoalveolar lavage fluid of the mice. Formaldehyde fixed paraffin lung tissue thin cuts were prepared. To assess overall lung inflammation the thin cuts were H&E-stained and evaluated under a microscope, while lung eosinophilia and mucus production of the lung epithelium were assessed after LUNA- and PAS-staining, respectively. Furthermore, allergen specific IgG1 and IgG2a-antibody-titers were assessed via enzyme-linked immunosorbant assay (ELISA).

We confirmed the results from our previous experiments, in which we observed a stronger allergic reaction to milk than to OVA in C57BL/6 mice, but not in BALB/c mice. We also found that higher doses of LPS (between $10^{-3}\mu\text{g}$ and $1\mu\text{g}$) suppressed eosinophilic airway and lung inflammation in milk-challenged BALB/c mice, while LPS doses less or equal to $10^{-4}\mu\text{g}$ did not influence eosinophilic airway and lung inflammation in this model of allergic asthma. Finally, addition of increasing doses of LPS to LPS-free OVA did not induce the strong eosinophilic reaction observed in C57BL/6 mice challenged with milk.

Altogether, the data from my thesis suggest that the influence of LPS on immunological reactions to intranasally administered OVA and milk is not responsible for the observed discrepancies in allergic responses to those allergens between BALB/c and C57BL/6 mice. The role of other mechanisms, including MHC haplotypes or other adjuvants, should be addressed in future research.

ZUSAMMENFASSUNG

Allergisches Asthma ist eine chronische entzündliche Erkrankung der Lungen charakterisiert durch chronische Atemwegs- und Lungenentzündung, Hyperreagibilität der Atemwege auf üblicherweise harmlose Stimuli, insbesondere spezifische Allergene, außerdem überschießende Schleimproduktion des Lungenepithels und das Auftreten von allergenspezifischen IgE-Antikörpern. Obwohl Asthma häufig effektiv mit inhalierten Corticosteroiden behandelt werden kann, stellt es doch eine hohe Belastung für den Patienten dar und benötigt massive Ressourcen des weltweiten Gesundheitswesens. Ein komplexes Zusammenspiel der individuellen genetischen Ausstattung und von Umweltfaktoren beeinflussen Entstehung, Entwicklung und Endpunkte der Erkrankung. Mausmodelle des allergischen Asthmas können verwendet werden, diese Mechanismen zu verstehen um letztendlich neue präventive und kurative Therapieoptionen zu entwickeln.

In vorhergehenden Experimenten unseres Labors beobachteten wir eine durch eosinophile Granulozyten dominierte immunologische Reaktion von C57BL/6 Mäusen auf intranasal appliziertes entfettetes Milchpulver (milk). Eine vergleichbare Reaktion dieser Mäuse auf Ovalbumin aus Hühnereiern (OVA) blieb aus. BALB/c Mäuse zeigten keine differenzierte Reaktion zwischen milk und OVA. Unser Ziel war es, diese Beobachtung eines genetischen Einflusses auf unser Mausmodell zu bestätigen. Darüber hinaus wollten wir das Potenzial von Lipopolysaccharid (LPS) als Additiv zu milk in Dosen von $10^{-4}\mu\text{g}$ bis $1\mu\text{g}$ als immunologischen Modulator der Reaktion auf milk-sensitivierte BALB/c Mäuse untersuchen. Abschließend wollten wir die eosinophiledominierte immunologische Reaktion von C57BL/6 Mäusen auf milk, unter Verwendung von LPS-gereinigtem OVA mit LPS als Additiv in Dosen von $10^{-5}\mu\text{g}$ bis $10^{-2}\mu\text{g}$, reproduzieren.

In dieser Arbeit verwendeten wir ein akutes Mausmodell, basierend auf der exklusiven respiratorischen Applikation von Allergenen. Das experimentelle Asthma wurde mit Hilfe von differenziellen Zellzählungen der Bronchalveolarlavageflüssigkeit der Mäuse beurteilt. Formaldehyd fixierte Paraffindünnschnitte des Lungengewebes wurden hergestellt. Um diese Schnitte hinsichtlich der allgemeinen Lungenentzündung zu beurteilen wurden sie mit H&E

angefärbt, und dann mikroskopisch betrachtet. Außerdem wurden Eosinophilie der Lungen mit Hilfe von LUNA-angefärbten und Schleimproduktion des Lungenepithels mit Hilfe von PAS-angefärbten Lungengewebsdünnschnitten unter dem Mikroskop beurteilt. Darüber hinaus wurden allergenspezifische IgG1 und IgG2a-Antikörpertiter via Enzyme-linked Immunosorbant Assay (ELISA) gemessen.

Wir konnten unsere Beobachtungen einer stärkeren immunologischen Reaktion gegen milk verglichen mit OVA von C57BL/6- aber nicht BALB/c-Mäuse bestätigen. Weiters konnten wir zeigen, dass höhere Dosen von LPS (zwischen $10^{-3}\mu\text{g}$ und $1\mu\text{g}$), welche milk als experimentellem Allergen hinzugefügt wurden, als Suppressor der eosinophilen Lungen- und Atemwegsentzündung von BALB/c-Mäusen agieren kann. Niedrigere LPS-Dosen (unter $10^{-4}\mu\text{g}$) hatten scheinbar keinen Einfluss auf eosinophile Lungen- und Atemwegsentzündung in unserem Modell. In unserem abschließenden Experiment konnte durch Zugabe von steigenden Dosen von LPS ($10^{-5}\mu\text{g}$ bis $10^{-2}\mu\text{g}$) zu LPS-gereinigtem OVA nicht die starke eosinophile Reaktion von C57BL/6 Mäusen reproduziert werden, die wir in C57BL/6 Mäusen, welchen milk administriert wurde, beobachtet haben.

Meine Studie legt nahe, dass der Einfluss von LPS auf die immunologische Reaktion auf intranasal administriertes OVA oder milk nicht für den beobachteten Unterschied der Reaktionen von BALB/c und C57BL/6 Mäusen verantwortlich ist. Andere Ursachen, zum Beispiel potenzielle in milk vorkommende Adjuvantien oder der Einfluss von MHC-Haplotypen sollten in folgenden Studien untersucht werden.

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1 INTRODUCTION

1.1 ALLERGIC ASTHMA: A BASIC OVERVIEW

1.1.1 DEFINITION OF ALLERGIC ASTHMA (INCLUDING PHENOTYPES)

Asthma is etymologically derived from “ἄσθμα“, a word going back as far as ancient Greek literature. More specifically, it was firstly recorded in Homers famous Iliad and originally referred to gasping, panting or a short drawn breath. Used as a medical term, asthma appeared around 2000 years ago in the Corpus Hippocraticum, and B. Aretaeus the Cappadocian was probably the first who colorfully described an acute asthma attack in his documentations and acknowledged it as an “*autonomous clinical entity*” [1]. Published in 1698, Floyer’s classic monograph “a treatise of the asthma” recommends smoking leaves from *Datura stramonium* and other solanaceae to ease asthma symptoms [2].



Figure 1.1. A photograph by the author of Datura stramonium at “Botanischer Garten der Universität Wien” in Vienna. It is a traditional asthma remedy. Its leaves were smoked to release bronchospasms. The active ingredient is the tropane alkaloid atropine.

In 1917, Orville Harry Brown outlined many different hypothesis on asthma of his time [3], demonstrating the difficulty to understand its complex pathology back in the age of enlightenment. Although medical science has made vast progress since then, a complete understanding of the mechanisms that lead to and maintain asthma still remains elusive. According to the GINA¹ report 2014 asthma is defined as:

“Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation.”[4]

This definition implies, that asthma presents itself as a disease of many different phenotypes. For the allergic asthma phenotype an atopic status is obligatory, meaning a tendency to allergic immune responses and hypersensitivity of patients towards normally harmless antigens. This results in airway eosinophilia and allergen specific IgE-antibodies in their blood. Allergic asthma usually starts in childhood and often goes along with allergic rhinitis and/or atopic eczema. An early skewing of the adaptive immunity towards Th2² cells and cytokines is believed to precede the development of this phenotype. The cytokine IL-4 can prime naïve Th0 cells to become Th2 cells and therefore, is a marker that can be found in bronchoalveolar lavage (BAL)³ fluid of allergic asthmatics [5]. Although featuring Th2 driven eosinophilia, there is no sufficient evidence for the involvement of exogenous allergens in intrinsic asthma and in BAL fluid of intrinsic asthmatics IL-2⁴ and IFN γ ⁵ is present instead of IL-4 [6]. It is not clear whether non-eosinophilic or “neutrophilic” asthma represents another phenotype, or just a more severe stage of allergic asthma. Other described asthma phenotypes include (i) aspirin intolerant asthma which might be due to an altered eicosanoid metabolism, (ii) extensive remodeling asthma, which is characterized by only very little inflammation, but a pronounced tendency to pathological changes of the airways [5], (iii) asthma with fixed airflow limitation and (iv) asthma with obesity [4]. To further complicate the matter, asthma phenotypes can be divided into many distinct endotypes according to specific underlying molecular and cellular mechanisms. Understanding

¹ Global Initiative for Asthma

² Type 2 T-helper cells

³ Bronchoalveolar Lavage

⁴ IL - interleukin

⁵ IFN γ – Interferon gamma

the phenotypes and their mechanisms might play a crucial role in the optimization of asthma treatments to improve patient lives. The focus of this work will be on allergic asthma.

1.1.2 PREVALENCE AND INCREASING INCIDENCE OF ALLERGIC ASTHMA

According to a GINA report, 300 million people are burdened with asthma worldwide [7]. Affecting at least 5% of any investigated country, asthma is one of the most common chronic diseases and derogates people at all ages [8]. Large studies on adult asthma, e.g., ECRHS⁶, WHS⁷ and GA2LEN⁸, are hard to reconcile due to their heterogeneous approaches and definitions of asthma. Therefore, it is difficult to draw conclusions whether there is any change in the prevalence within the last 20 years or not, although ECHRS and GAL2EN point to an increase. All three studies mentioned show considerable geographical deviation in the frequency of adult asthma amongst all reviewed nations [9]. Results from the 20-year ISAAC⁹ program revealed that childhood asthma is very common in high GDP¹⁰ as well as lower GDP countries, but in the latter the severity of the condition appears to be higher [10]. Compared to 1990, where the global mortality rate for asthma was about 25 out of 100,000 men and 17 out of 100,000 women, in 2010 it was almost halved to 13 out of 100,000 men and 9 out of 100,000 women, respectively [11]. Nevertheless, the annual health costs due to asthma in 2012 were 1583€ for every patient in the European Union and appear to be growing [12-14].

1.1.3 SYMPTOMS

As mentioned before, asthma is a heterogeneous disease. The allergic asthma phenotype is characterized by certain hallmark symptoms.

⁶ the European Community Respiratory Health Survey carried out between 1991-1994

⁷ World Health Survey carried out between 2002-2003

⁸ Global Allergy and Asthma Network of Excellence carried out between 2008-2009

⁹ International Study of Asthma and Allergy

¹⁰ Gross Domestic Product

1.1.3.1 ALLERGIC AIRWAY INFLAMMATION

Bronchial biopsies of asthmatics characteristically reveal mucosal infiltrations of inflammatory cells. The specific cell composition depends on the particular phenotype or endotype of the disease. In many cases Th2 cells, mast cells, basophils and eosinophils are predominant, but also other cell types such as neutrophils can occur [15]. It has also been suggested, that events in the local tissues play an essential part in allergic airway inflammation. Dendritic cells¹¹ and allergen specific Tregs¹² appear to modulate tolerance. Their malfunction seems to promote immunological defenses leading to inflammation [16]. Persistent and progressive allergic airway inflammation promotes airway remodeling, makes the airways frail to irritation and contributes to narrowing them [17].

1.1.3.2 MUCUS HYPERSECRETION

The compounds of respiratory mucus are secreted by submucous glands, epithelial cells and airway goblet cells. The latter secrete mucins. Mucins are glycoproteins that form viscoelastic gel-structures in the aqueous environment of the outer respiratory tract. The mucins derived from *MUC5AC* and *MUC5B* genes present a main compound of mucus. Cellular proteins as well as lipoids and serum are also present in mucus. In conjunction with ciliar movement in the lungs the secretion creates a constant outward stream of mucus. The physiological function of this mucosal stream is to clear the airways from pathogens, waste products and cell debris. Coughing out sputum also aids in clearing the airways. In patients with allergic asthma goblet cells are hyperplastic, their mucus production is excessive and the mucus is richer in mucin. This results in pathological amounts of mucus and changes in the rheological properties. These changes are hindering the clearance of the lungs. IL-13 and secretory phospholipase A2 (PLA2) can facilitate goblet cell hyperplasia and cause an increase in mucin release. Both IL-13 and PLA2 are produced at high levels in the lungs of allergic asthmatics [18, 19].

¹¹ Physiology and pathophysiology of dendritic cells will be explained later

¹² Physiology and pathophysiology of regulatory T cells will be explained later in detail

1.1.3.3 AIRWAY HYPERRESPONSIVENESS

An increased tendency to constriction of the bronchial muscles resulting from the inhalation of nonspecifically irritating chemicals or a physical stimulus such as cold air, is called airway hyperresponsiveness (AHR). AHR presents a risk factor for developing asthma on its own but moreover is a main feature of asthma. It is positively associated with worse outcomes for the reduction in FEV1¹³ and asthma severity. There is also a correlation between AHR and the grade of inflammation. To quantify the extent of airway hyperresponsiveness, patients can be challenged with aerosolized metacholine. Spirometric measurement of their FEV1 before and after the challenge can then serve as an indicator for the extent of AHR [17].

1.1.3.4 ALLERGEN SPECIFIC ANTIBODIES

IgE¹⁴ is an important part in the pathology of allergic asthma. There is 10⁴ times more IgE negative B-cells, than IgE positive B-cells in healthy humans. In contrast asthmatics have 4% IgE-expressing B-cells in total and approximately 13% of plasma cells of their nasal mucosa express IgE [20]. Most of the IgE is taken up by cells and remains surface bound. The presence of IgE induces upregulation of IgE receptors [21]. These receptors are high-affinity-IgE receptor (FcεRI), which can be found on mast cells and basophils, and low-affinity-IgE receptor (FcεRII, which is also called CD23). Activation of FcεRI on mast cells and basophils leads to the release of effector molecules and contribute to hypersensitivity reactions in allergic diseases [21]. Beside mast cells, dendritic cells, eosinophils and various other antigen presenting cells can bind IgE, which appears to activate these cells and influences their specific functions by not fully understood mechanisms [20].

¹³ Forced Expiratory Volume in 1 Second

¹⁴ Immunoglobulin E

1.1.4 AVAILABLE TREATMENTS FOR ALLERGIC ASTHMA

The modern approach for asthma treatment is a control-based management of the disease. The non-pharmacological interventions have a significant impact on the successful management of the disease. Smoking cessation and less exposure to irritants and allergens are beneficial [4]. A healthy amount of physical activity is also recommended. Patients should also be countenanced to avoid allergens and noxious medication (e.g., NSAIDs) [22]. However, asthmatics require regular medication regimes to fully control the disease. This means periodic monitoring of patients and continuous adjustment of the therapy to obtain optimal symptom control, while minimizing the side effects and less undesired future outcomes, i.e. fixed airflow limitation.

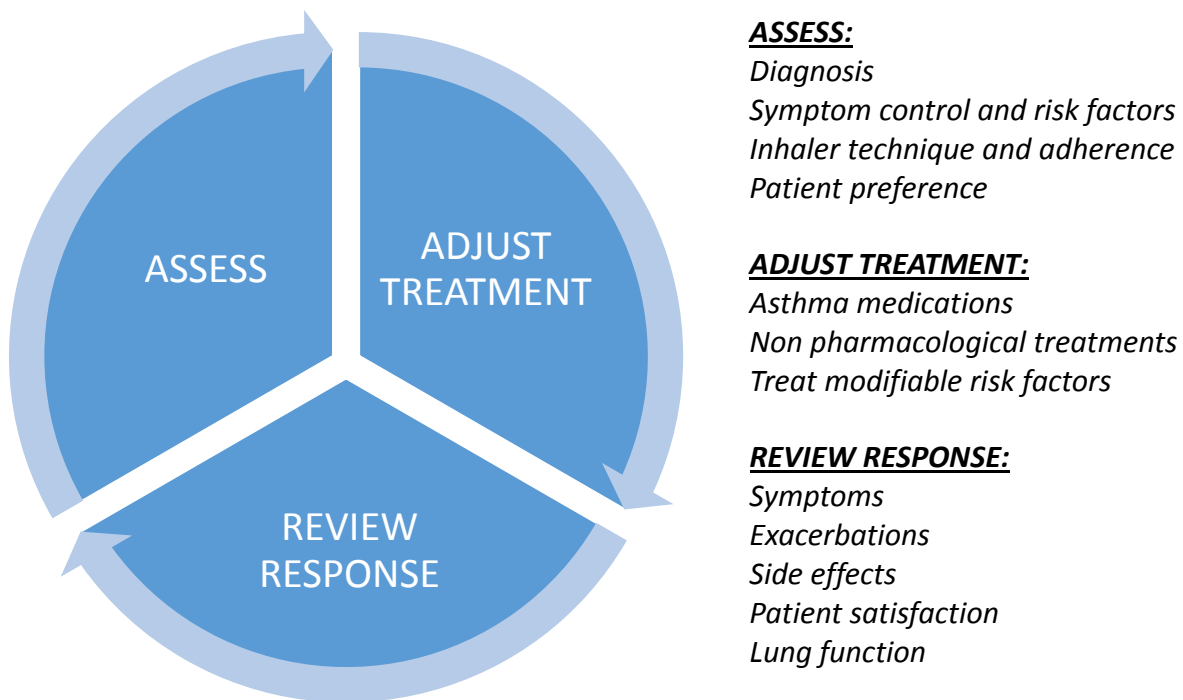


Figure 1.2. Adaptive treatment for asthma is key. (GINA Report 2014)

Asthma medication can be divided into controllers, relievers and add on therapies [4]. Controllers are intended to act against the underlying chronic inflammation and prohibit exacerbations. Corticosteroids (CS) are potent anti-inflammatory agents used for this purpose. Although highly efficacious, systemic therapy with CS is prohibited to very severe asthma and for short term therapy only, because of their long known adverse effects, including increased susceptibility to infections, osteoporosis, weight gain and central obesity, striae cutis distensae, impaired wound healing and behavioral changes [23, 24]. For mild to moderately severe asthma, inhalation of corticosteroids, such as fluticasone propionate, beclometasone dipropionate, mometasone furoate and budesonide [25], presents an effective local therapy with fewer side effects. However, long term therapy with inhaled corticosteroids can also lead to reduction in bone density and growth, oral candidiasis, dysfunction of the hypothalamo-pituitary-adrenal axis, eye pathology, tendency to respiratory infections and changes of the skin [25]. Preparations with inhalable CSs interfere with nuclear factor kappa B (NF- κ B), which is a central mediator of the expression of inflammation associated molecules, e.g., TNF α , interleukin-1 and leukotriens. CSs are ligands of an intracellular receptor called glucocorticoid receptor (GR). In the cytosol, the CS activates the GR and then forms a dimer with it (here referred to as CS|GR). The CS|GR-dimer translocates to the cell's nucleus and binds a specific region on the DNA. CS|GR upregulates the expression of the inhibitor of NF- κ B (I κ B). This increase of I κ B leads to the retention of NF κ B in the cytosol, preventing NF- κ Bs downstream effects. The CS-activated GR can also inhibit NF- κ B activity directly. Furthermore, CS can inhibit phospholipase A2, preventing an early step in the production of prostaglandin- and leukotrien- inflammatory mediators [24]. In some asthma patients CS are counter indicated, others are unwilling to take CS-medication. These patients might benefit from treatment with specific leukotriene receptor antagonists e.g. montelukast [4]. To a limited degree mast cell stabilizers, namely chromoglycinic acid and nedocromil can be used for seasonal asthma [4]. In very severe cases of asthma oral corticosteroids are indicated [4].

Relievers are used to reduce acute symptoms in asthmatics. The frequency of their use is, therefore, an indicator for asthma control [4]. Recommended reliever drugs for mild to moderate asthma are short acting β 2-agonists (SABAs). These β 2-agonists are designed to specifically bind to and activate β 2-receptors on the surface of smooth muscle cells of the bronchi. This leads to the activation of the β 2-receptor coupled G-protein. In turn, a subunit of the G-protein activates the adenylyl cyclase. With increased adenylyl cyclase activity the cytosolic concentration of

cyclic adenosine monophosphate (cAMP) increases, activating the protein kinase A (PKA). PKA blocks calcium release in the cytosol and also inhibits the calcium-dependent activation of myosin light chain kinase (MLCK). Both effects prohibit the smooth muscle cell from contraction and therefore lead to relaxation of the bronchial muscles [26]. For more severe asthma, long acting β 2-agonists (LABAs) have to be part of the regimen to maintain good lung function [4]. If β 2-agonists are not well tolerated by the patient, inhaled theophylline and anticholinergics can be administered. However, due to the small therapeutic index of theophylline and slower onset of anticholinergics such as ipratropium they are not first choice medications. Altogether, controllers provide on demand and in the case of LABAs long-term relief for the patient, but do not affect the inflammatory aspect of asthma.

Subcutaneous immunotherapy can also be of benefit for atopic individuals with asthma [4]. Omalizumab, a specific anti-IgE-antibody is recommended as an add-on-therapy for very severe, therapy resistant asthma and special cases [4]. Under Omalizumab treatment systemic IgE is reduced considerably and also the receptors for IgE are getting downregulated, which prevents the pathological effects of IgE. However, lately concerns have been raised about the side effects of Omalizumab, including anaphylaxis and malignant tumors [27] so its use should be prohibited to experienced specialists.

Very recently sb010, started phase III-pharmacological study. Sb010 is an oligomer or so called “DNAzyme” that targets GATA-3 [28]. GATA-3 is important for T-cell development and functions as a Th2-differentiation factor [29]. Another promising approach uses calcilytics, to target calcium sensing receptors. This can potentially reduce airway inflammation and hyperresponsiveness in asthma patients [30, 31]. Further studies are needed to determine the value of this new agents to treat asthma.

1.2 A MISPLACED IMMUNE RESPONSE LEADS TO ALLERGIC ASTHMA

1.2.1 INITIATION OF ALLERGIC ASTHMA

It has been widely accepted that allergic asthma often starts in childhood with the sensitization to normally harmless antigens. These antigens can originate from animal dander, house dust mite, fungi, cockroaches, pollens and many other sources [32, 33]. Initially, such an antigen is taken up by an antigen presenting cell (APC), often a dendritic cell (DC). Sampling the airways for potentially dangerous agents, DCs function as airway sentinels. The DCs can catch and phagocytize antigens. After phagocytosis, antigens are stored in separate compartments, so called phagosomes. Thereafter, the phagosomes fuse with lytic vesicles and form a phagolysosome. Within the phagolysosome, the antigen gets broken down to peptide fragments. These fragments can be mounted to MHC-class-II molecules and presented to other immune cells. After migration to a draining lymph node, an APC can interact with lymphocytes causing T- and B-cell differentiation to antigen specific subtypes [34]. In the absence of a danger signal from potentially harmful microbes, healthy humans mucosal DCs tend to induce regulatory T-cells and maybe certain subtypes of B-cells with tolerogenic properties [35-37], localizing the adaptive processes in the mucosal tissues and suppressing a systemic immune response. The microbiotic flora greatly influences these processes [38]. However, as a reaction to potentially dangerous agents, DCs can also induce a defensive immunological reaction. This is partially achieved through priming of lymphocytes to mature into other subtypes with distinct effector functions and long-lived memory cells that influence future encounters with this particular antigen. Ideally, this results in clearing the host from harmful agents after the first encounter and creating immunological memory of this particular antigen rendering the host immune to this particular agent [34]. Despite extensive research, it is still poorly understood, how defective signaling from DCs promote the complex asthma syndrome [35, 39, 40].

Individuals with allergic asthma seem to favor a defensive immune response over tolerance to specific, usually innocuous antigens. The response looks very much like an immunological reaction to large parasites. As such, it lacks the necessary effector functions to fight off viral and bacterial pathogens. That might explain the high susceptibility of allergic individuals to them [41-43]. Typically, IgE antibodies play a dominating role in these inflammatory events. The presence

of allergen specific IgE-antibodies implies, that immunological memory to these antigens had to be obtained somehow. An initial skewing of the adaptive immune system towards a humoral, “type-2”- or “Th2”-immune response early in life could lead to symptoms seen in atopy and asthma [16]. The Th2-hypothesis stated, that an early disruption in the regulation of the balance between Th1 and Th2 cells was the cause for this tendency [44, 45]. In the hygiene hypothesis, it was proposed, that this imbalance was due to a lack of early exposure to microbial compounds. This assumption is based on the observation, that children raised on a farm have a much lower prevalence for asthma [46]. Indeed, Th2 cells are capable to provide with a cytokine cocktail associated with the pathogenesis and maintenance of allergic asthma, including IL-4, IL-5 and IL-13 [47, 48]. IL-4 is necessary for isotype switching of B-cells to produce IgE [49]. IL-5 activates and attracts eosinophils and is central to their maturation and survival [32]. IL-13 ultimately activates the transcription of the Muc5AC gene in airway mucosal-cells that is required to build mucus [19]. However, the Th2 hypothesis does not provide a complete theoretical model for asthma, especially as only 50% of asthma patients show the Th2-dominant endotype of the disease [50]. In some asthmatics neutrophilia can be observed and Th1- and Th17-cells and their derived cytokines dominate rather than Th2 cytokines [51]. Furthermore, within the Th2-hypothesis alone there is no convincing explanation for the initial occurrence of IL-4 starting the Th2-differentiation to skew the immune system towards a type-2 response [52]. But lately, there has been new developments in the research of asthma onset. Clinical evidence was growing that viral infections combined with allergen exposure might be an important coincidental factor in the development of human asthma [53]. It was shown that AHR, airway inflammation and mucosal hyperplasia can persist in post-viral mouse models of chronic airway disease for many weeks after allergen challenge [44]. Newer studies indicate, that airway epithelial cells (AECs) can be reprogramed through viral pathogens such as respiratory syncytial virus (RSV) to produce IL-33 [53]. Moreover, airway epithelial progenitor cells (APECs) can act as a renewable, long-lived source for IL-33 after they have been virally reprogramed. IL-33 in turn can stimulate innate lymphoid cells type 2 (ILC2s) to produce Type-2 cytokines e.g. IL-5 and IL-13 [54]. IL-33 also favors the development of Th2-cells from naïve T-cells [34]. Furthermore, pattern recognition receptors (PRR) on AECs and innate immune cells can be activated by viral compounds, inducing cytokine and prostaglandin secretion [19] which in succession orchestrate changes in immune functions. Under certain conditions, this can favor allergy over tolerance [44].

1.2.2 FORMATION OF IGE-ANTIBODIES SPECIFIC TO ALLERGENS

As stated by Coombs and Gell, hypersensitivity can be roughly divided into 4 subtypes. General asthma has lots of the characteristics of the cellularly mediated type IV hypersensitivity. The allergic phenotype of asthma however also features type I hypersensitivity, which is mediated primarily by IgE-antibodies and its effects [34]. The physiological function of IgE is probably to initiate mechanisms that damage large parasitic worms. It creates an increase in tissue permeability and secretions to expel the parasites through their entrance routes, often the mucosal surfaces of the body [34, 43]. In healthy people, IgE-antibodies are typically localized within peripheral tissues, tightly attached to Fc-receptors, FcεRI or FcεRII that are expressed by numerous cell types, e.g. mast cells, basophils and eosinophils and DCs [34]. Multivalent ligands capable of crosslinking many of these receptors can activate the cell to carry out different effector functions [55]. Certain factors favor IgE-production, most strikingly a Type-2-cytokine environment, but also the chemical nature of the antigen, its quantity and the mucosal route of antigen presentation. The naïve B-cell has to be primed to become an IgE secreting B-cell. This priming happens in the so-called germinal center reaction. Germinal centers are usually developmentally predetermined secondary lymphoid tissues, but can also form in inflamed mucosal tissues. These structures enable close contact between antigen laden APCs that were chemotactically attracted from the periphery and antigen specific naïve T-cells, coming from the bloodstream. Through the contact of the MHC-II-peptide complex presented by the APC and the T-cell receptor, but also other costimulatory signals, namely IL-4 or IL-13, antigen specific naïve T-cells are primed to mature into Th2-cells. Th2 cells in turn, can give the differentiation signal to an IgM-producing immature B-cell that presents a specific antigen to the aforementioned Th2-cell. Then this B-cell undergoes isotype switching and matures into a plasma cell. An obligatory costimulatory signal is given by the CD40 ligand on the Th2-cell [34]. The receptor of CD40 ligand, CD40 is on the surface of B-cells and recognizes the signal. IL-4 or IL-13 gives a secondary signal to this B-cell via the Jak-pathway. Janus family kinases Jak1 and Jak3 get activated. This pathway gives the final signal to phosphorylate STAT6 in this B-cell, to ultimately become an IgE secreting B-cell [34]. DCs and possibly eosinophils as well, activated by their FcεRI reinforce Th2-differentiation. Other cell types, e.g. basophils and also mast cells can express CD40 ligand at the site of inflammation and secrete IL-4, after they were activated via FcεRI. This can make additional B-cells undergo isotype switching and express IgE at the site

of inflammation. This process suggest a plausible mechanism for the self-sustainability of IgE generation [34, 56].

1.2.3 ALLERGIC ASTHMA: PROGRESSION, PATHOPHYSIOLOGY AND IMMUNOLOGICAL MEMORY TO ALLERGENS

After the disease has been initiated, changes in the organization of the lung tissue manifest through the effects of inflammatory cells, the effects of IgE-antibodies and other processes. For instance, mast cells accumulate under the surface of the airway mucosa. They are maintained by IL-9 and express the high affinity IgE receptor (FcεRI) on their surface. In the atopic individual FcεRI is upregulated, making the individual more sensitive to IgE [34]. IgE, previously produced by antigen specific B-cells, is attached to FcεRI. If these IgE covered mast cells bind antigens, that crosslinks their IgE receptors, the mast cells get activated. In succession, many different proinflammatory molecules are released upon the tissue, e.g. histamine, eicosanoids (prostaglandins, thromboxans and leukotriens), platelet activating factor, TNFα, serine esterases, proteases (e.g. chymase and tryptase). Histamine is a neurotransmitter but also a local mediator. To this day, it has 4 known receptors, H1-H4 [26]. Signaling through H1 is known to rapidly activate a proinflammatory pathway, causing an increase in tissue permeability and more local blood flow. This is associated with local skin reactions and itching in many allergic and pseudoallergic reactions [34]. TNFα is a very potent cytokine that acts on endothelial cells to upregulate cellular adhesion molecules, trapping more leukocytes at the site of inflammation. Mast cell derived proteases cleave matrix metalloproteinases. The metalloproteinases cause tissue damage, by cleaving molecules in the extracellular matrix. The mast cell derived leukotriens C₄, D₄ and E₄ are involved in prolonged tissue inflammation. The chemotactic prostaglandin D₂ recruits cells through PTGDR-receptor signaling, predominantly eosinophils, Th2s and basophils [34]. In post mortem derived lung tissues, basophils are much more abundant in asthmatic individuals than in non-asthmatics. Although still poorly understood, the basophil is very likely to contribute to asthma pathology. Basophils can be activated through many routes. IgE, but also cytokine signaling and proteolytic allergens such as Der p1 can lead to their activation. They can produce IL-4, IL-5 and IL-13 and therefore reinforce the Th2-cytokine environment [57]. IL-13 is important for the recruitment of eosinophils. Coming from the bone marrow eosinophils need IL-

5 to maintain their survival and initiate their maturation amongst IL-3 and GM-CSF. Their apoptosis is regulated by siglec-8- and siglec-F-signaling. Eosinophils are a hallmark of the Th2-inflammatory pathway. They also express FcεRI on their surface. When activated through FcεRI, eosinophils release eosinophilic cationic protein, eosinophil derived neurotoxin and many different cytokines. Interestingly, eosinophils can also release whole, intact granula [58-63]. This causes again inflammation and direct tissue damage [34, 54]. Persistent inflammation and injury of the airway epithelium locks the tissue to constant “repair mode”. More local growth factors get released than under normal conditions and upregulation of the EGF-receptor-pathway takes place. TGF-β, fibroblast growth factor, endothelin and other profibrotic mediators cause myofibroblasts and fibroblasts to deposit different collagens, tenascin and fibronectin. In turn the lamina reticularis of the airway epithelium continuously thickens. Interactions between epithelial cells, mast cells and eosinophils make contributions to this process as well. The resulting pathological changes also include smooth muscle hypertrophy, thickening of the basement membrane, increase of angiogenesis and lymphangiogenesis and goblet cell metaplasia. Through the myofibroblast activity alterations in the connective tissue result in loss of elastic capabilities and hyperinflation of the lungs occurs. Cysteinyl leukotrienes by eosinophils and TGF-β and PDGF increase the mass of airway smooth muscle. This increase contributes to remodeling, the likeliness for bronchoconstriction and possibly exacerbations. In addition, the pathologically hypertrophied smooth muscle can gain secretory function for mediators such as IL-5, IL-13, TGF-β, IL-1β and TNFα that are all associated with asthma pathophysiology [54]. Furthermore, epithelial cells lose polarity and cell adhesion capabilities, while tight junctions in the airway epithelium tend to collapse, leaving a leaky mucosal barrier more easily penetrable for allergens and pathogens. All these changes can be summarized under the term airway remodeling [54] and lead to the reduced lung function in asthmatics.

1.3 GENETIC PREDISPOSITION TO ALLERGIC ASTHMA

1.3.1 DEFINITION OF ATOPY

Derived is the word “atopy” from the Greek “ἀτοπία”. Its original meaning was “misplaced” “out of place” or “unusual” [64]. Besides its use in medical science, atopy is a term of philosophy, probably established by Plato. Referring to his high originality and genius, Socrates was often called “atopos”. Interestingly, in the philosophical context, atopy cannot be described, in fact its actual meaning is “something that eludes description” but is yet there [65]. Within the 1920s, Coca and his colleague Cooke, introduced “atopy” as a general term to describe what at that time was known as dermal hypersensitivity-reactions or “*wheal and flare*” [66]. In 1921, Küstner and Prausnitz demonstrated that immediate hypersensitivity was transferable by serum. It was not until 1968, that WHO's International Reference Center for Immunoglobulins acknowledged the existence of IgE antibodies. Later, in 1970, Pepys defined atopic allergy as “*IgE-mediated allergic reactions to inhalant allergens*” [66]. A modern definition taken from Allergy Frontiers: Classification and Pathomechanisms (Johansson, S.G.O.) states: “*Atopy is a personal and/or familial tendency, usually in childhood or adolescence to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis or eczema...*” [66]. Considering all different phenotypes and endotypes of asthma, atopy by definition only describes a distinct quality of but not the disease as a whole. What exactly predisposes an individual to the development of atopy and asthma is still under heavy debate. Genetic and environmental factors have to be considered.

1.3.2 GENES INVOLVED IN THE SUSCEPTIBILITY TO ASTHMA

According to many GWAS¹⁵ studies done on asthmatics, genes involved in the regulation of IgE expression have very little impact on asthma susceptibility. Atopy is likely not a prerequisite for asthma but an optional consequence [67]. It was not possible to pinpoint asthma to one single gene. On the contrary, there are at least 100 candidates, that are likely to contribute to the asthma

¹⁵ Genome-wide association study

pathology [68]. Examples of candidate genes include orosomucoid like 3 (ORMDL3), which is overexpressed in some asthmatics [69]. These variations in ORMDL3 expression were linked to AHR, early onset asthma in children and maybe to a lower degree adult asthma. These findings have been reproduced many times. Interestingly, there was no correlation between allergic sensitization and ORMDL3 variations. Very little is known about the actual physiological function of ORMDL3 in humans. However, it seems plausible that ORMDL3 influences calcium release. It also has been implicated in the proper protein folding in the cell. Moreover, it possibly mediates the metabolism of sphingolipids. This might ultimately have an effect on bronchial reactivity. In mice it was shown, that ORMDL3 expression can be upregulated via lipopolysaccharide, tobacco smoke and allergens. In vitro studies indicate, that IL-4 and IL-13 are potent stimulators of ORMDL3 expression. Both these findings have to be reproduced in humans though [69]. Other investigated genes code for thymic stromal lymphopoietin (TSLP), IL-33 and IL1RL1. All three revolve around the activation and attraction of inflammatory cells [70] and their variants seem to correlate with basophil and eosinophil numbers [71]. Originating from AECs, TSLP-signaling increases the expression of OX40L on DCs. OX40L in turn fortifies the humoral pathway in Th2-cells, influencing eosinophils and IgE antibody-release as a consequence [70, 72]. Variants in HLA-genes, IL-2RB, DENND1B and IL6R possibly contribute to asthma via their influence on the differentiation of T-cell subtypes and their effector functions. PDE4D, SMAD3 and CDHR3 are genes that are involved in the modulation of certain aspects of cell signaling were also correlated in GWAS studies to asthma [73]. Genetic data, thus, reflect the complexity of asthma pathophysiology.

1.4 INFLUENCE OF ENVIRONMENTAL FACTORS ON ALLERGIC ASTHMA

1.4.1 ANTIGENS ACTING AS ALLERGENS

1.4.1.1 ALLERGEN DEFINITION

The AAAAI¹⁶ defines an allergen as follows: “*An allergen is a usually harmless substance capable of triggering a response that starts in the immune system and results in an allergic reaction*” [74], while in “*Immunology*”, Fifth Edition. 2002 by W. H. Freeman: „*...an allergen is an antigen capable of stimulating a type-I hypersensitivity reaction in atopic individuals through Immunoglobulin E (IgE) responses.*” [75]. It seems, an allergen is defined primarily by its effects on the immune system, rather than its intrinsic properties. Hence, it only functions within the context of biological organisms capable of allergic immune responses. This might explain, why so far it was very difficult to predict the potential to induce allergy of a given molecule from typical structural, biochemical or functional features [76]. Nevertheless, there has been extensive research on molecules that were known for their allergenicity. It revealed some intrinsic features that might cause their allergenicity or at least increase the propensity to induce allergic reactions [77]. It is way beyond the scope of this work to thoroughly discuss all types of allergenic substances, which is why I will focus on a few well researched specimen only, excluding smaller molecules as well as pseudoallergens, haptens, biogenic amines, and so forth.

1.4.1.2 TYPICAL ALLERGENS AND THEIR CHARACTERISTICS

Allergens can be classified by their function. Many allergens are enzymes, in contrast, some have enzyme-inhibiting properties. Others act as transporters. Also, allergens can function as regulatory proteins and naturally there are those, which have biochemical features that do not fit in any of the previously mentioned categories. Structurally, allergens can be extremely variable. *In silico* searches to identify common surface features of allergens were at best modestly successful. However, the antigenic epitopes of allergens very often have homologous structures

¹⁶ American Academy of Allergy, Asthma & Immunology

to compounds found in parasites such as helminths. These include specific glycosylation patterns that can be recognized via the mannose receptor. For instance, Ara h1, a glycosylated allergen present in peanuts resembles similarity to such helminthic compounds. Paradoxically there seems to be fewer allergies, when more parasites are around.

A very well researched allergen of the house dust mite *Dermatophagoides pteronyssinus* is a glycoprotein called Der p1. As many other allergens, Der p1 is a protease, more precisely a cysteine protease. This activity seems to have a tremendous impact on its allergenicity [76, 77]. By cleavage of ZO-1 and occludin, which are both necessary for tight junction integrity in lung tissue cells, Der p1 digs its way through the airway epithelium. Der p1 also facilitates the degradation of surfactant proteins A and D, making the mucosal barrier more permeable for itself but also other allergens. This facilitation of the entrance of other allergens is often referred to as the bystander effect. Der p1 also affects map-kinase signaling of airway smooth muscle. Interestingly Der p1 maintains its proteolytic ability even when bound to Der p1-specific IgE. Der p1 also affects cells of the innate as well as the adaptive immune system. It induces degranulation without the need of IgE cross-linking FcεRI in basophils, and mast cells. Furthermore, Der p1 facilitates IL-4 secretion in these cells. Through cleavage of CD40 on DCs, Der p1 changes DC-function, which ultimately impairs the production of extracellular thiols. Der p1 also interferes with DC-SIGN, a C-type lectin. All this results in defective DCs signaling polarizing naive T-cells towards Th2-cells and possibly less Th1 and T-regulatory cells. Lastly, Der p1 can degrade FcεRII on B-cells, which might play a role in downregulation of their IgE synthesis [77]. Despite this impressive array of effects one has to bear in mind that allergens do not have to have protease activity per se to be effective.

1.4.1.3 OVALBUMIN AND ITS USE AS A TEST ALLERGEN

Exposure to dried egg powder has been correlated with the development of occupational asthma (“bakers’ asthma”) and food allergy with allergen specific IgE to egg compounds. Although there are potentially allergenic compounds in egg yolk, egg whites are believed to contain most of the allergens of egg allergy. Ovalbumin (OVA) represent 54% of proteins present in egg white. In the

1980s, ovalbumin was identified by cross-radio-immunoelectrophoresis and RAST¹⁷ assays to be one of the major allergens in eggs. OVA is made up of 385 amino acids and has a relative molecular mass of 45,000Da. Ovalbumin has structural homology with the superfamily of serin protease inhibitors (serpins) but lacks their protease inhibitory function. Since OVA was widely available, it was established as the main test allergen for the mouse model of allergic asthma [78].

1.4.1.4 MILK AS AN ALLERGEN AND ITS USE AS A TEST ALLERGEN

Some of the compounds of cow's milk are frequent food allergens in children. Approximately, 2.5% of children suffer from this allergy, but mostly lose it before they are 3 years old. Nevertheless, cow's milk allergy is correlated with the risk to develop asthma. Cow's milk contains up to 35g of protein per liter. Some of these proteins are derived from the mammary gland, but others actually come from the blood. After acidification of cow's milk, the proteins split into 2 fractions. The larger fraction being the coagulum (=curd), containing 80% of the proteins and the smaller fraction, the lactosum (=whey). Whey makes up about 20% of the proteins. In the coagulum, α s1-caseins, α s2-caseins, β -caseins and κ -caseins are the most abundant. The whey is mostly comprised of globular proteins, e.g. α -lactalbumin, β -lactoglobulin, bovine serum albumin, lactoferrin and immunoglobulins. It is very common that patients with cow's milk allergy, are sensitized to multiple milk-proteins. More than 50% of patients are specifically sensitized to caseins, α -lactalbumin and β -lactoglobulin. It has to be noted, that the procession of milk alters the chemical structures of milk compounds. Maillard reactions, glycation and denaturations among others can occur. These processes can also affect the allergenicity of milk compounds. To date, it is very poorly understood how structural changes from food processing influence allergenicity [79, 80].

¹⁷ Radio-Allergo-Sorbent Test

1.4.2 THE HYGIENE HYPOTHESIS

From the 1970s to the 1990s, there has been a noticeable increase in the prevalence of allergic diseases. Data from epidemiological studies revealed, that having older siblings was a strong protective factor against the risk to develop hay fever. Living on a farm and the consumption of unprocessed cow's milk early in life are also protective against atopy [46]. Based on these observations, David Strachan proposed in 1989 the hygiene hypothesis claiming that an excessive cleanliness correlated with the risk of atopy [46]. In a more refined version of the hygiene hypothesis, it was assumed that due to the lack of infections at a certain developmental stage of a child, the so called "window of opportunity", Th0-cells were more likely to differentiate into Th2-cells than Th1-cells and therefore, facilitate the development of an atopic phenotype [81]. In 2004, Graham Rook took it a step further in the "*old friends*"-hypothesis, which describes our dependency on the microbiome that developed in a coevolution going on since the beginning of mankind. He stated that the composition and diversity of the microbiome inhabiting the inner and outer surfaces of the body was crucial for immune tolerance and a functional immune system. The zealous use of antiseptics and antibiotics and the western industrialized life style in general, probably aggrieved our ancestral commensal flora, which in turn, facilitates the development of atopy [82].

1.4.3 BACTERIAL LIPOPOLYSACCHARIDE (LPS) AND ITS ROLE IN ALLERGIC ASTHMA

LPS is a part of gram-negative bacterial cell walls. It was classified by Johannes Friedrich Pfeiffer as an endotoxin, but this is actually misleading, because it can also be secreted via vesicles of the outer membrane [83], which makes it an ubiquitous agent even in many sterile environments. LPS is very important to uphold the structural integrity of bacterial cell walls and also contributes in the stabilization of the bacterial membrane. Furthermore, it protects the organism against harmful chemicals. LPS has negative charges that are vital for its proper function. All LPS molecules are built out of 3 parts. Exposed to the outer surface is a glycan polymer called the O-antigen, which is unique to the specific strain of bacteria it is derived from. Interestingly, antibodies can bind the O-antigen specifically [84]. The next building block is the core oligosaccharide. It is made of sugars, e.g. 3-deoxy-D-mannooctulosonic acid and heptose

but also non-sugar compounds. The core oligosaccharide is covalently bound to the last of the 3 parts, namely Lipid A [85], which is a glucosamine disaccharide with chains of fatty acids attached to it. It also has multiple phosphorylation sites. The lipophilic fatty acid-chains are necessary to anchor the LPS molecule in the bacterial phospholipid bilayer. The other parts of Lipid A stick out the surface and are responsible for many of the toxic effects of LPS [86].

1.4.3.1 MECHANISMS OF LPS DETECTION IN HUMANS AND MICE

For LPS signal transduction of mammalian cells, two TLR-4 monomers are necessary [34]. Additionally, the molecules lymphocyte antigen 96 (often called MD-2) and CD14 have to cooperate with TLR-4, to recognize LPS and activate the signaling pathway [34]. DCs, neutrophils and macrophages express CD14, which is a phagocytic receptor for LPS or can act as accessory protein for TLR-4. MD-2 is another accessory protein that is obligatory to transport TLR-4-molecules to the cell membrane. There MD-2 binds the curved ectodomain of one TLR-4 molecule and creates a hydrophobic binding site. 5 lipid chains of LPS indirectly bind to this pocket on the first TLR-4 molecule. A sixth lipid chain and parts of the residue of the bound LPS-molecule can now bind directly on the convex part of the second TLR-4-molecule [34]. This enables dimerization of two TLR-4-monomers. That, in turn, activates a signal transduction cascade. Apparently, also other molecules such as opioids, certain fatty acids and nickel ions can activate TLR-4 signaling independently from LPS [34]. On the N-terminus of the TLR-4 molecule, is a leucine rich repeat (lrr). The lrr is attached to a transmembrane domain, which connects it to the Toll/IL-1R/resistance (TIR) domain, essential for the intracellular signal transduction [87]. The activated TLR-4-dimer interacts with its TIR domains with a number of adaptor proteins, among them, MyD88. In turn, multiple signaling pathways get activated, facilitating different responses of the cell. This often leads to inflammation [88].

1.4.3.2 LPS EFFECTS ON ALLERGIC ASTHMA

Although LPS is involved in proinflammatory signaling pathways, data from the 1990s and early 2000s indicated that LPS might be a protective factor against the development of atopy and asthma. At that time, occupational studies have already linked livestock farming to exposure of

high levels of bacterial LPS [89, 90]. The induction through TLR-signaling of the Th1-skewing cytokines IL-12 and IFN γ was a suggested mechanism for the protective properties of LPS. This seemed compatible with the hygiene hypothesis, since the immune system can detect potentially harmful gram-negative bacteria via LPS. It was also compatible with the so-called “window of opportunity” hypothesis: studies indicated a reduction in the risk for allergic diseases if the LPS-exposure happened within the neonatal or perinatal stage of development of an individual, the “window of opportunity”. If encountered within the neonatal or perinatal period of life, LPS might program the immune system towards a Th1 phenotype or tolerance. If an individual lacks this exposure, its immunity “defaults” to Th2-driven, leading to atopy [34, 89, 91, 92]. This was also supported by data derived from mouse models of asthma. In these experiments, the protective features against allergic airways disease of neonatal exposure to LPS could be demonstrated [93]. In contrast, LPS induced or worsen respiratory symptoms associated with occupational asthma, which is characteristically very severe in its clinical appearance [34, 89, 91, 92]. How these apparently paradox findings can be reconciled is still not fully understood. But a few hints were provided, when it was demonstrated that in a mouse model of asthma low doses of LPS (0.1 μ g) added to intranasally administered allergen could facilitate airway eosinophilia, whereas high doses (10-100 μ g) of LPS added to the allergen induced airway neutrophilia. Mice that did not receive any LPS at all, appeared to be immunologically tolerant to OVA [94]. Furthermore, newer studies in asthma patients linked mild and moderate asthma to eosinophilia and more severe asthma to neutrophilia [94]. Eosinophilic (mild to moderate) asthma and neutrophilic (severe) asthma can be interpreted as two different phenotypes, rather than one single phenotype that only differs in symptom severity. On the other hand, in another mouse model of allergic airway inflammation, exposure to an antibody capable of activating TLR-4 signaling and therefore mimicking LPS, before allergen sensitization and challenge was associated with downregulation of LPS-receptors resulting in endotoxin tolerance, which in turn facilitated immunological tolerance to an afterwards administered allergen [95]. These findings point to a dose dependent effect of LPS as well as to a dependence on when in the history of an individual the exposure to LPS took place. Interestingly, the house dust mite allergen Der p2 is a structural homologue to MD-2 [96] and capable of activating TLR-4 signaling autonomously in the absence of LPS [97]. Short ragweed pollen is another allergen that has been shown to have TLR-4 agonistic properties. The TLR-4 activity of allergens might add to their allergenic potential [98]. Differences in genetic backgrounds of mice have also been linked to the

differential effects of TLR-4 signaling on allergies [99]. Till this day many questions about the role of LPS in asthma remain unanswered.

1.4.4 OTHER ENVIRONMENTAL FACTORS THAT INFLUENCE ALLERGIC ASTHMA

1.4.4.1 CIGARETTE SMOKE

The countless abominable effects of tobacco smoking include dental decay and bad breath, coronary heart and artery disease, collagen damage, skin problems and impaired wound healing, osteoporosis and degenerative pathology of the locomotor system, infertility, chronically cold hands and feet due to insufficient blood circulation, cancer and numerous respiratory problems, such as emphysema and permanent loss of lung function. And this is only the tip of the iceberg. According to a statement by the WHO,

“Smoking is the most important and preventable cause of morbidity and premature mortality in the developed and developing world” [100, 101].

Besides the aforementioned adverse health effects, smoking also impacts the inflammatory status of the body, suggesting itself to promote many different chronic inflammatory diseases, including asthma. It is now widely accepted, that smoking increases the Th2-pathway, facilitates allergic sensitization and increases airway hyperresponsiveness [102]. Many studies have been conducted, that link smoking to asthma exacerbations in adults and increased severity [103, 104]. Active and passive smoking is also clearly associated with adult onset asthma in a dose dependent way [105, 106]. Maternal smoking appears to have a more pronounced impact than paternal smoking. Further prenatal exposures to the toxins of tobacco smoke have a stronger effect than postnatal exposure [102, 107]. Tobacco smoke has been shown to reduce the Th1 cytokines IFN γ and the activity of NK-cells. It also reinforces the Th2-inflammatory pathway by increasing the secretion of IL-4 and IL-5, as well as the production of several more cytokines associated with allergic diseases [108, 109].

1.4.4.2 AIR POLLUTION

There are many different kinds of air pollutants relevant to asthma. One is, of course, tobacco smoke. Another pollutant is particulate matter (PM), which is composed of chemically heterogeneous microscopic particles, mixed with tiny liquid droplets. PM can be classified by size. Abraded from soil, from construction debris, dust from roads or formed through aggregation of combustion particles, “coarse” particles range from 10 μ m – 2.5 μ m. “fine” and “ultrafine” PM mostly originate from combustion of fossil fuels, e.g. diesel. Fine PM ranges from 5 μ m – 2 μ m. Ultrafine particles are smaller than 0.1 μ m. All PM, but particularly PM made of particles smaller than 2.5 μ m easily pass through the nose and throat to ultimately arrive at the lungs. PM exposure has been linked to pulmonary inflammatory processes, decreases in lung function and exacerbations of allergic asthma in children [102, 110, 111]. PM can harm the body indirectly, as an inductor of reactive oxygen species, which are able to damage cells directly [102]. Aluminum particles found in PM are known to have adjuvant effects and can favor a Th2 immune response, with increase IL-4, and IL-5, and IgE- and IgG1-antibody formation [112]. Similarly, crystalline silica facilitates Th2-responses and isotype switching of B-cells towards IgE, and IgG1 [113]. Prenatal as well as neonatal exposure of children to diesel exhaust facilitates the development of asthma [102]. Similarly, in OVA-sensitized mice that were previously exposed to diesel exhaust developed high IgE levels in serum and had more Th2- and Th17-cytokines in their system after OVA-challenge, compared to mice without previous diesel exhaust exposure. Combined exposure of diesel exhaust and products derived from house dust mites to mice resulted in increased Th2- and Th17-cytokine secretion compared to mice that were exposed to house dust mite-products alone. Moreover, children suffering from allergic asthma in conjunction with exposure to high levels of diesel exhaust particles incurred more frequent asthma symptoms and had 5 times more IL17A in their serum, than asthmatic children with low levels of diesel exhaust exposure. The levels of IL17A are believed to correlate with asthma severity [102, 114, 115]. Pesticides are also frequent air pollutants. Some are known to increase the incidence of occupational wheezing and coughing. Pesticides can influence airway inflammation. *In utero* exposure to pesticides is also correlated with allergic asthma [102, 116, 117].

1.4.4.3 RESPIRATORY INFECTIONS

It has been generally recognized that asthmatics have an increased risk to contract bacterial or viral respiratory infections that tend to be more severe in asthmatics than non-asthmatics [118]. Moreover, it was discovered that the long thought to be sterile lower respiratory tract is actually colonized with a microflora beneficial to the host [119]. Further investigation revealed that this bacterial microflora was very likely to interact with contracted pathogens [118]. Interestingly, the presence of *Haemophilus influenzae*, *Mycobacterium catarrhalis* and *Streptococcus pneumoniae* within the respiratory microflora was positively correlated with asthma risk [120]. Over the last years, evidence has accumulated that viral lower respiratory tract infections are associated with asthma. In particular, respiratory syncytial virus (RSV) was shown to be associated with a higher risk to develop asthma, whereas rhinovirus seemed to facilitate exacerbations [118, 121-124]. Many studies now indicate, that bronchial cells from asthmatic and healthy persons react differently to viral infections [125, 126]. These differences comprise mucus production and clearance of AECs, macrophage activity and altered cytokine secretion. Usually, in asthmatic patients, the Th2-inflammatory pathway is pronounced and sometimes the IFN γ response to viral pathogens is defective [125-130]. Because of this, the asthmatic may be incapable of the elimination of viral infections, possibly leading to prolonged inflammatory pathology in the respiratory tract. This possibility was explored in several experimental models. In such, AHR and changes of the immune system were observed resembling asthma like symptoms [127, 131]. Results from post viral mouse models of airway diseases indicate that virally reprogramed AECs and also ILC2s and the activation of the iNKT-macrophage-IL-13-axis to be possible underlying mechanisms of the persistent physiological changes [39, 44, 53, 132]. RSV infection in mice has been demonstrated to compromise Treg function through induction of GATA-3. These Tregs established a Th2-cytokine environment and their tolerogenic capabilities were attenuated [133]. Additionally, Th17 cytokines were found to be elevated in RSV infected asthmatic patients compared to asthmatic patients without RSV-infection [134] and increased deposition of collagens and endothelial growth factors were observed in cultured human, RSV-infected AECs [135]. Studies on antiviral agents such as pavilizumab, an IgG-type antibody against the fusion F glycoprotein of RSV and the antiviral drug ribavirin also link the treatment of viral infections to a reduction in recurrent wheezing in children [136, 137].

1.5 MOUSE MODELS OF ALLERGIC ASTHMA

Although horses can suffer from the heaves and cats from eosinophilic bronchiolitis, conditions that are very similar to asthma, one could argue, that in nature only humans develop true asthma [138-141]. This presents the researcher with fundamental difficulties to find a suitable host organism to study the disease as a whole. However, since it is possible to induce asthma like symptoms in numerous animals, it is possible to study aspects of asthma in animal models to get a better understanding of the underlying mechanisms [142, 143]. Not least thanks to these efforts, our understanding of immunological pathways has vastly improved over the years [144]. Many of these advances are due to studies in mouse models, which also transferred to asthma. Mice are cheap and easy to handle and maintain. They can be kept under very well controlled conditions. This makes them suitable for animal experiments. But one has to bear in mind, that the data derived from such studies, although valuable, cannot be applied to human diseases without critical assessment. The respiratory tract of the mouse is set up differently than in humans, which can lead to differences in pulmonary conduct. There are discrepancies between the human and murine immune cells, molecules and all their functions [141]. Furthermore, there is striking differences in the magnitude of immunological reactions between distinct mice strains to identical stimuli [145, 146]. On the other hand, these differences in immunological reactions could be a chance to study the influence of genetic variability on the immune system. BALB/c mice are most often chosen for today's mouse models of asthma. The strain is known for its propensity to Th2 biased immune reactions. C57/BL6 although less likely to react in this way are also frequently used [147, 148].

1.5.1 ACUTE MODELS BASED ON INTRAPERITONEAL IMMUNIZATION AND AEROSOL CHALLENGE

Most protocols start by sensitization to an antigen. For this, the mice can be intraperitoneally injected with the desired antigen, combined with an adjuvant. The adjuvant is usually alum, because it is known to enhance phagocytosis and facilitates a Th2-biased immune response, depending on the route of administration [149, 150]. After successful sensitization, the mice are repeatedly “challenged” by exposure to the same antigen on different days. The mice inhale the

antigen and develop inflammation in their lungs. One day after the last challenge-procedure, peribronchially located inflammation and goblet cell hyperplasia of the airway epithelial mucosa as well as antigen-specific antibodies in their blood and short temporary AHR can be observed [151, 152]. These physiological changes resemble events typically observed during the acute asthma attacks in humans and therefore allow to study these aspects of the disease [147].

1.5.2 ACUTE MODELS BASED ON EXCLUSIVE RESPIRATORY DELIVERY OF ALLERGENS

An alternative protocol employs only multiple intranasal administrations of the desired antigen dissolved in phosphate buffered saline, for sensitization and challenge as well. Results of this induce a similar type of reaction in mice like the intra peritoneal sensitization and aerosol challenge [153]. Moreover, this approach allows a more specific delivery of the allergen to the respiratory tract via the route of the nasal and bronchial mucosa, making this model is particularly important for investigation of mechanisms that lead to allergic asthma initiation. [154]. Another advantage of this approach is that there are less expenditures on special equipment than for the aerosol challenge-approach.

1.5.3 CHRONIC MODELS

Chronic models of allergic asthma were developed to study certain characteristics of the disease that are absent in acute models of allergic asthma. In these models, mice are usually intraperitoneally sensitized to an allergen with an adjuvant and then repeatedly exposed to rather small amounts of the allergen for up to 12 weeks. This allows the development of persistent AHR and airway remodeling to study these aspects of allergic asthma [155-157].

1.6 AIM OF THIS STUDY

Previous studies conducted in our lab indicated, that intranasal application milk or OVA induced eosinophilic airway inflammation in BALB/c mice of comparable type and magnitude. On the other hand, intranasal challenge with milk induced a more severe eosinophilic airway inflammation than OVA in C57BL/6 mice. One of differences between OVA and milk used in our studies was amount of LPS, with OVA containing at least 200 times higher contamination than the powdered milk. Thus, we hypothesized that the high amounts of LPS contamination of the OVA was responsible for a suppression of the induction of airway eosinophilia in C57BL/6 mice to OVA and that BALB/c mice less sensitive to the suppressive effects of LPS [158]. The aim of this study was to 1) reproduce the previous findings of the response of BALB/c and C57BL/6 mice to intranasal administrations of OVA and powdered milk: 2) to examine the potential of LPS as an immunological modulator in milk-challenged BALB/c mice and OVA-challenged C57BL/6 mice. Specifically, we tested A) whether in BALB/c mice addition of ascending doses of LPS changes the immune response to milk and B) whether addition of ascending doses of LPS to LPS-free OVA induce allergic inflammation comparable to milk challenge in C57BL/6 mice.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 MICE USED FOR ALL EXPERIMENTS:

- 4 weeks old female BALB/c mice purchased from Charles River, Germany
- 4 weeks old female C57B6 mice purchased from Charles River, Germany

2.1.2 ANESTHETICS:

- Ketanest S 25mg/ml (Pfizer, Esketamin-hydrochlorid, Cat# 0036126, ONP002-1)
- Rompun 2% (Bayer, Xylazin-hydrochloride, Ch-B KPO3L8D)
- Demineralized water (Apotheke AKH Wien)

2.1.3 MATERIALS FOR SENSITIZATION AND CHALLENGE:

- 10xPBS, Gibco, Cat# 12400
- 1xPBS Gibco, Cat# 14190
- Anesthetic to sleep, prepared as described above
- Bacterial lipopolysaccharide (LPS) for all studies: Lipopolysaccharide from Escherichia coli 011:B4, Sigma, Cat# L4391-1MG, Lot#:0664142
- Milk for all studies: Fixmilch®, Magermilchpulver, unter 1.5% Fett, Maresi, Lot# 4251
- Needles 0.6mm (BD Microlance™ 3)
- Ovalbumin (OVA) for all studies: Albumin from chicken egg, Sigma, Cat# A5503-5G, Lot#: 038K7012
- Pipettes and tips for intranasal challenge
- Solutions/suspensions for sensitization/challenge
- Syringes 1ml (Soft-Jekt, Lot# OD190048)

2.1.4 SOLUTIONS FOR INTRANASAL ADMINISTRATION OF ALLERGENS:

- OVA (uncleaned, LPS content as declared by the manufacturer)
- OVA cleaned from LPS with LPS removal columns (cleaned OVA)
- Solution of LPS in 1xPBS, concentration: 1µg LPS/50µl in PBS
- Solution of 1µg of cleaned OVA /µl with added 10⁻⁴µg LPS/50µl in PBS
- Solution of 1µg/µl cleaned OVA with added 10⁻³µg LPS/50µl in PBS
- Solution of 1µg/µl cleaned OVA with added 10⁻²µg LPS/50µl in PBS
- Solution of 1µg/µl cleaned OVA with added 10⁻¹µg LPS/50µl in PBS
- Solution of 1µg/µl cleaned OVA with added 1µg LPS/50µl in PBS
- Solution of 1µg/µl powdered milk in PBS
- Solution of 1µg/µl powdered milk with added 10⁻⁵µg LPS/50µl in PBS
- Solution of 1µg/µl powdered milk with added 10⁻⁴µg LPS/50µl in PBS
- Solution of 1µg/µl powdered milk with added 10⁻³µg LPS/50µl in PBS
- Solution of 1µg/µl powdered milk with added 10⁻²µg LPS/50µl in PBS

2.1.5 MATERIALS FOR THE ASSESSMENTS:

- 4% paraformaldehyde solution
- 70% Ethanol, Apotheke AKH Wien,
- Buckets with ice
- Cassettes for the lungs (Simport)
- Corkboards
- Cytospin 4 centrifuge (Thermo Shandon; centrifugation at 800 rpm for 3 min).
- DiffKwik kit (Thermo Scientific Shandon, Cat# 9990700)
- 1.5ml Eppendorf-Tubes
- Needles 0.6 mm (BD Microlance™ 3)
- Pins, scissors, forceps, clip
- butterfly infusion set
- Syringes 1ml (Soft-Jekt)
- Tissue paper

2.1.6 MATERIALS FOR TOTAL AND DIFFERENTIAL CELL COUNTS IN BAL:

- Air-dried cytospin slides
- Demineralized water
- DIFFKWIK staining set, Thermo Electron Corporation, Cat#: 9990700
- 4 glass chambers
- Trypan blue Trypan blue solution 0.4% Sigma,

2.1.7 MATERIALS FOR H&E-, PAS- AND LUNA- STAINING:

- Paraffin
- Microtome used for tissue thin cuts: Microm HM400R
- 5% (wt/vol) periodic acid
- 1% (vol/vol) HCl water,
- 50% ethanol
- 70% ethanol
- Eosin Y: (Eosin Y solution alcoholic, Sigma, Cat# HT110132-1L)
- Ethanol Absolute (AustroAlco Österr. Alkolhandel GmbH, 2104 spillern. 5L bottle)
- Fuming HCl (HCl, 37% Merck, Ref: K32970717 409, 1.00317. 1000)
- Haematoxylin powder (Merck, Cat. 1.05680.0250)
- Haematoxylin (Sigma Aldrich Haematoxylin solution, 1LSigma Aldrich Chemie GmbH, Gill No. 3, Ref: GHS 332)
- Iron (III) chloride hexahydrate (Sigma, Cat. F2877-250G, Batch Nr. 035 KO 180)
- Lithium carbonate (Merck, Cat. 1.05680.0250)
- Mix 175 ml of absolute ethanol and 75 ml of demineralized water
- Mounting media: Eukitt (O. Kindler GmbH and Co., Germany)
- N-butyl acetate (Merck, Ref: 1.09652.1000)
- Papanicolaus solution 1a Harris Haematoxylin solution (Merck, Ref: OB546994, 1.09253.0500)
- Periodic acid (FLUKA Chemika, Ref: 380218/1 12299, 77310, 25g)
- Ponceau S practical grade (Sigma, Cat. P3504-10G, Lot. 067K0098)

- Schiff's reagent (Merck, Ref: OB04602, 1.09033.0500)
- Sodium disulfite (Merck, Ref: K36593928 684, 1.06528. 1000),
- Xylene (Fluka, Chemika, Ref: 95690)

2.1.7.1 SOLUTIONS THAT HAD TO BE FRESHLY PREPARED FOR H&E-, PAS- AND LUNA- STAINING:

- SO₂ water
- 1% acid alcohol solution in 70% ethanol
- 1% haematoxylin solution:
- 30% ferric chloride solution:
- 1% Ponceau S solution (Biebrich Scarlet solution):
- Lithium carbonate solution:
- Haematoxylin-Biebrich scarlet solution:

2.1.8 MATERIALS FOR ELISA:

- Anti IgE, Southern Biotech, cat#:02122D, lot#:M047644
- Anti IgG1, Southern Biotech, cat#:1070-08, lot#:H017-9287
- Anti IgG2a, Southern Biotech, cat#:1080-08, lot#:F456
- Demineralized water, AKH Wien Apotheke, lot#: 138465
- Bottles for mixing and storing solutions
- Bovine Serum Albumin (BSA), Sigma-Aldrich, Cat# A2153-100G
- Control samples, kindly provided by Berislav Bosnjak
- ELISA plates: Iwaki ELISA 96 well plate, flat bottom, cat#: 38010-096
- 1.5ml Eppendorf tube
- Ice buckets, ice
- NaOH, Merc, cat#: 9137
- OVA solution (10µg/mL)
- PBS (x1)

- PBS (x10)
- Pipette tips
- Pipettes
- Plastic wells for use with a multipipette
- Plate plastic covers
- Stop solution: 2N H₂SO₄, Roth, Ch.050152977 prepared as needed
- Streptavidin-HRP, Southern biotechnology associates Inc. USA, in 1:8000 (in 1%BSA)
dilution lot#:A2805BA2053
- Styrol boxes for covering the plates from light while
- TMB Substrate (3,3', 5,5'-tetramethylbenzide Cat# 555214)
- Tris, Tris (hydroxymethyl)-aminomethan, MP Biomedicals, cat#: 819623
- Tween 20, Bio-Rad, Cat# 170-6531
- Washing buffer 1L contains 10mL 0.1% Tween 20 BIO-RAD
- Opsys MR (Dynex Technologies)

2.2 METHODS:

2.2.1 PREPARATION OF ANESTHETIC TO SLEEP:

To prepare anesthetic for intranasal administrations to mice, 10mL of Ketanest solution were put in a falcon tube and 0.75mL of Rompun 2% solution were added. Then autoclaved water was added to a volume of 25mL. The falcon tube was afterwards turned multiple times to properly mix the solutions. To put the mice to sleep, 0.1mL of this anesthetic was injected intraperitoneally per mouse, giving final dose of 0.04mg of Ketanest/g and 0.00012mg of Rompun/g. An average weight of 25g/mouse was assumed.

2.2.2 PREPARATION OF TERMINAL ANESTHESIA:

At first, 10mL of Ketanest solution were put in a falcon tube. 1.50mL of Rompun 2% solution were added. Then autoclaved water was added to a volume of 25mL. The falcon tube was afterwards turned multiple times to properly mix the solutions. To kill mice, 0.5mL of this anesthetic was injected intraperitoneally per mouse, giving final dose of 0.2mg of Ketanest/g and 0.0006mg of Rompun/g. An average weight of 25g/mouse was assumed.

2.2.3 PREPARATION OF SOLUTIONS THAT HAD TO BE FRESHLY PREPARED FOR H&E-, PAS- AND LUNA- STAINING:

- 1% (vol/vol) HCl water

Preparation: 2.5ml of fuming HCl were added to 248.5 ml of demineralized water

- SO₂ water

Preparation: 1g of Na₂SO₂O₅ were dissolved in 200ml demineralized water. Then 1920μl of fuming acid were added.

- 1% acid alcohol solution in 70% ethanol

Preparation: 175 ml of absolute ethanol were mixed with 75 ml of demineralized water, and then 2 ml of fuming acid were added.

- 1% haematoxylin solution:

Preparation: 1g of haematoxylin were dissolved in 100 ml of 95% ethanol solution.

- 30% ferric chloride solution:

Preparation: 2g of Iron (III) chloride hexahydrate were dissolved in 6.7 ml of demineralized

- 1% Ponceau S solution (Biebrich Scarlet solution):

Preparation: 0.3 g of Ponceau S were dissolved in 30 ml of demineralized water

- Lithium carbonate solution:

Preparation: Dissolve 0.5 g of Lithium carbonate in 500 ml of demineralized water by mixing on a magnetic stirrer.

- Haematoxylin-Biebrich scarlet solution:

Preparation: The following compounds were mixed in this order: 95ml of demineralized water, 4ml of 30% ferric chloride solution, 100ml of 1% haematoxylin solution through a filter, 22.5ml of 1% Ponceau S solution through a filter and 1ml of fuming acid.

2.2.4 ADMINISTRATION OF ALLERGENS FOR SENSITIZATION AND CHALLENGE:

On days 0, 2, 4, 14, 16 and 18 of each experiment, challenge solutions were administered to the mice with a pipette intranasally according to the respective table of the experiment as shown below. For the administration, each mouse was anesthetized with intraperitoneal (i.p.) administration of 0.1mL of anesthesia to sleep. After waiting for the mice to fall asleep, each mouse was held in an upright position while the respective fluid for sensitization and challenge was pipetted on one nostril. Each mouse was carefully watched to soak in the fluid and to recover normal breathing patterns. Afterwards, the mouse was put back in its cage. Then, seventy-two hours after the last allergen challenge, on day 21 all mice were sacrificed via injection of terminal

anesthetic to perform cardiac blood puncture, bronchoalveolar lavage fluid collection and lungs dissection. On day 21 (seventy-two hours after the last allergen challenge), all mice were sacrificed via injection of terminal anesthetic to perform cardiac blood puncture, bronchoalveolar lavage fluid collection and lungs dissection.

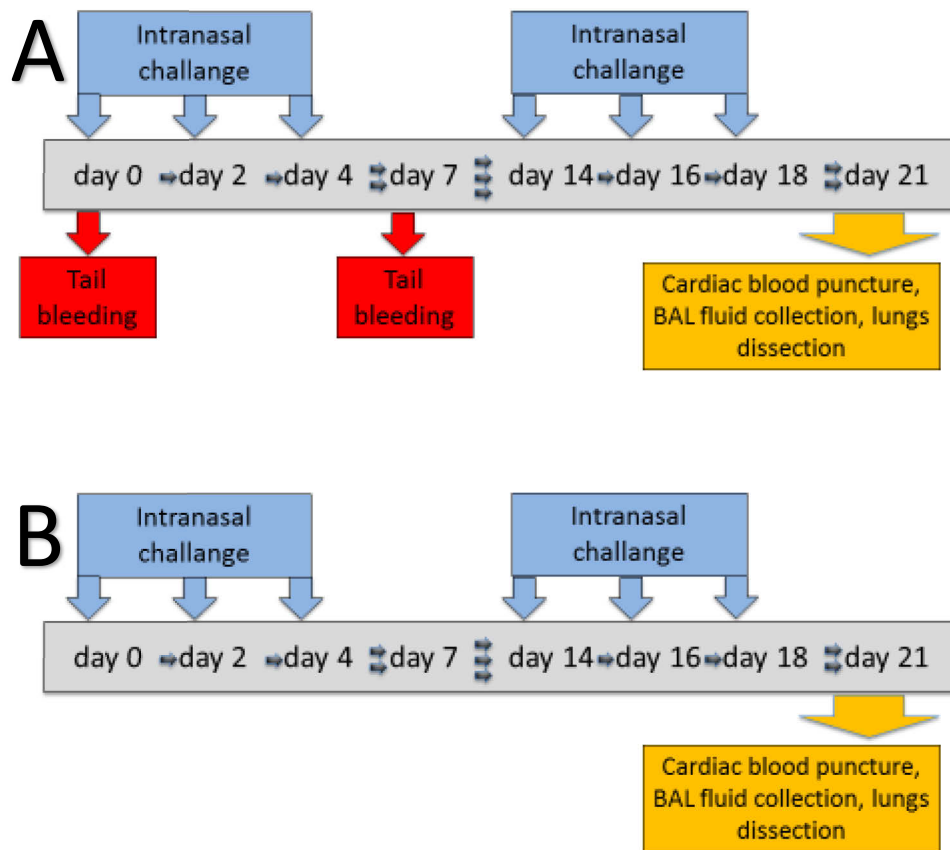


Figure 2.1 Timeline of the administration of allergens in the experiments:

A: “Immune response to OVA and milk in BALB/c and C57BL/6 mice”.

B: “Allergic responses to milk with added LPS in doses from $10^{-2}\mu\text{g}$ to $1\mu\text{g}$ ”

“Allergic responses to milk with added LPS in doses between 10^{-4} and $10^{-1}\mu\text{g}$ ”

“Comparison between the immunological response of C57BL/6 mice to milk, OVA, and LPS depleted OVA with added doses of LPS”

2.2.4.1 IMMUNE RESPONSE TO OVA AND MILK IN BALB/C AND C57BL/6 MICE

Either 50µg of OVA or 50µg of milk dissolved in 50µl of PBS were administered to either BALB/c or C57BL/6 mice respectively. As a control, BALB/c as well as C57BL/6 mice received 50µl of PBS alone. Each group comprised of 12 mice except the groups that received PBS which were comprised of 5 mice. Additionally, blood from the tail was extracted from all mice on days 0 and 7 of the experiment (Figure 2.1 A).

Table 2.1 Administration scheme of OVA or milk to BALB/c and C57BL/6 mice. As a control, BALB/c as well as C57BL/6 mice received PBS.

Mice strain	BALB/c			C57BL/6		
Allergen challenge	50 µl PBS	50µg OVA	50µg milk	50 µg PBS	50µg OVA	50µg milk
Number of mice	5	12	12	5	12	12

2.2.4.2 ALLERGIC RESPONSES TO MILK WITH ADDED LPS IN DOSES FROM 10⁻²µG TO 1µG

Either 1µg of LPS, 50µg of milk, 50µg of milk with added 10⁻²µg of LPS, 50µg of milk with added 10⁻¹µg of LPS or 50µg of milk with added 1µg of LPS, dissolved in 50µl of PBS were administered to BALB/c mice. As a control, BALB/c mice received 50µl of PBS alone. Each group comprised of 7 mice except the group that received PBS, which was comprised of 4 mice.

Table 2.2 Administration scheme of milk with added incremental doses of LPS ranging from 10⁻²µg of LPS to 1µg of LPS. The control group received 50 µl of PBS or 1µg of LPS.

Mice strain	BALB/c					
Allergen challenge	50 µl PBS	1µg LPS	50µg milk	50µg milk + 10 ⁻² µg LPS	50µg milk + 10 ⁻¹ µg LPS	50µg milk + 1µg LPS
Number of mice	4	7	7	7	7	7

2.2.4.3 ALLERGIC RESPONSES TO MILK WITH ADDED LPS IN DOSES BETWEEN 10^{-4} AND $10^{-1}\mu\text{G}$

Either 50 μg of OVA, 50 μg of milk, 50 μg of milk with added $10^{-4}\mu\text{g}$ of LPS, 50 μg of milk with added $10^{-3}\mu\text{g}$ of LPS, 50 μg of milk with added $10^{-2}\mu\text{g}$ of LPS or 50 μg of milk with added $10^{-1}\mu\text{g}$ of LPS, dissolved in 50 μl of PBS were to BALB/c mice. As a control, BALB/c mice received 50 μl of PBS alone. n=7.

Table 2.3 Administration scheme of milk with added incremental doses of LPS ranging from $10^{-4}\mu\text{g}$ of LPS to $1\mu\text{g}$ of LPS. The control group received 50 μl of PBS or 50 μg OVA.

Mice strain	BALB/c						
Allergen challenge	50 μl PBS	50 μg OVA	50 μg milk	50 μg milk + $10^{-4}\mu\text{g}$ LPS	50 μg milk + $10^{-3}\mu\text{g}$ LPS	50 μg milk + $10^{-2}\mu\text{g}$ LPS	50 μg milk + $10^{-1}\mu\text{g}$ LPS
Number of mice	7	7	7	7	7	7	7

2.2.4.4 COMPARISON BETWEEN THE IMMUNOLOGICAL RESPONSE OF C57BL/6 MICE TO MILK, OVA, AND LPS DEPLETED OVA WITH ADDED DOSES OF LPS

Either 50 μg of OVA, 50 μg of milk, cleaned OVA, cleaned OVA with added $10^{-5}\mu\text{g}$ of LPS, cleaned OVA with added $10^{-4}\mu\text{g}$ of LPS, cleaned OVA with added $10^{-3}\mu\text{g}$ of LPS or cleaned OVA with added $10^{-2}\mu\text{g}$ of LPS dissolved in 50 μl of PBS were to C57BL/6 mice. As a control BALB/c mice received 50 μl of PBS alone. Each group comprised of 7 mice except the group that received PBS which were comprised of 6 mice (one mouse died before the end of the experiment).

Table 2.4 Administration scheme of cleaned OVA with added incremental doses of LPS ranging from 10^{-5} μ g of LPS to 10^{-2} μ g of LPS. The control group received 50 μ l of PBS, 50 μ g of milk or 50 μ g OVA.

Mice strain	C57BL/6						
Allergen challenge	50 μ l PBS	50 μ g Milk	50 μ g OVA	50 μ g cleaned OVA + 10^{-5} μ g LPS	50 μ g cleaned OVA + 10^{-4} μ g LPS	50 μ g cleaned OVA + 10^{-3} μ g LPS	50 μ g cleaned OVA + 10^{-2} μ g LPS
Number of mice	6	7	7	7	7	7	7

2.2.5 ASSESSMENT OF MICE

2.2.5.1 COLLECTION OF BLOOD, BAL AND LUNGS

Seventy-two hours after the last challenge, mice were terminally anesthetized with i.p. administration of 0.5mL of terminal anesthesia. For further processing, the mice were restrained on corkboards and sprayed with 70% ethanol.

2.2.5.2 CARDIAC BLOOD PUNCTURE:

After the mice have been anesthetized, cardiac blood puncture was performed. For this, the skin of each mouse was cut from the abdomen to the trachea. The skin was then pulled aside to expose the chest and the trachea. Afterwards, blood from the heart was extracted with a syringe needle. The blood was then collected in a 1.5ml Eppendorf tube for further processing. To remove clotting factors from the blood, the samples were kept on RT for at least 3 hours and then spinned in a centrifuge at 13.000 rpm for 10 min. The supernatants were collected to obtain the sera of the mice.

2.2.5.3 BRONCHOALVEOLAR LAVAGE FLUID COLLECTION:

After the cardiac blood puncture, the neck glands of each mouse were pulled aside to expose the trachea. The trachea was then nicked, so that a catheter could be inserted and fixed with a clamp. Lungs were then flushed with 0.4 mL of PBS. After waiting for about 5 seconds, the fluid was soaked back inside the syringe. The collected BAL fluid was stored in a 1.5ml Eppendorf tube. This process was repeated twice more, each time with 0.3 mL of PBS. The Eppendorf tube with the BAL was temporarily stored in bucket of ice for further processing. The syringe and catheter was then washed twice with 1mL PBS for reuse.

2.2.5.4 LUNGS DISSECTION:

After BAL fluid collection, the lungs were filled with 4% buffered neutral formalin fixative solution through the previously fixed catheter for inflation and fixation of the lungs. The chest bones of each mouse were cut on both sides for easy lung removal. Then the lungs were put into a plastic cassette. The filled cassettes were put in 4% buffered neutral formalin fixative solution for 24 hrs.

The formalin fixed lungs were then embedded in paraffin, frozen at -20°C and cut to 3µm slices. The 3µm thick cuts were then put on glass microscope slide in pairs for histological staining.

2.2.6 ANALYSIS OF TOTAL CELLS IN BAL FLUID:

To enumerate cells, I mixed 50µl of the cell suspension from the BAL fluid with 50µl of a trypan blue solution in 96 well plate. An appropriate amount of the mixture was put in a Neubauer chamber to count total cells under a microscope using 10x magnification. If the cell number was too high for efficient counting, the BAL fluid was diluted appropriately.

2.2.7 DIFFERENTIAL CELL COUNTS IN BAL VIA DIFFKWIK STAINING

Using the estimated number of cells in the cell suspension, the cell suspensions were diluted so that 10^5 were in 200 μ l of BAL fluid. BAL-cell cytospins were prepared in a Cytospin 4 centrifuge at 800 rpm for 3 min. BAL cytospins were then stained with DiffKwik staining according to protocol.

This staining allows differential cell identification and counting of the cells. Lymphocytes, erythrocytes, macrophages, eosinophils and neutrophils from BAL fluid can be identified with a microscope after the staining. The cytospins with the BAL-cells were put in slide racks. These racks were dipped in Solution 1 from the DiffKwik staining kit 10 times for 1 second each dip. Excess stain was soaked up with tissue paper afterwards. Then the racks were dipped in Solution 2 from the DiffKwik staining kit 10 times for 1 second each dip. Excess stain was soaked up with tissue paper afterwards. Then the racks were dipped in Solution 3 from the DiffKwik staining kit 10 times for 1 second each dip. Excess stain was soaked up with tissue paper afterwards. Afterwards, cytospin slides were washed by dipping the slides 3 times in demineralized water. Then the slides were left to dry on air. After drying, the slides were examined with a microscope. Differential BAL-cell counts of each mouse, of Lymphocytes, erythrocytes, macrophages, eosinophils and neutrophils from BAL fluid were performed, using the estimated total cell numbers from total cell count of trypan blue stained cells.

2.2.8 PREPARATIONS OF TISSUE SAMPLES FOR H&E, PAS AND LUNA STAINING:

Microscopic slides with 3 μ m thick cuts were placed in a slide holder rack and incubated in an oven at 65°C for 40 minutes to melt the paraffin on the tissue samples. The racks were afterwards put into o-xylene for 10 min to remove the paraffin from the tissue. This step was repeated with fresh o-xylene to properly remove the paraffin. Then the slides were put into 100 % ethanol for 1 min, afterwards into 70 % ethanol for 1 min and then into 50 % ethanol for 1 min. The slides were then rinsed with demineralized water for 1 min, and then incubated in fresh demineralized water for another 9 minutes on a mixing plate. This step is rehydrating the tissue samples.

2.2.9 HAEMATOXYLIN AND EOSIN (H&E)–STAINING OF 3µm LUNG TISSUE CUTS:

After removal of paraffin and rehydration, the slides are put into haematoxylin for 5 minutes. Then the slides were carefully rinsed with tap water to remove excess stain. Next, the slides were put into 1% hydrochloric acid for only 3 to 5 seconds. This ensures, that the tissues are not over stained and transparent. Then the acid was washed off the tissues by leaving them into tap water for 5 minutes. This step changes the colour of the tissue. For fixation of the colour, the slides were then put into demineralized water for another 5 minutes. Afterwards, the tissues were dehydrated again by putting them into 50% ethanol for 30 seconds, followed by putting them into 70% ethanol for 30 seconds. Then slides were put into Eosin Y for 30 seconds. Then the slides were quickly dipped into tap water in order to prevent Eosin over staining. Then another dehydration was performed by putting the slides into 50% ethanol for 30 seconds, followed by 70% ethanol for 30 seconds and 100% ethanol for 30 seconds. Afterwards, the tissue samples were left to dry on air. After drying, mounting media was put on the tissue samples and cover slips were placed on the tissue samples. When the mounting media was hardened, the slides were ready for examination with a microscope.

2.2.10 PAS–STAINING OF 3µm LUNG TISSUE CUTS:

After removal of paraffin and rehydration, slides were put in 0.5% periodic acid for 10 minutes. Then, the tissue samples were washed by dipping in them in demineralized water 5. Then the Tissue samples were put into cold Schiff's reagent. Afterwards, the slides were put in an oven at 60°C for 30 minutes. After that, the tissue samples were put into SO₂ water for 6 minutes to fix the stain. After fixation, the slides were put into tepid hot tap water for 15 minutes while the water was changed multiple times within the 15 minutes. Afterwards, the slides were put in Papanicolaus solution 1a Harris haematoxylin solution for 19 seconds. After that the slides were dipped in tap water and the water was changed frequently to remove excess Eosin. The washing was stopped, when no more blue colour came out of the tissue samples. After that, the samples were dehydrated by putting them into 50% ethanol for 5 minutes, followed by 70% ethanol for 5 minutes and lastly 100% ethanol for 5 minutes. Afterwards, the slides were put into in n-butyl acetate for 5 minutes. Afterwards, the tissue samples were left to dry on air. After drying,

mounting media was put on the tissue samples and cover slips were placed on the tissue samples. When the mounting media was hardened, the slides were ready for examination with a microscope.

2.2.11 LUNA-STAINING OF 3 μ m LUNG TISSUE CUTS:

After removal of paraffin and rehydration, the slides were put into working solution for 7 minutes and 30 seconds. Then they were dipped into 1% acid alcohol solution 3 times. Afterwards, the slides were washed in tepid hot tap water for 5 minutes under running tap water and then in a vessel for another 5 minutes. Then the tissue samples were dipped into a lithium carbonate solution. After they changed color from pink to blue the slides were put once more into the lithium carbonate solution and then washed with tap water. After that, the slides were washed again in fresh tepid hot tap water for 5 minutes under a running tap water and then in a vessel for 5 more minutes. Following was a dehydration step by putting the slides into 50% ethanol for 10 minutes, then 70% ethanol for 10 minutes and lastly into 100% ethanol for 10 minutes. Afterwards, the tissue samples were left to dry on air. After drying, mounting media was put on the tissue samples and cover slips were placed on the tissue samples. When the mounting media was hardened, the slides were ready for examination with a microscope.

2.2.12 GRADING OF H&E STAINED LUNG SECTIONS:

H&E staining allows the assessment of the cell infiltrates in lung parenchyma, airways and near the blood vessels.

The lung inflammation was evaluated using the following grading system:

0: no inflammatory infiltrates.

1: inflammatory infiltrates around the central airway area

2: medium inflammatory infiltrates, reaching from the central area to the middle airways

3: extensive inflammatory infiltrates reaching from the central area to the periphery airways

2.2.13 RANKING OF PAS STAINED LUNG SECTIONS:

PAS staining allows the evaluation of mucus production in the central airways.

The mucus production was evaluated using the following ranking system:

All PAS stained slides were assessed.

The slides with the most mucus producing cells were assigned the rank 3.

Healthy mice were assigned a rank of 0.

All other mice were ranked between 0 and 3 according to their mucus production.

2.2.14 EVALUATION LUNA STAINED LUNG SECTIONS:

LUNA staining specifically marks the granula of eosinophils red, allowing to count eosinophils within the lung sections. Therefore, LUNA staining allows the evaluation of eosinophilic inflammation. For this, 10 random microscope screens using 40x magnification were selected and all eosinophils were counted on these screens. The numbers were then added up. The sums were used for comparison of eosinophilic inflammation in the tissue.

2.2.15 ELISA FOR MILK SPECIFIC IGG1, IGG2A AND IGE:

On day 1 of the experiment, milk coating solution was prepared according to the specifications listed above. Then 50µl of the milk coating solution was pipetted in each well of a 96 well maxisorb plate. The plates were stacked and sealed with a plastic cover and then stored in a fridge over night at 4°C. On day 2 of the experiment, the washing- and blocking-buffers for milk ELISA were prepared according to the specifications listed above. Then all plates were washed with the milk washing buffer 3 times. Afterwards, 200µl of the milk blocking buffer were pipetted in each well of each plate. The plates were stacked and sealed with a plastic cover and then put on shaker plate (I don't know the manufacturer, model...), set to speed 2.5 for 2h at room temperature. While the plates were incubating, previously prepared sera of mice were diluted 1:50 with milk blocking buffer for IgG1-detection and IgG2a-detection but were 1:5 diluted for IgE-detection respectively. Then the plates were washed 3 times with milk washing buffer. Afterwards, 80µl of milk blocking buffer were put in each well of the plates, except the

wells of the first row of each plate. 100 µl of the previously prepared serum dilution were then put in the referring wells of the first rows of the plates according to scheme A and then mixed with the pipette. Then, 20µl from each well of the first of the plate were put in following row and then mixed with a pipette. This process was repeated consecutively until the row of the plate before the last row. The last 20µl of the dilutions were discarded and not put into the last row. Therefore, the last row was left out and didn't receive dilutions. Through this procedure, a 1:5 dilution gradient of the mice sera was created from row to row. The plates were stacked and sealed with a plastic cover. Then, the plates were incubated in a fridge at 4°C over night. On day 3 of the experiment, the plates were washed 3 times with milk washing buffer. Solutions of anti IgG1, anti-IgG2a and anti-IgE-antibodies were prepared according to specifications above. Then, 100µl of either one of these solutions were pipetted in each well according to scheme A. Afterwards, the plates were incubated at room temperature for 2 hours. Within the 2 hours a solution of Streptavidin-Horseradish-Peroxidase was prepared according to specifications above. After the 2 hours, each plate was washed 3 times with milk wash8ing buffer. Then 100µl of SA-HRP were pipetted in each well and the plates were afterwards incubated for 1 hour at room temperature. Within this hour TMB and stop solution were prepared according to specifications above. Then the plates were washed 3 times with milk washing buffer. Afterwards, 100µl of TMB were pipetted in each well of each plate. A timer was set to 10 minutes and the plates were put into a light depraved environment. After the 10 minutes, 100µl of the stop solution was pipetted into each well of each plate. Finally, the plates were read with an ELISA a reader at 450 nm.

2.2.16 ELISA FOR OVA SPECIFIC IGG1, IGG2A AND IGE:

On day 1 of the experiment, OVA coating solution was prepared according to the specifications listed above. Then 50µlof the OVA coating solution was pipetted in each well of a 96 well maxisorb plate. The plates were stacked and sealed with a plastic cover and then stored in a fridge over night at 4°C.

On day 2 of the experiment, the serum-, washing- and blocking-buffers for OVA ELISA were prepared according to the specifications listed above. Then all plates were washed with the washing buffer 3 times. Afterwards, 200 µl of the OVA-blocking buffer were pipetted in each well of each plate. The plates were stacked and sealed with a plastic cover and then put on shaker

plate (I don't know the manufacturer, model...), set to speed 2.5 for 2h at room temperature. While the plates were incubating, previously prepared sera of mice were diluted 1:500 with serum-buffer for IgG1-detection and IgG2a-detection but were 1:5 diluted for IgE-detection respectively. Then the plates were washed 3 times with the washing buffer. Afterwards, 80µl of the serum-buffer were put in each well of the plates, except the wells of the first row of each plate. 100 µl of the previously prepared serum dilution were then put in the referring wells of the first rows of the plates according to scheme A and then mixed with the pipette. Then, 20µl from each well of the first of the plate were put in following row and then mixed with a pipette. This process was repeated consecutively until the row of the plate before the last row. The last 20µl of the dilutions were discarded and not put into the last row. Therefore, the last row was left out and didn't receive dilutions. Through this procedure, a 1:5 dilution gradient of the mice sera was created from row to row. The plates were stacked and sealed with a plastic cover. Then, the plates were incubated in a fridge at 4°C over night. On day 3 of the experiment, the plates were washed 3 times with the washing-buffer. Solutions of anti IgG1, anti-IgG2a and anti-IgE-antibodies were prepared according to specifications above. Then, 100µl of either one of these solutions were pipetted in each well according to scheme A. Afterwards, the plates were incubated at room temperature for 2 hours. Within the 2 hours a solution of Streptavidin-Horseradish-Peroxidase was prepared according to specifications above. After the 2 hours, each plate was washed 3 times with the washing buffer. Then 100µl of SA-HRP were pipetted in each well and the plates were afterwards incubated for 1 hour at room temperature. Within this hour TMB and stop solution were prepared according to specifications above. Then the plates were washed 3 times with the washing buffer. Afterwards, 100µl of TMB were pipetted in each well of each plate. A timer was set to 10 minutes and the plates were put into a light depraved environment. After the 10 minutes, 100µl of the stop solution was pipetted into each well of each plate. Finally, the plates were read with an ELISA reader at 450nm.

2.2.17 STATISTICAL ANALYSIS AND GRAPHS

GraphPad Prism5 was used for visualization and statistical analysis of all shown data. Data sets are expressed as means $\pm$ SEM. Significant differences between groups were assessed via One-Way-Anova. p-values lower than 0.05 were considered as significant differences.

3 RESULTS

3.1 IMMUNE RESPONSE TO OVA AND MILK IN BALB/C AND C57BL/6 MICE

Previous experiments conducted in our lab indicated that the immunological response in the airways and lungs of C57BL/6 mice differs depending on whether OVA or milk was used as a test allergen in the intranasal mouse model of allergic asthma. In contrast, this specific differential response to OVA or milk seen in C57BL/6 mice was not observed in BALB/c mice. This suggests that the genetic differences between these two mice strains can result in different reactions to allergens. Initially, we confirmed and expanded those results in an independent set of experiments.

3.1.1 TOTAL CELL NUMBER IN BAL

Seventy-two hours after the last of 6 intranasal administrations of OVA or milk, BALB/c mice as well as C57BL/6, develop airway inflammation. To assess the airway inflammation, we performed counted total inflammatory cells in the airways (Figure 1). C57BL/6 mice that received PBS only had approximately $61,000 \pm 17,060$ total cells in their BAL fluid. In C57BL/6 mice treated with milk 8 times as many cells were counted ($490,000 \pm 84,650$ cells). In comparison, BALB/c mice treated with milk had only a 1.5 times increase in total cells in BAL compared to the PBS-challenged BALB/c mice. OVA induced a similar increase in the number of airway cells, 3 times and 2.7 times in BALB/c and C57BL/6 mice, respectively. In contrast, the response to milk was significantly higher in C57BL/6 (8-fold) compared to BALB/c mice (1.5-fold).

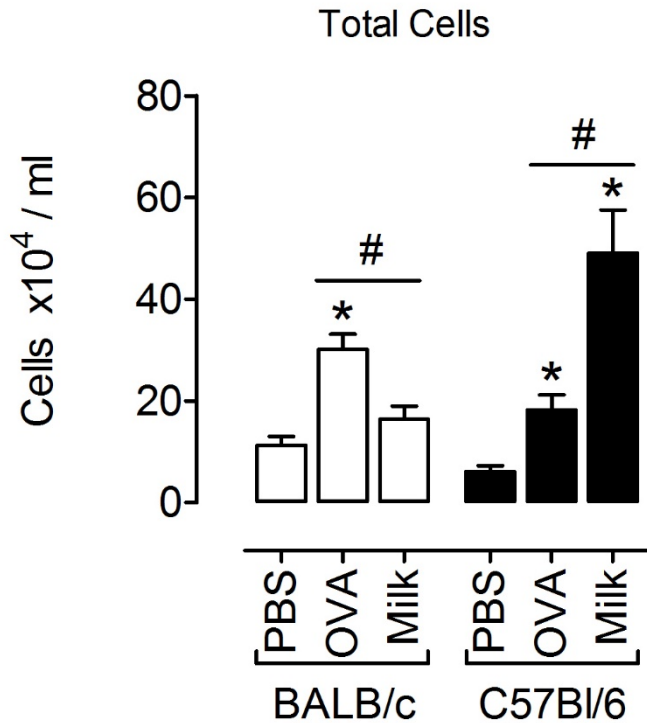


Figure 3.1. Total cell number in BAL in BALB/c and C57BL/6 mice after 6 intranasal administrations of 50ul of PBS, 50μg of ovalbumin (OVA) or 50μg of Fixmilch®, Magermilchpulver, unter 1.5% Fett, manufacturer: Maresi, Lot# 4251 (milk). Results are pooled from 2 independent experiments (n= 7-12) and shown as mean group value ± SEM and analyzed with One-way ANOVA. * $p < 0.05$ vs. the PBS-treated group from the same respective strain. # $p < 0.05$ vs. OVA and milk treated mice of the same strain.

3.1.2 DIFFERENTIAL CELL COUNTS IN BAL

To determine the type of inflammation in the airways, we stained the BAL with a DiffKwik staining kit to count macrophages, eosinophils, neutrophils and lymphocytes. Regardless of the strain, mice that received milk showed almost no change in total macrophages compared to mice of the respective strain that received PBS. In BALB/c mice that received OVA, we observed a 1.8-fold increase in total macrophages. In C57BL/6 mice that received OVA, again a 1.8-fold increase in total macrophages was observed. Compared to controls that had 486 ± 163 eosinophils in their BAL, total eosinophils were significantly increased in BALB/c mice that received OVA, which had $52,671 \pm 15,850$ eosinophils or milk to ($32,276 \pm 12,480$ eosinophils),

respectively. C57BL/6 mice that received OVA had $29,530 \pm 11,750$ eosinophils in their BAL, which is significantly more than control animals. Strikingly, milk-treated C57BL/6 mice had $367,909 \pm 78,940$ eosinophils, which was 12-fold the eosinophils of C57BL/6 mice that received OVA. OVA-treated BALB/c mice had 6 times more neutrophils compared to controls that only had 900 ± 215 , but the number of neutrophils in BAL of milk-treated BALB/c mice was not significantly different compared to the control. C57BL/6 mice, on the other hand, had significantly more neutrophils when treated with OVA or milk ($3,573 \pm 732$ and $3,897 \pm 1,368$ neutrophils, respectively) compared to controls with only 242 ± 100 . BALB/c mice receiving OVA had $48,394 \pm 10,088$ lymphocytes, a 17-fold increase in lymphocytes compared to controls that had $2,829 \pm 306$ lymphocytes. BALB/c mice receiving milk with $23,689 \pm 4900$ lymphocytes only had 8-fold increase of lymphocytes. In contrast C57BL/6 mice had $44,847 \pm 9,286$ lymphocytes or 42-fold increase in lymphocytes when treated with OVA and $55,640 \pm 11,660$ lymphocytes or a 52-fold increase when treated with milk. In summary, we observed eosinophil accumulation in BAL of all allergen-challenged animals that was the most prominent in C57BL/6 mice challenged with milk.

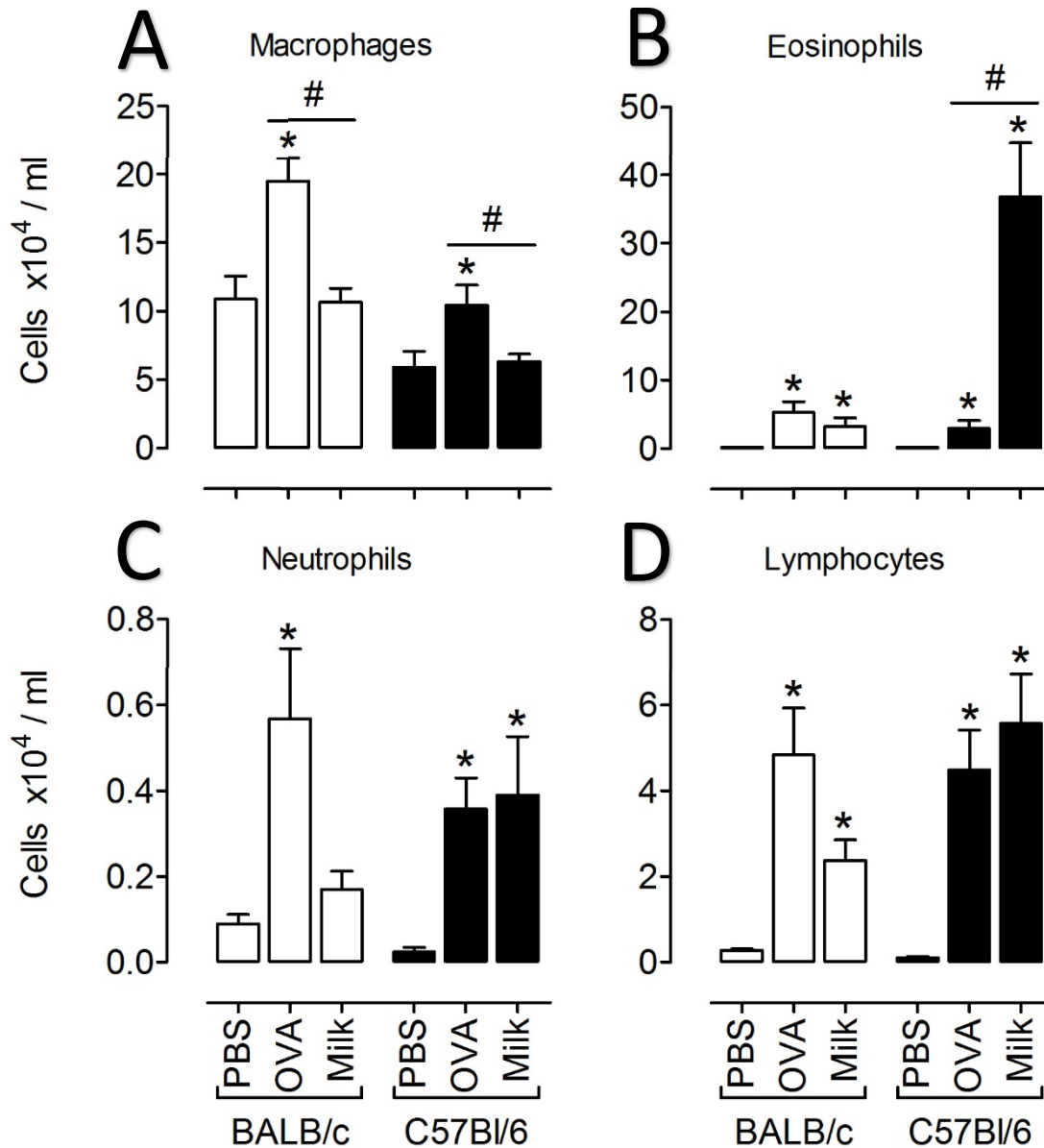


Figure 3.2. Total macrophages (A), eosinophils (B), neutrophils (C) and lymphocytes (D) in BAL in BALB/c and C57BL/6 mice after 6 intranasal administrations of 50ul of PBS, 50μg of ovalbumin (OVA) or 50μg of milk. Results are pooled from 2 independent experiments (n= 7-12) and shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. PBS-treated group from the same strain. # $p < 0.05$ vs. OVA and milk treated mice of the same strain.

3.1.3 CHARACTERIZATION OF THE TYPE OF AIRWAY INFLAMMATION BASED ON THE PERCENTAGES OF MACROPHAGES, EOSINOPHILS LYMPHOCYTES AND NEUTROPHILS.

As shown in Figure 3, BAL of BALB/c and C57BL/6 mice that received PBS have approximately the same BAL composition containing mostly macrophages. In the BAL of BALB/c mice that received PBS, there were over $95.8 \pm 0.6\%$ macrophages but only $0.5 \pm 0.2\%$ eosinophils, $0.8 \pm 0.1\%$ neutrophils and $2.9\% \pm 0.6$ lymphocytes. C57BL/6 mice that received PBS had $96.7 \pm 0.6\%$ macrophages, $0.9 \pm 0.3\%$ eosinophils, $0.5 \pm 0.1\%$ neutrophils and $1.9 \pm 0.5\%$ lymphocytes. We observed $68.5 \pm 5.7\%$ macrophages in OVA and $64.8 \pm 4.8\%$ in milk treated BALB/c mice, as well as $61.3 \pm 4.8\%$ in OVA treated C57BL/6 mice, while C57BL/6 mice that received milk had only $16.5 \pm 2.5\%$ macrophages in their BAL. Decrease in macrophage percentage was induced by influx of other cells, especially eosinophils. In BALB/c mice that received OVA or milk, the percentage of eosinophils was $15.3 \pm 3.7\%$ and also $15.3 \pm 3.5\%$, respectively. C57BL/6 mice that received OVA had roughly the same eosinophil percentage ($12.4 \pm 2.8\%$) as these two groups. In contrast, the BAL of C57BL/6 mice that received milk had with $70.5 \pm 3.6\%$ the most eosinophils of all groups displaying the eosinophil-dominated inflammatory response of this group. Besides eosinophils, the BAL of OVA or milk treated BALB/c mice had $14.5 \pm 2.4\%$ or $13.7 \pm 1.7\%$ lymphocytes respectively, while the BAL of OVA- or milk-treated C57BL/6 mice contained $24.2 \pm 3.5\%$ or $12.2 \pm 1.7\%$ lymphocytes respectively. Finally, we observed some neutrophils, but their relative ratio was less than $2 \pm \%$ in both strains regardless of administered allergen. Altogether, these data confirm that BALB/c mice develop a similar type of inflammation in their BAL regardless of whether OVA or milk was administered. In contrast, C57BL/6 mice showed divergent responses to OVA and milk: while the response to OVA was similar to BALB/c mice, accumulation of eosinophils was highest in C57BL/6 mice challenged with milk compared to all other test groups.

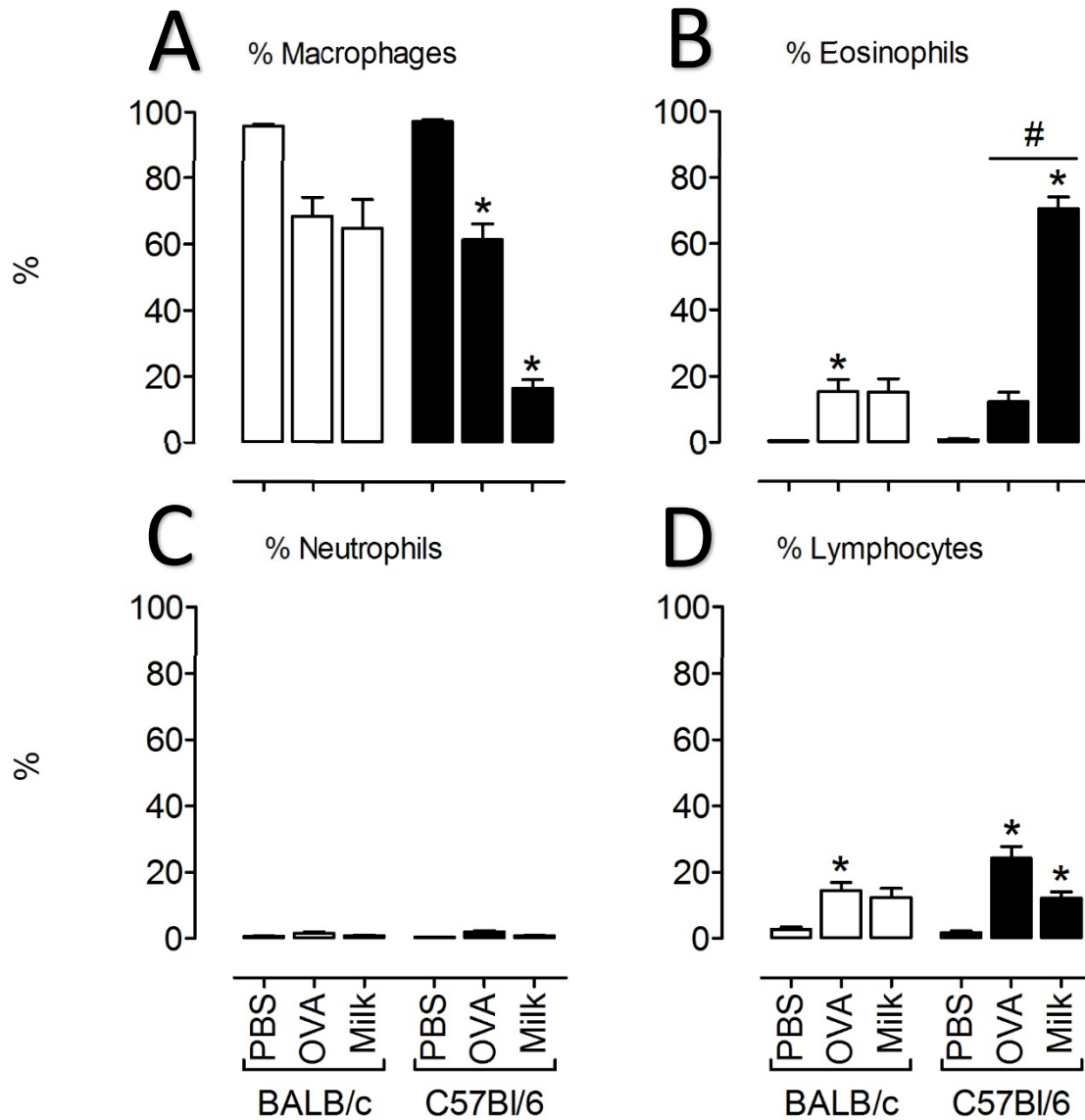


Figure 3.3. Percentages of macrophages (A), eosinophils (B), neutrophils (C) and lymphocytes (D) in the BAL in BALB/c and C57BL/6 mice after 6 intranasal administrations of 50ul of PBS, 50μg of ovalbumin (OVA) or 50μg of milk. Results are pooled from 2 independent experiments (n= 7-12) and shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. PBS-treated group from the same strain. # $p < 0.05$ vs. OVA and milk treated mice of the same strain.

3.1.4 LUNG INFLAMMATION OF ALLERGEN SENSITIZED BALB/C AND C57BL/6 MICE

Intranasally allergen-sensitized mice develop inflammatory infiltrates in their lungs that do not occur in PBS treated mice. To compare the severity of overall lung inflammation, thin cuts of the lungs were stained via H&E staining and then assessed as described in the materials and methods section. The severity of the inflammation was not significantly different regardless of whether OVA or milk was used as a test allergen, or whether BALB/c or C57BL/6 mice received the test allergens (Figure 4).

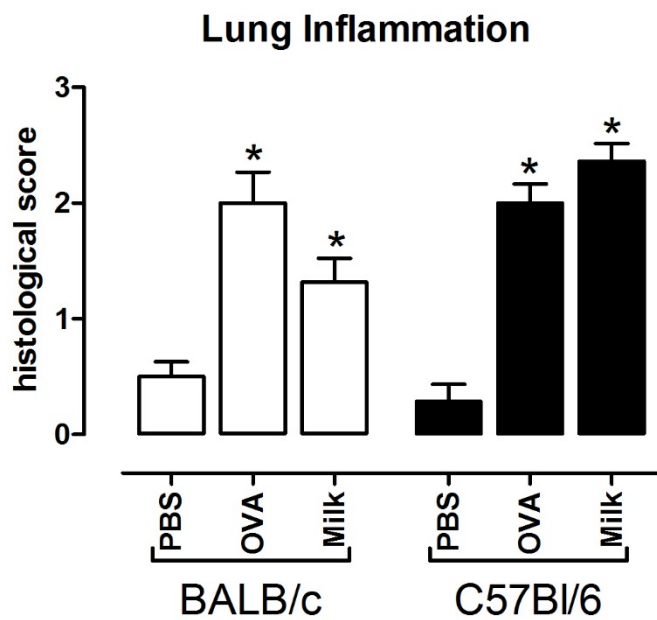


Figure 3.4. Assessment of the lung inflammation of H&E stained lung sections from BALB/c and C57BL/6 mice after 6 intranasal administrations of 50ul of PBS, 50µg of ovalbumin (OVA) or 50µg of milk. Results are pooled from 2 independent experiments (n= 7-12) and shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. PBS-treated group from the same strain. # $p < 0.05$ vs. OVA and milk treated mice of the same strain.

3.1.5 EOSINOPHIL ACCUMULATION IN THE LUNGS

In order to assess lung eosinophilia, paraffin lung tissue sections were stained with LUNA staining and eosinophils were counted in 10 randomly chosen microscope screens from each sample (Figure 5). As expected, we detected only few eosinophils in PBS treated BALB/c and C57BL/6 mice. In contrast, number of eosinophils increased for 14.7-fold and 8.7-fold in BALB/c mice that received OVA or milk, respectively. In contrast, OVA-treated C57BL/6 mice had lowest eosinophil counts and milk treated C57BL/6 mice had the most eosinophils of all groups. Overall, these results confirmed our airway inflammation data indicating that milk and OVA induce similar extent of allergic response in BALB/c mice, but divergent in C57BL/6 mice.

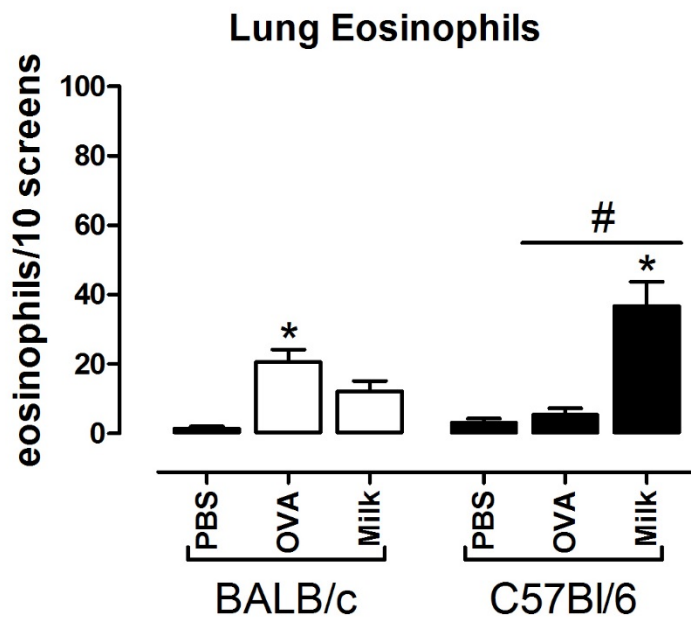


Figure 3.5. Number of eosinophils per randomly chosen 10 microscope screens of LUNA stained lung sections from BALB/c and C57BL/6 mice after 6 intranasal administrations of 50ul of PBS, 50µg of ovalbumin (OVA) or 50µg of milk. Results are pooled from 2 independent experiments (n= 7-12) and shown as mean group value ± SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. PBS-treated group from the same strain. # $p < 0.05$ vs. OVA and milk treated mice of the same strain.

3.1.6 MUCUS PRODUCTION

Beside airway and lung inflammation, another important hallmark of allergic asthma is mucus hypersecretion by goblet cells. For determination of mucus production, paraffin lung sections were stained with PAS staining and evaluated as described in material and methods (Figure 6). Regardless of the strain, mice sensitized with intranasal administrations of OVA or milk develop mucosal hyperplasia, which is not observed in PBS treated mice. There was no significant difference between the score of mucosal hyperplasia of BALB/c mice that received OVA or milk, since both had a score of ~ 2 (± 0.1 and ± 0.2 respectively). On the other hand, the hyperplasia of C57BL/6 mice that received OVA only had a mean score of 1.4 ± 0.1 whereas C57BL/6 mice that received milk had a mean score of 2.7 ± 0.1 of mucosal hyperplasia. The extent of mucus hypersecretion is, in alignment with airway and lung inflammation, indicating that the overall allergic response is highest in C57BL/6 mice challenged with milk, followed by BALB/c mice challenged with OVA or milk and lowest with C57BL/6 mice challenged with OVA.

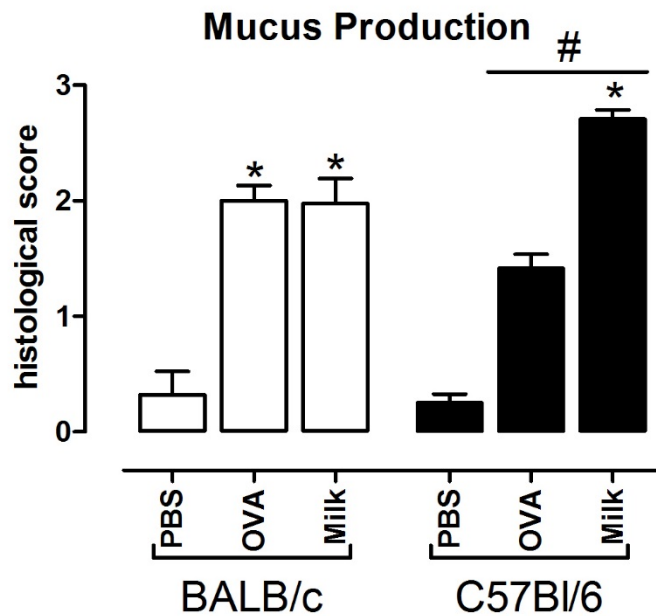


Figure 3.6. Scores of mucosal hyperplasia of PAS stained lung sections from BALB/c and C57BL/6 mice after 6 intranasal administrations of 50 μ l of PBS, 50 μ g of ovalbumin (OVA) or 50 μ g of milk. Results are pooled from 2 independent experiments ($n = 7-12$) and shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. PBS-treated group from the same strain. # $p < 0.05$ vs. OVA and milk treated mice of the same strain.

3.1.7 SERUM IGG1- AND IGG2A-TITERS

For isotype switching from IgM to IgG (or IgE), allergen specific Th-cells and B-cells have to be formed beforehand and then cooperate. The presence of allergen-specific IgG confirms the development of immunological memory to that allergen. Therefore, measurements of IgGs allow the monitoring of immunological memory. Serum titers of OVA- and milk-specific IgG1 and IgG2a were evaluated via ELISA. The milk- or OVA-specific IgG1 and IgG2a levels in sera of mice that received OVA or milk increase depending on how many times the animals have been challenged with the test allergen (Figure 3.7 and 3.8). OVA treated mice develop very similar OVA-specific IgG1 and IgG2a titers in their sera regardless whether the strain of mice was BALB/c or C57BL/6 (Figure 3.7). Interestingly, milk treated BALB/c mice had lower milk-specific IgG1 titers and milk-specific IgG2a-titers compared to their respective group of milk treated C57BL/6 mice (Figure 3.8).

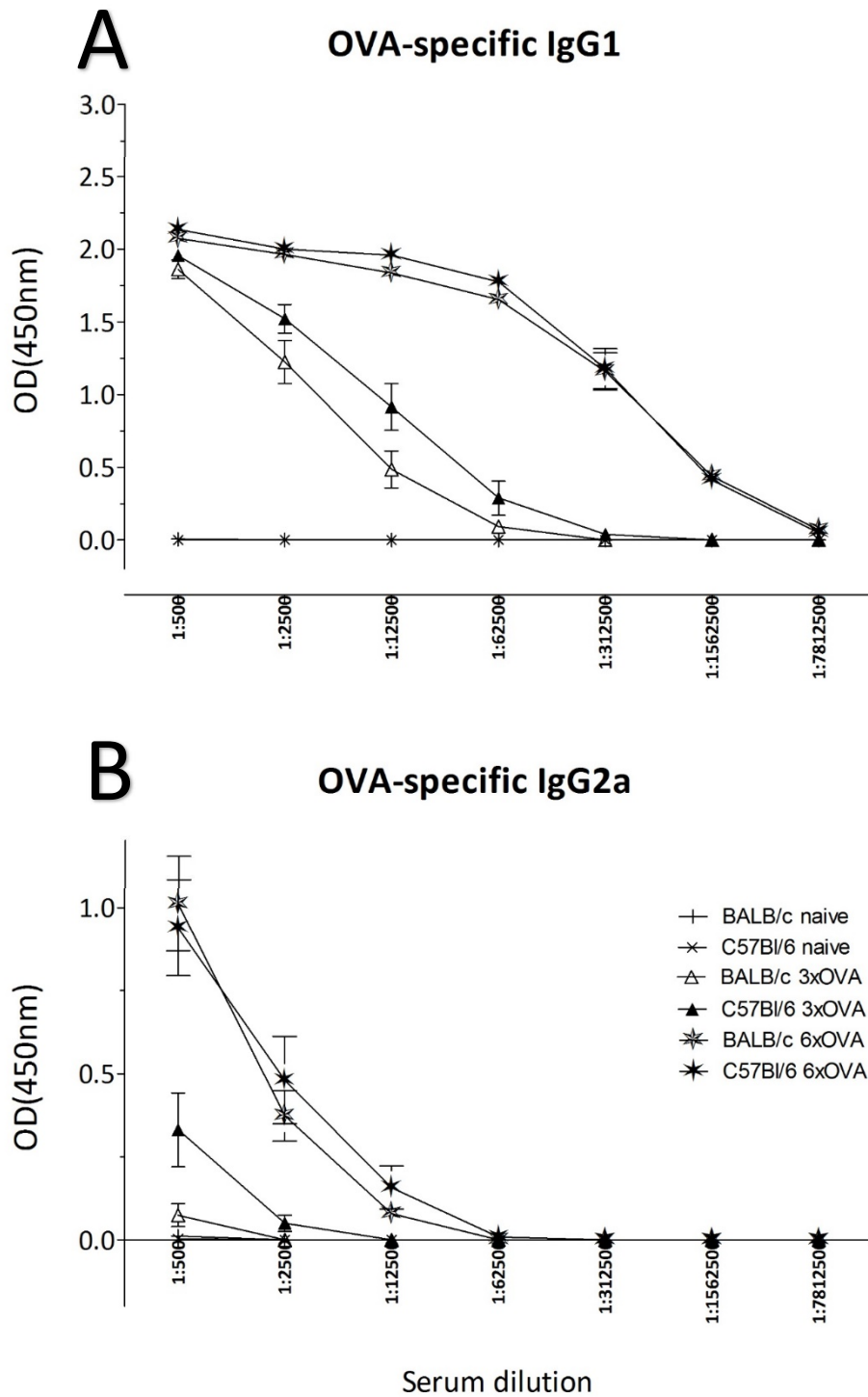


Figure 3.7. OVA-specific titers of IgG1 (A) and IgG2a (B) after 3 or 6 intranasal administrations of 50ul of PBS, 50 μ g of ovalbumin (OVA) or 50 μ g of milk. Results are pooled from 2 independent experiments ($n = 7-12$) and shown as mean group value $\pm$ SEM.

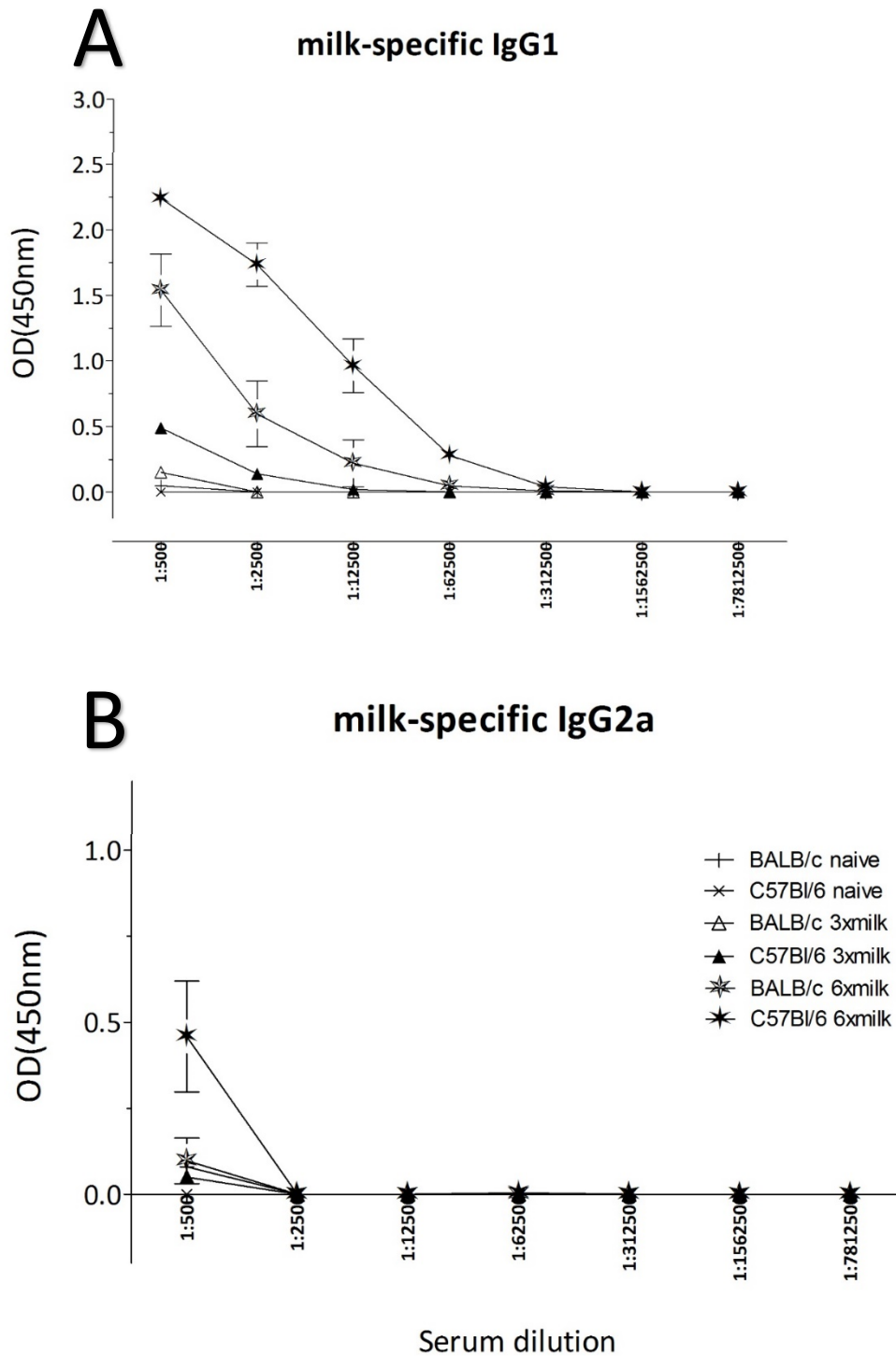


Figure 3.8. Milk-specific titers of IgG1 (A) and IgG2a (B) after 6 intranasal administrations of 50ul of PBS, 50 μ g of ovalbumin (OVA) or 50 μ g of milk. Results are pooled from 2 independent experiments ($n = 7-12$) and shown as mean group value $\pm$ SEM.

3.2 LPS AS AN IMMUNOLOGICAL MODULATOR IN MILK-CHALLENGED BALB/C MICE AND OVA-CHALLENGED C57BL/6 MICE

Our previous results indicated that milk and OVA solutions differ markedly in the LPS content. In our hands, 1% OVA solution contains >200ng/mL of LPS, while 1% solution of the milk had an LPS content of 2ng/ml. This translates to a LPS content of 10^{-3} µg per administration of OVA and 10^{-5} µg of LPS per administration of milk respectively in our intranasal mouse model of asthma. It has also been demonstrated that OVA deprived from LPS fails to induce the lung and airway inflammation in a mouse model of asthma. Instead, immunological tolerance to OVA was induced in these mice [94]. OVA without LPS lacks an appropriate danger signal and hence is considered innocuous by the immune system. Furthermore, it has been suggested that TLR-4-expression in spleen derived DCs [159] and bone marrow derived macrophages [160] is higher in BALB/c mice than C57BL/6 mice. But higher receptor expression does not necessarily translate to higher downstream effects linked to the receptor. For instance, in case of the NO production to stimulation of the TLR4-pathway it has been shown, that C57BL/6 mice with their lower receptor density than BALB/c mice actually have a stronger response due to increased activation of IFN-β and further downstream STAT-1 after stimulation of TLR-4 via LPS, ultimately leading to an increased expression and activation of the inducible NO-synthetase [160]. Taking all this into consideration, we hypothesized that LPS might act as a dose-dependent adjuvant for the allergic response or as a modulator to switch between eosinophilic and non-eosinophilic inflammation. The differential response to allergens in BALB/c and C57BL/6 mice might be due to the different LPS sensitivities of the two strains and the different LPS contents of these two allergens. To test this hypothesis, we conducted 2 sets of experiments.

Firstly, we added divergent doses of LPS to milk and evaluated the airway and lung inflammation in BALB/c mice. Milk-treated C57BL/6 mice had 3.0-fold more eosinophils in their lungs compared to milk-treated BALB/c mice. Since milk contains more than 100 times less LPS than OVA and antigen-presenting cells in BALB/c mice are less sensitive to LPS than C57BL/6 mice [155, 156, 159, 160], we assumed that milk LPS content might not be sufficient to boost the eosinophilia in BALB/c mice to levels observed in milk treated B6 mice.

In a second set of experiments, we purified OVA from endogenous LPS and then added defined doses of LPS to boost allergic response to OVA in C57BL/6 mice. We hypothesized that endogenous LPS level in OVA might be too high to act as an adjuvant in C57BL/6 mice.

3.2.1 ADDITION OF INCREMENTAL DOSES OF LPS AS AN ADJUVANT AND IMMUNOLOGICAL MODULATOR TO MILK IN BALB/C MICE

3.2.1.1 ALLERGIC RESPONSES TO MILK WITH ADDED LPS IN DOSES FROM $10^{-2}\mu\text{G}$ TO $1\mu\text{G}$

3.2.1.1.1 AIRWAY INFLAMMATION

We found $1,005,140 \pm 186,900$ total cells in the BAL of BALB/c mice treated with $1\mu\text{g}$ of LPS. This is a 6-fold increase compared to mice that received PBS, which served as a control group. The total BAL cells in milk treated BALB/c mice increased depending on the dose of LPS added to milk. Ranging from $444,286 \pm 70,440$ total cells for mice treated with milk alone, $985,000 \pm 108,500$ for $10^{-2}\mu\text{g}$ of LPS added, $1,888,570 \pm 169,000$ total cells for $10^{-1}\mu\text{g}$ and $1,947,140 \pm 218,400$ total cells for $1\mu\text{g}$ of LPS.

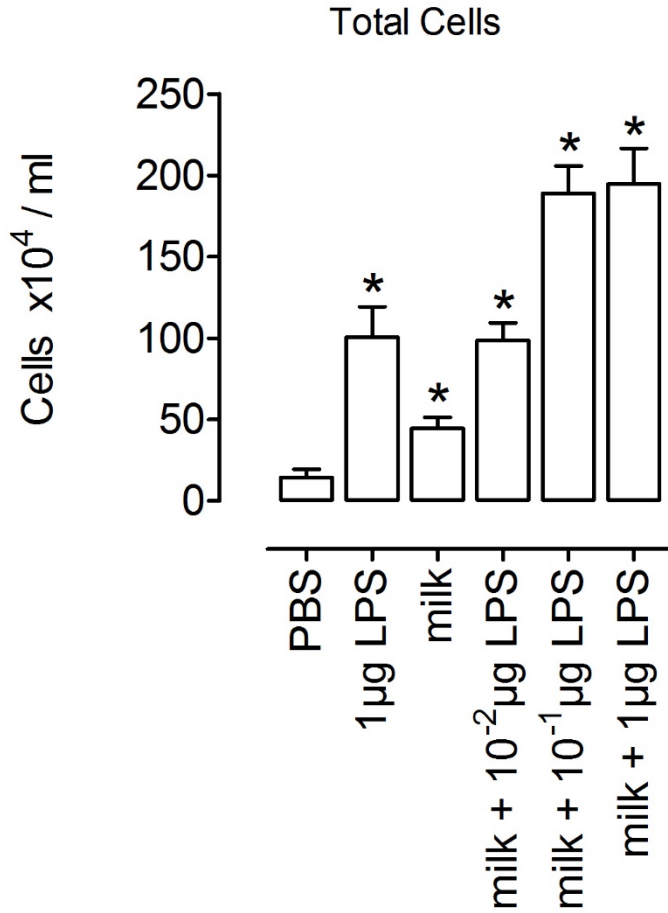


Figure 3.9. Total cell number in BAL of BALB/c mice after 6 intranasal administrations of 50µl of PBS, 1µg of LPS, 50µg of milk, 50µg of milk + 10⁻²µg of LPS, 50µg of milk + 10⁻¹µg of LPS, 50µg of milk + 1µg of LPS. (n= 7) shown as mean group value ± SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.1.2 DIFFERENTIAL CELL COUNTS IN BAL

We observed a 5-fold increase in the number of macrophages in BALB/c mice challenged with 1µg of LPS compared to mice that received PBS. In contrast, the number of macrophages in BALB/c mice that received milk only did not-significantly increase compared to PBS-challenged controls, confirming the findings of previous experiments. BALB/c mice that received milk with added LPS had macrophages ranging from $538,643 \pm 87,000$ for 10⁻²µg of LPS added, $106,252 \pm$

77,850 for $10^{-1}\mu\text{g}$ and $971,362 \pm 79,810$ for $1\mu\text{g}$ of LPS added. BALB/c mice that were challenged with PBS or $1\mu\text{g}$ of LPS did not develop airway eosinophilia, but milk challenged BALB/c mice developed eosinophilia. We counted $161,207 \pm 25,160$ eosinophils in their BAL. The airway eosinophilia was suppressed through LPS addition in a dose dependent way, ranging from $161,529 \pm 21,140$ eosinophils for milk challenged mice with added $10^{-2}\mu\text{g}$ of LPS, $148,771 \pm 15,620$ eosinophils for $10^{-1}\mu\text{g}$ of added LPS and $610,143 \pm 14,650$ eosinophils for $1\mu\text{g}$ of added LPS. We counted 483 neutrophils in the BAL of mice that received PBS only and $19,117 \pm 7,842$ neutrophils when mice were challenged with $1\mu\text{g}$ of LPS. Only 680 neutrophils were found in the BAL of mice challenged with milk only. Addition of LPS to milk challenge increased the number of BAL neutrophils in a dose dependent way: from $6,902 \pm 2,638$ neutrophils in group challenged with $10^{-2}\mu\text{g}$ of LPS added to milk to $174,167 \pm 63,850$ neutrophils in group challenged with $1\mu\text{g}$ of LPS added to milk. While BALB/c mice that received PBS had only $7,941 \pm 3,315$ lymphocytes, lymphocytes were increased to $217,479 \pm 34,960$ in mice that received $1\mu\text{g}$ of LPS. Mice that received milk only had $49,611 \pm 10,110$ lymphocytes. The addition of $10^{-2}\mu\text{g}$ of LPS increased the number of lymphocytes to $279,760 \pm 18,910$, while the addition of $10^{-1}\mu\text{g}$ of LPS increased the number of lymphocytes to $692,286 \pm 103,800$ and the $1\mu\text{g}$ of added LPS increased the number of lymphocytes to $744,143 \pm 128,600$. This also indicates increasing migration of lymphocytes in the airways depending on the dose added to milk.

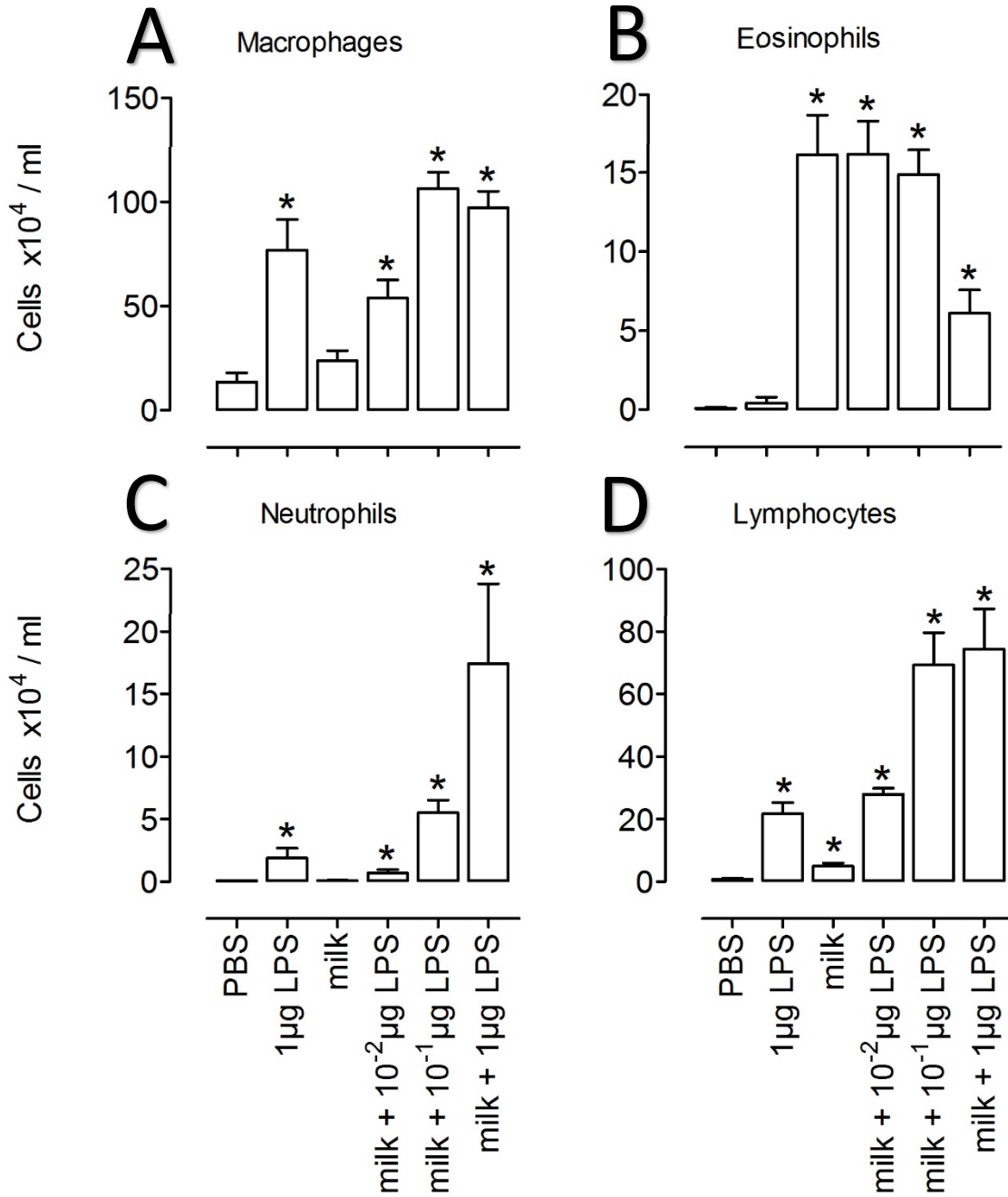


Figure 3.10. Total macrophages (A), eosinophils (B), neutrophils (C) and lymphocytes (D) in the BAL of BALB/c mice after 6 intranasal administrations of 50µl of PBS, 1µg of LPS, 50µg of milk, 50µg of milk + 10⁻²µg of LPS, 50µg of milk + 10⁻¹µg of LPS, 50µg of milk + 1µg of LPS. (n= 7) Shown as mean group value ± SEM and analyzed via One-way ANOVA. * *p* < 0.05 vs. the PBS-treated BALB/c mice.

3.2.1.1.3 CELL PERCENTAGES IN BAL

As shown in previous experiments, the BAL of BALB/c mice that received PBS alone is characterized by a macrophage dominated cell composition. In PBS challenged BALB/c mice $94.0 \pm 2.0\%$ macrophages can be observed, while there is only $0.3 \pm 0.2\%$ eosinophils, $0.2 \pm 0.2\%$ neutrophils and $5.3 \pm 1.9\%$ lymphocytes. BALB/c mice challenged with $1\mu\text{g}$ of LPS had $76.4 \pm 1.4\%$ macrophages in their BAL. In all other test groups macrophage percentage decreased to $\sim 50\%$, regardless of the LPS dose added to milk. In contrast to macrophages, the percentage of eosinophils in BAL appears to be suppressed in a LPS-dose dependent way. Ranging from $37.2 \pm 6.4\%$ for milk only, $16.6 \pm 1.8\%$ for added $10^{-2}\mu\text{g}$ of LPS to milk, $8.0 \pm 0.8\%$ for added $10^{-1}\mu\text{g}$ of LPS and $3.0 \pm 0.7\%$ for added $1\mu\text{g}$ of LPS. The percentage of neutrophils is $2.0 \pm 0.6\%$ in BALB/c mice that received $1\mu\text{g}$ of LPS. Milk challenged mice had $0.1 \pm 0.1\%$ neutrophils. The percentage of neutrophils increased in a dose dependent way when LPS was added to milk, ranging from $0.7 \pm 0.3\%$ for added $10^{-2}\mu\text{g}$ of LPS, $2.9 \pm 0.4\%$ for added $10^{-1}\mu\text{g}$ of LPS and $8.9 \pm 3.5\%$ for added $1\mu\text{g}$ of LPS. The percentage of lymphocytes was $21.91.7\%$ in BALB/c mice that received $1\mu\text{g}$ of LPS. Milk challenged mice had $10.7 \pm 1.0\%$ of lymphocytes. The percentage of lymphocytes increased in a dose dependent fashion when LPS was added to milk, ranging from $29.5 \pm 2.3\%$ for added $10^{-2}\mu\text{g}$ of LPS, $36.7 \pm 4.3\%$ for added $10^{-1}\mu\text{g}$ of LPS and $37.1 \pm 2.3\%$ for added $1\mu\text{g}$ of LPS.

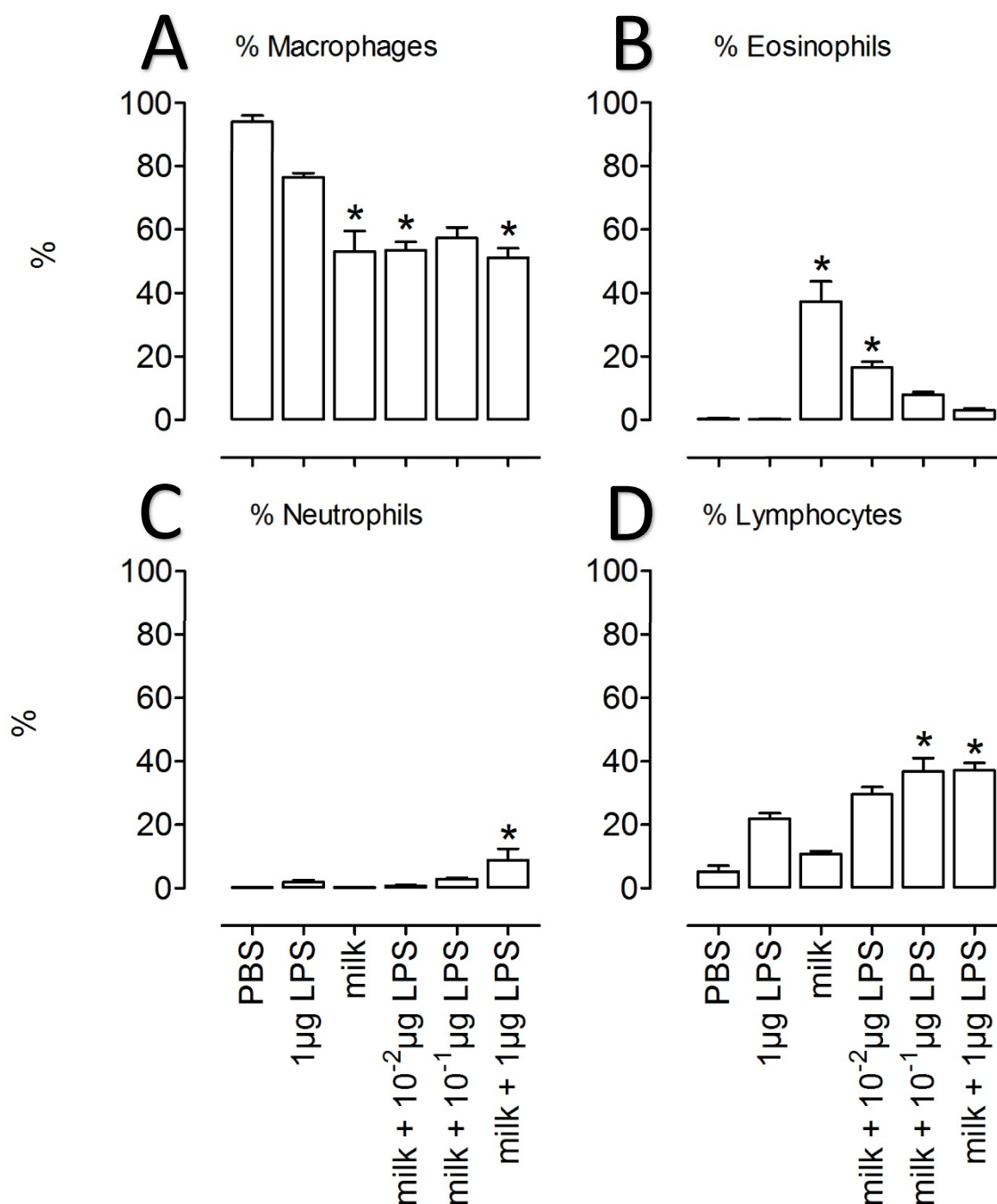


Figure 3.11. Percentages of macrophages (A), eosinophils (B), neutrophils (C) and lymphocytes (D) in the BAL of BALB/c mice after 6 intranasal administrations of 50µl of PBS, 1µg of LPS, 50µg of milk, 50µg of milk + 10⁻²µg of LPS, 50µg of milk + 10⁻¹µg of LPS, 50µg of milk + 1µg of LPS. (n= 7) Shown as mean group value ± SEM and analyzed via One-way ANOVA. * *p* < 0.05 vs. the PBS-treated BALB/c mice.

3.2.1.1.4 LUNG INFLAMMATION

As described above, lung tissue sections were stained with H&E staining to assess lung inflammation. We found, that mice challenged with PBS only did not develop lung inflammation. BALB/c mice that received 1 μ g of LPS or milk only developed a low grade of lung inflammation both with scores around 1. The addition of LPS to our test allergens induced a significant increase in lung inflammation to scores around 2.5, but we did not observe a dose dependent effect in this experiment.

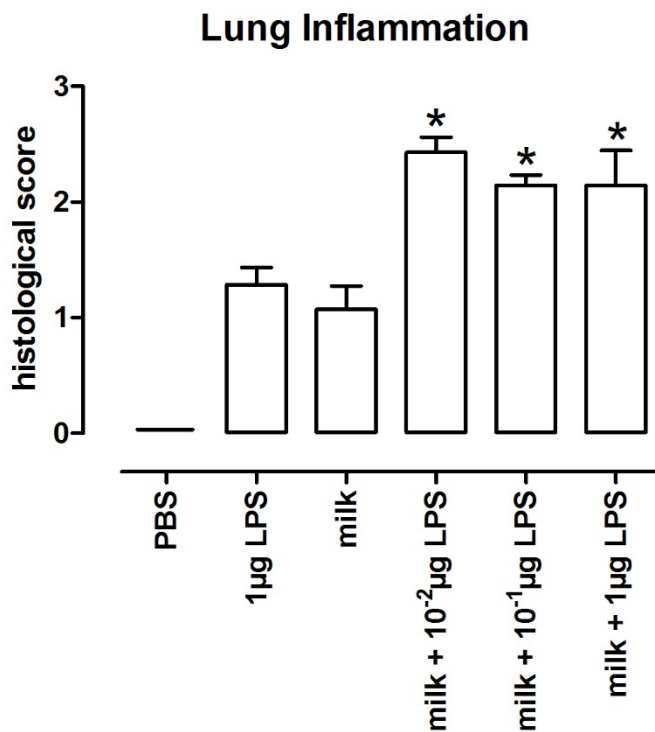


Figure 3.12. Assessment of the lung inflammation of H&E stained lung sections from BALB/c mice after 6 intranasal administrations of 50 μ l of PBS, 1 μ g of LPS, 50 μ g of milk, 50 μ g of milk + 10⁻² μ g of LPS, 50 μ g of milk + 10⁻¹ μ g of LPS, 50 μ g of milk + 1 μ g of LPS. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.1.5 EOSINOPHIL ACCUMULATION IN THE LUNGS

Lung sections were stained with LUNA staining to count eosinophils in the lung tissues. BALB/c mice that received PBS only had a mean of 1.8 ± 0.3 eosinophils per 10 screens. We counted 2.9 ± 0.9 eosinophils in mice that received $1\mu\text{g}$ of LPS. The highest count of eosinophils had mice that received milk with 7.1 ± 1.5 eosinophils per 10 screens. With the addition of LPS, lung eosinophils were suppressed in a dose dependent way. We counted 5.9 ± 1.5 eosinophils/10 screens when $10^{-2}\mu\text{g}$ of LPS was added to milk, 3.6 ± 1.4 eosinophils/10 screens when $10^{-1}\mu\text{g}$ of LPS was added to milk and 2.4 ± 0.7 eosinophils/10 screens when $1\mu\text{g}$ of LPS was added to milk.

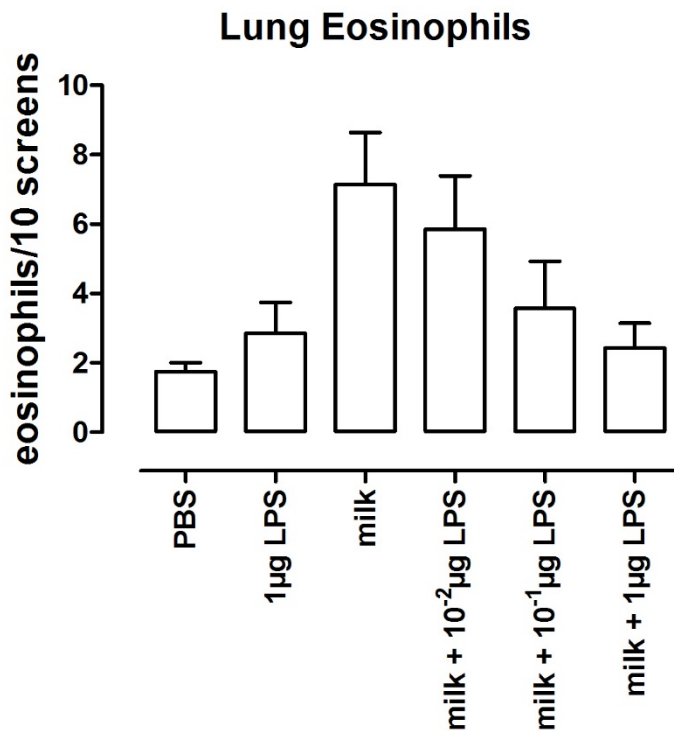


Figure 3.13. Number of eosinophils per randomly chosen 10 microscope screens of LUNA stained lung sections from BALB/c after 6 intranasal administrations of 50ul of PBS, $1\mu\text{g}$ of LPS, $50\mu\text{g}$ of milk, $50\mu\text{g}$ of milk + $10^{-2}\mu\text{g}$ of LPS, $50\mu\text{g}$ of milk + $10^{-1}\mu\text{g}$ of LPS, $50\mu\text{g}$ of milk + $1\mu\text{g}$ of LPS. ($n=7$) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.1.6 MUCUS PRODUCTION

For comparison of mucosal hyperplasia, paraffin lung sections were stained with PAS staining and evaluated as described above. BALB/c mice that received PBS only had a score of 0.0 ± 0.0 . Mice that received $1\mu\text{g}$ of LPS had a score of 0.1 ± 0.1 . Mice that received milk only had a score of 2.5 ± 0.1 . The highest score of 2.7 ± 0.2 had mice that received milk with added $10^{-2}\mu\text{g}$ of LPS. With the addition of more LPS, mucosal hyperplasia suppressed in a dose dependent way. We scored 1.5 ± 0.2 when $10^{-1}\mu\text{g}$ of LPS was added to milk and 0.5 ± 0.0 when $1\mu\text{g}$ of LPS was added to milk.

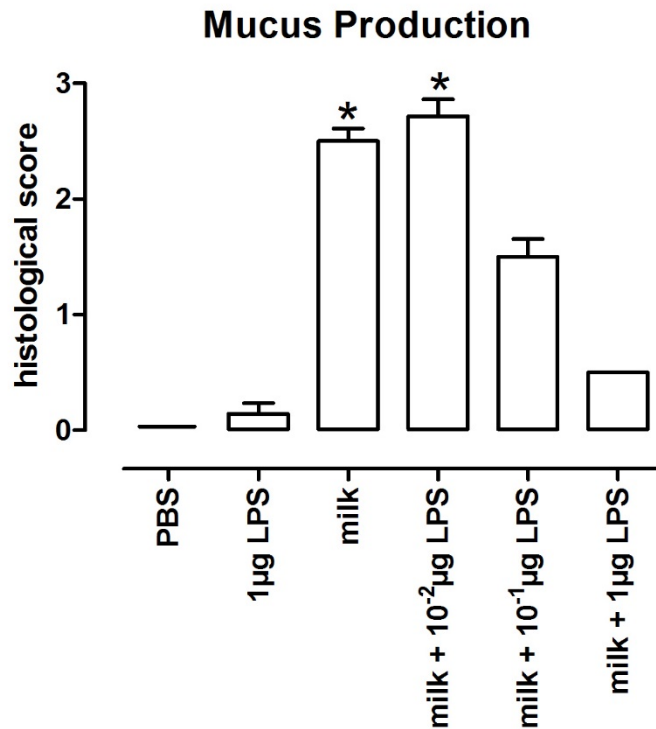


Figure 3.14. Scores of mucosal hyperplasia of PAS stained lung sections from BALB/c after 6 intranasal administrations of $50\mu\text{l}$ of PBS, $1\mu\text{g}$ of LPS, $50\mu\text{g}$ of milk, $50\mu\text{g}$ of milk + $10^{-2}\mu\text{g}$ of LPS, $50\mu\text{g}$ of milk + $10^{-1}\mu\text{g}$ of LPS, $50\mu\text{g}$ of milk + $1\mu\text{g}$ of LPS. ($n=7$) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.1.7 MILK-SPECIFIC SERUM IGG1- AND IGG2A-TITERS

Interestingly, LPS seems to increase the levels of milk-specific IgG1 and IgG2a-antibodies in a clearly dose dependent way. As expected, LPS alone did not induce the production of milk specific IgG1 or IgG2a.

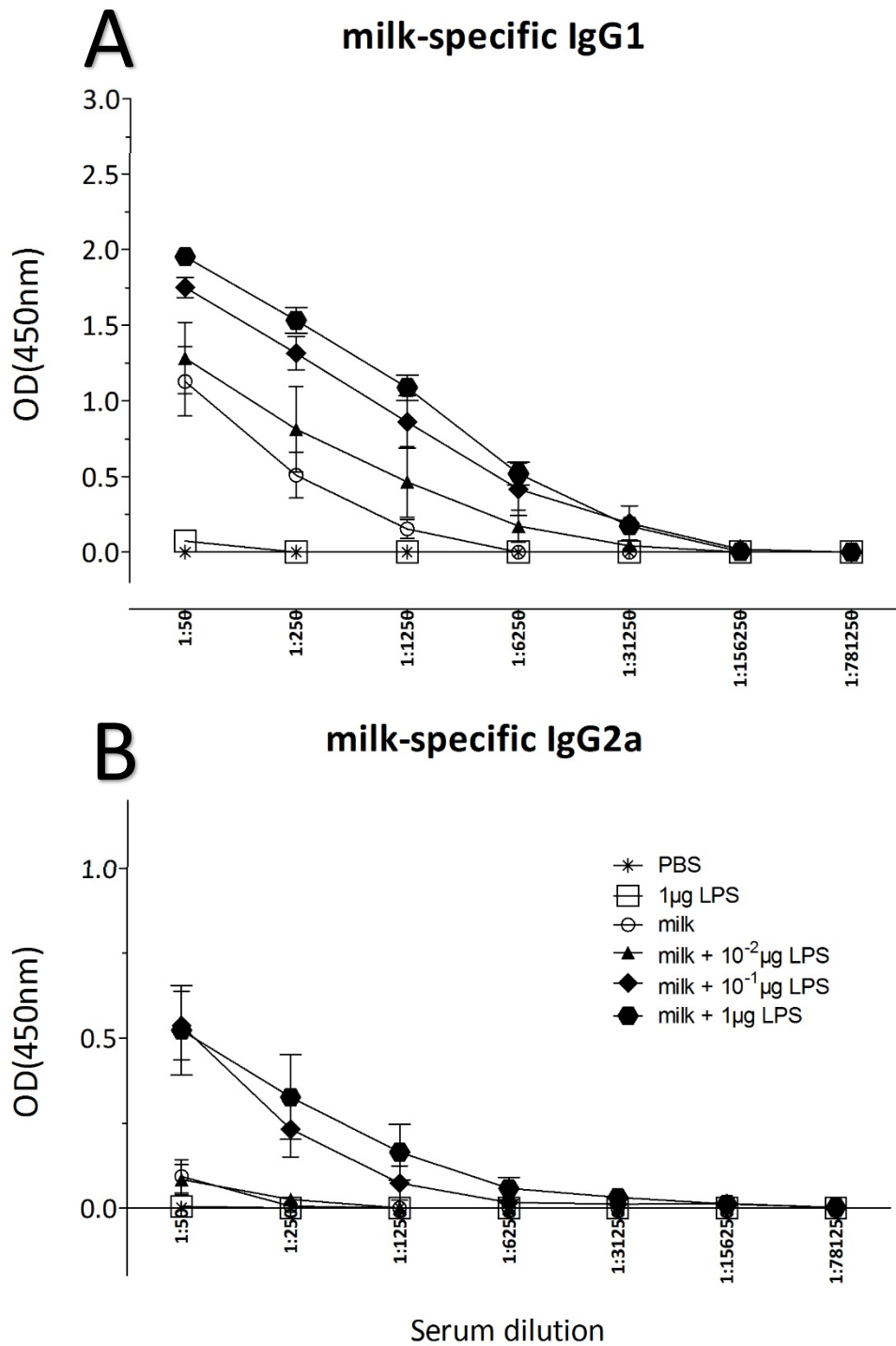


Figure 3.15. OVA-specific titers of IgG1 (A) and IgG2a (B) in BALB/c mice after 6 intranasal administrations of 50µl of PBS, 1µg of LPS, 50µg of milk, 50µg of milk + 10^{-2} µg of LPS, 50µg of milk + 10^{-1} µg of LPS, 50µg of milk + 1µg of LPS. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.2 ALLERGIC RESPONSES TO MILK WITH ADDED LPS IN DOSES BETWEEN 10^{-4} AND $10^{-1}\mu\text{G}$

To examine the possibility, that lower doses of LPS below $10^{-2}\mu\text{g}$ added to milk could still have a boosting effect on airway and lung eosinophilia and mucosal hyperplasia, BALB/c mice were challenged with milk with LPS between 10^{-4} and $10^{-1}\mu\text{g}$ added to it.

3.2.1.2.1 *TOTAL CELL COUNTS IN BAL*

As expected, BALB/c mice treated with PBS had with $91,428 \pm 19,110$ cells the lowest number of total BAL cells of all groups in this experiment. Milk treated mice had the most BAL cells with a count of $1,171,430 \pm 296,500$ cells. Mice that received milk with added $10^{-4}\mu\text{g}$ of LPS had $921,429 \pm 108,800$ BAL cells. Mice that received milk with added $10^{-3}\mu\text{g}$ of LPS had $627,857 \pm 106,800$ BAL cells. Neither $10^{-4}\mu\text{g}$ nor $10^{-3}\mu\text{g}$ of LPS added to milk had any boosting effect on total BAL cells compared to mice that were treated with milk alone. Mice that received milk with added $10^{-2}\mu\text{g}$ of LPS had $927,619 \pm 161,500$ BAL cells. Mice that received milk with added $10^{-1}\mu\text{g}$ of LPS had $495,476 \pm 136,500$ BAL cells.

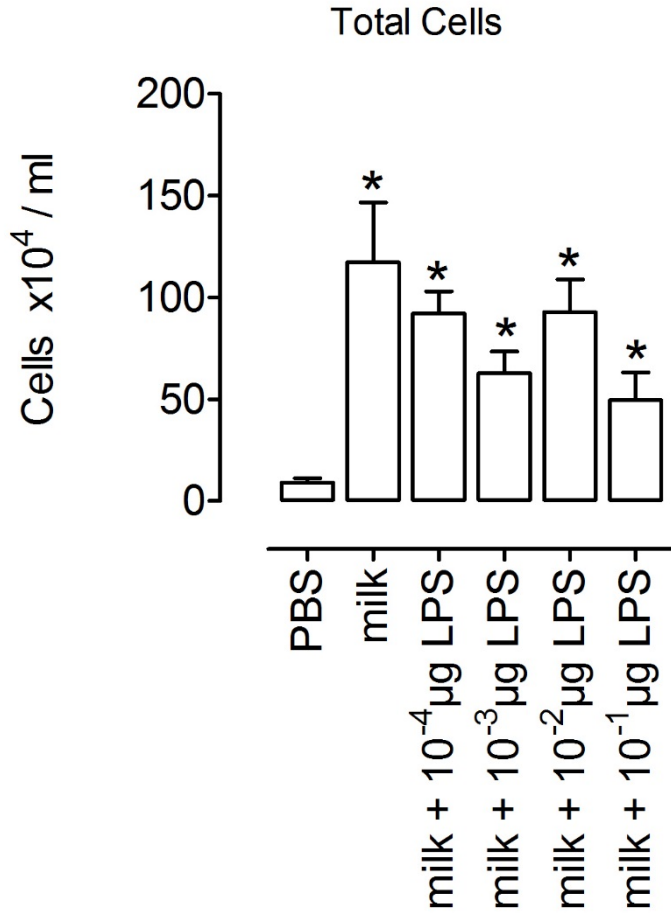


Figure 3.16. Total cell number in bronchoalveolar lavage fluid (BAL) of BALB/c mice after 6 intranasal administrations of 50µl of PBS, 50µg of milk, 50µg of milk + 10⁻⁴µg of LPS, 50µg of milk + 10⁻³µg of LPS, 50µg of milk + 10⁻²µg of LPS, 50µg of milk + 10⁻¹µg of LPS. (n= 7) Shown as mean group value ± SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.2.2 DIFFERENTIAL CELL COUNTS IN BAL

We observed an increase from $85,234 \pm 17,990$ to $227,610 \pm 30,670$ in the number of macrophages in BAL of BALB/c mice challenged with milk compared to mice that received PBS. Mice that received milk with added 10⁻⁴µg, 10⁻³µg, 10⁻²µg or 10⁻¹µg of LPS had all a very similar numbers of total macrophages ranging from $311,002 \pm 30,920$ to $279,105 \pm 78,410$. The airway eosinophilia developed in all groups that received milk regardless of the dose of LPS

addition, but it was not increased in any of the groups that received milk with added $10^{-4}\mu\text{g}$, $10^{-3}\mu\text{g}$, $10^{-2}\mu\text{g}$ or $10^{-1}\mu\text{g}$ of LPS, when compared to mice that received milk alone. We found no neutrophils in the BAL of mice that received PBS. $1,331 \pm 951$ neutrophils were found in the BAL of mice challenged with milk only, further, 324 neutrophils for $10^{-4}\mu\text{g}$ of LPS added to milk, $1,044 \pm 524$ neutrophils for $10^{-3}\mu\text{g}$ of LPS added to milk, $5,235 \pm 3841$ neutrophils for $10^{-2}\mu\text{g}$ of LPS added to milk and $9,869 \pm 3,930$ neutrophils for $10^{-1}\mu\text{g}$ of LPS added to milk. Whereas BALB/c mice that received PBS only had only $4,591 \pm 952$ lymphocytes, lymphocytes were increased to $93,510 \pm 22,030$ in mice that received milk. The group with added $10^{-4}\mu\text{g}$ of LPS to milk had $135,432 \pm 36,270$ lymphocytes. The group with added $10^{-3}\mu\text{g}$ of LPS to milk had $70,025 \pm 20,320$ lymphocytes. The group with added $10^{-2}\mu\text{g}$ of LPS to milk had $198,401 \pm 37,860$ lymphocytes. The group with added $10^{-1}\mu\text{g}$ of LPS to milk had $100,197 \pm 24,670$ lymphocytes.

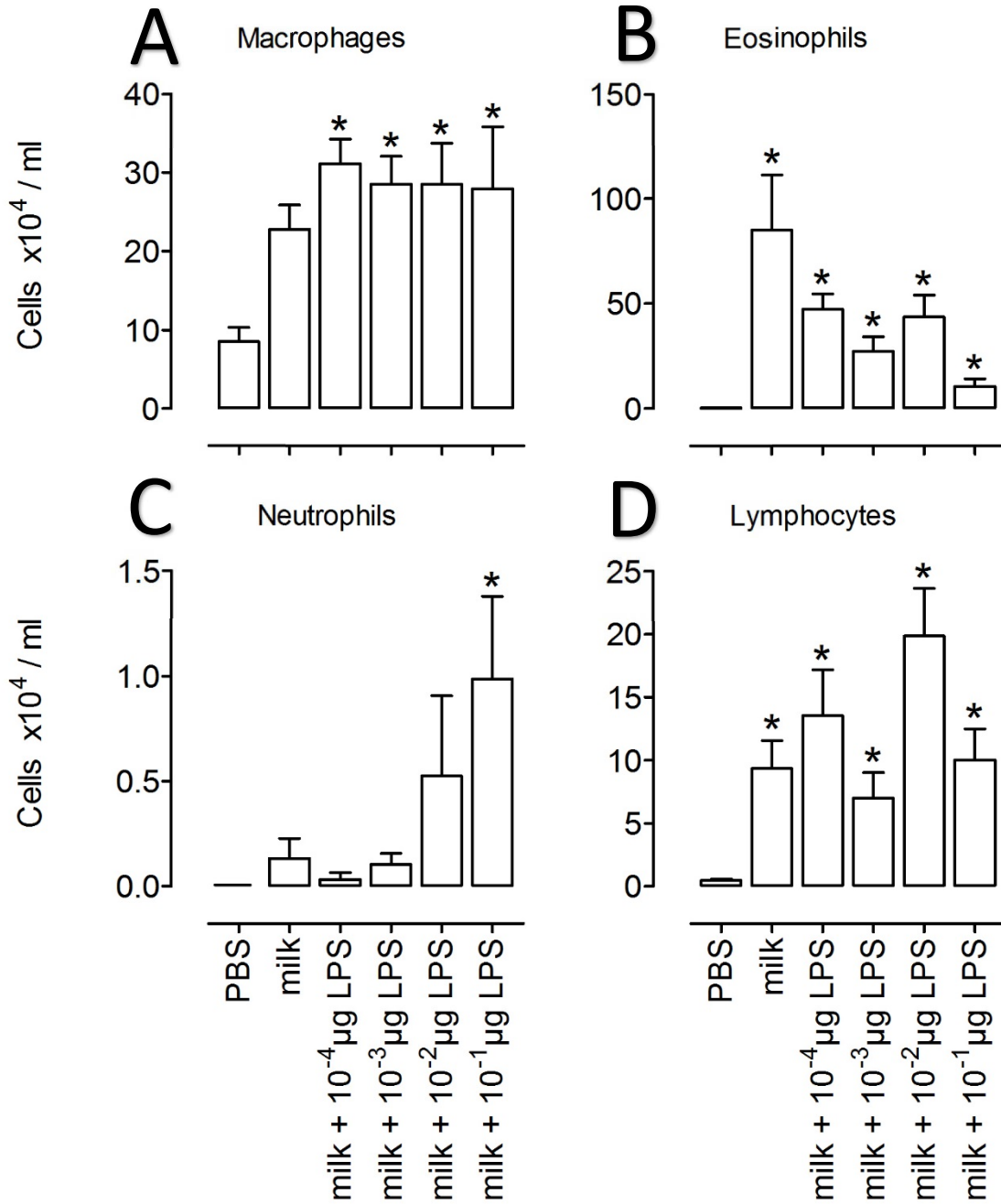


Figure 3.17. Total macrophages (A), eosinophils (B), neutrophils (C) and lymphocytes (D) in BAL of BALB/c mice after 6 intranasal administrations of 50ul of PBS, 50 μ g of milk, 50 μ g of milk + 10^{-4} μ g of LPS, 50 μ g of milk + 10^{-3} μ g of LPS, 50 μ g of milk + 10^{-2} μ g of LPS, 50 μ g of milk + 10^{-1} μ g of LPS. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.2.3 THE CELL COMPOSITION IN BAL

The lowest tested LPS dose of 10^{-4} μg does not significantly impact the cell composition in BAL compared to BALB/c mice treated with milk without any LPS added. In contrast, LPS doses equal or higher than 10^{-3} μg suppress eosinophils and induce migration of lymphocytes and neutrophils, as observed in the previous experiment.

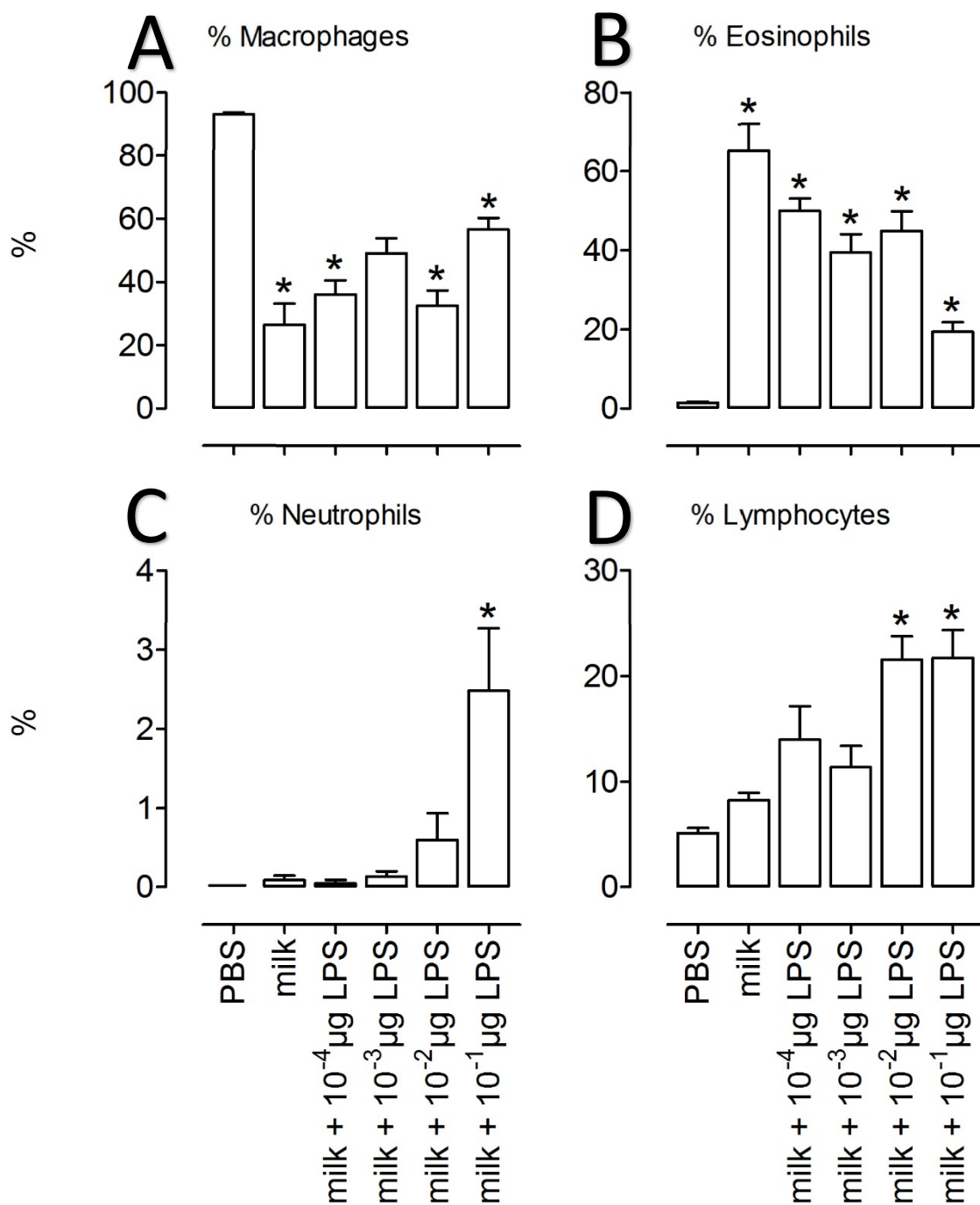


Figure 3.18. Percentages of macrophages (A), eosinophils (B), neutrophils (C) and lymphocytes (D) in BAL of BALB/c mice after 6 intranasal administrations of 50 μ l of PBS, 50 μ g of OVA, 50 μ g of milk, 50 μ g of milk + 10^{-4} μ g of LPS, 50 μ g of milk + 10^{-3} μ g of LPS, 50 μ g of milk + 10^{-2} μ g of LPS, 50 μ g of milk + 10^{-1} μ g of LPS. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.2.4 LUNG INFLAMMATION

Lung inflammation was evaluated via scoring of H&E stained lung tissue sections. Lung inflammation of mice challenged with PBS was scored 0.3 ± 0.1 . Lung inflammation of mice challenged with milk scored 2.1 ± 0.2 . This was consistent with our previous experiments. In the group where $10^{-4}\mu\text{g}$ of LPS was added to milk the score was 2.4 ± 0.1 . Mice challenged with milk with added $10^{-3}\mu\text{g}$, $10^{-2}\mu\text{g}$ or $10^{-1}\mu\text{g}$ of LPS were not significantly different between each other nor significantly different to mice that received PBS. The three groups had values between 1.4 ± 0.1 and 1.6 ± 0.2 .

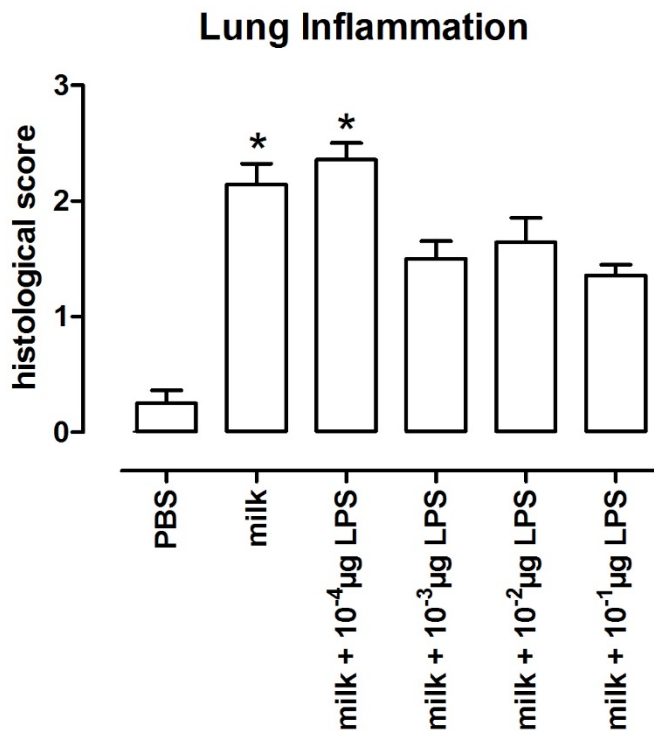


Figure 3.19. Assessment of the lung inflammation of H&E stained lung sections from BALB/c mice after 6 intranasal administrations of 50ul of PBS, 50 μg of milk, 50 μg of milk + $10^{-4}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-3}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-2}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-1}\mu\text{g}$ of LPS. ($n = 7$) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.2.5 EOSINOPHIL ACCUMULATION IN THE LUNGS

Lung eosinophilia was evaluated via counting of eosinophils in LUNA stained lung tissue sections. We counted 4.2 ± 1.7 eosinophils in mice challenged with PBS and 56.9 ± 7.8 eosinophils in mice challenged with milk. The most eosinophils, namely 60.4 ± 5.3 were found in mice challenged with milk with added $10^{-4}\mu\text{g}$ of LPS. Following a similar trend than overall lung inflammation, there was no significant difference in the number of eosinophils in the three remaining groups compared to the PBS-group. We found only 30.4 ± 3.2 eosinophils in mice challenged with milk with added $10^{-3}\mu\text{g}$ of LPS, 40.4 ± 4.9 eosinophils in mice challenged with milk with added $10^{-2}\mu\text{g}$ of LPS and only 16.3 ± 4.6 eosinophils in mice challenged with milk with added $10^{-1}\mu\text{g}$ of LPS.

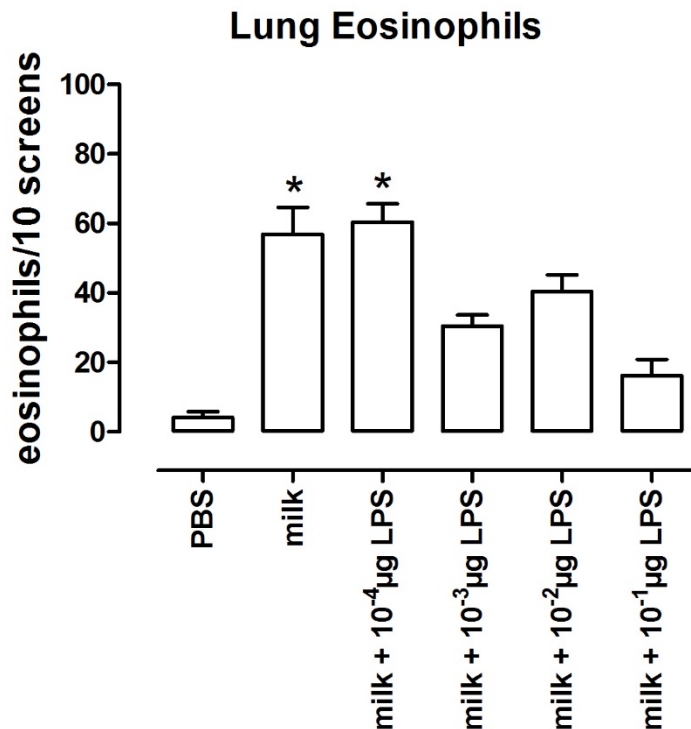


Figure 3.20. Number of eosinophils per randomly chosen 10 microscope screens of LUNA stained lung sections from BALB/c after 6 intranasal administrations of 50ul of PBS, 50 μg of milk, 50 μg of milk + $10^{-4}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-3}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-2}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-1}\mu\text{g}$ of LPS. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.2.6 MUCUS PRODUCTION

Mucosal hyperplasia was evaluated via scoring of PAS stained lung tissue sections as described above. As expected, the mucosal hyperplasia of mice challenged with PBS was scored only 0.5 ± 0.0 . The mice challenged with milk alone had an average score of 2.9 ± 0.1 . None of the doses of LPS added to milk induced any boost in mucus production compared to the milk-group. The highest dose of $10^{-1}\mu\text{g}$ of added LPS only scored 1.9 ± 0.2 on the scale. There was no significant difference compared to the milk-groups of any of the remaining groups where LPS between $10^{-4}\mu\text{g}$ and $10^{-2}\mu\text{g}$ was added. These groups scored between 2.6 ± 0.1 and 2.8 ± 0.1 .

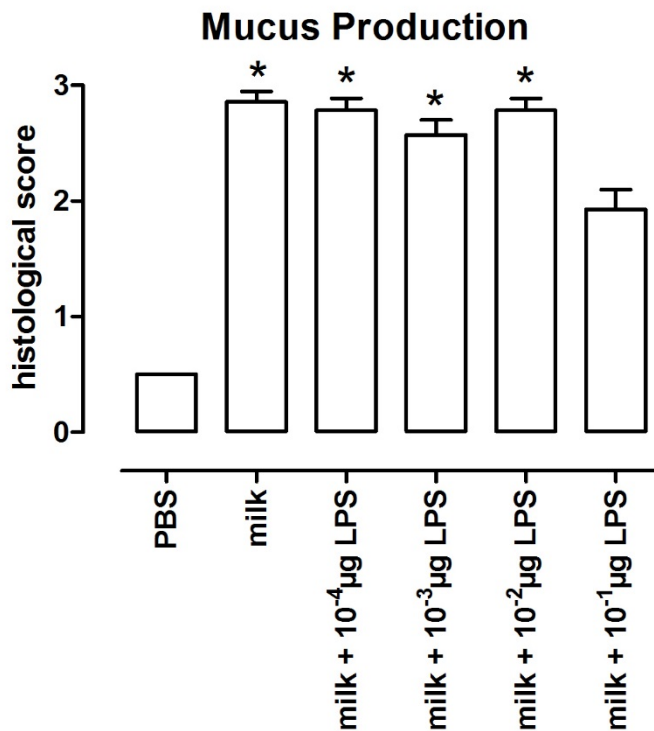


Figure 3.21. Scores of mucosal hyperplasia of PAS stained lung sections from BALB/c after 6 intranasal administrations of 50ul of PBS, 50 μg of milk, 50 μg of milk + $10^{-4}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-3}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-2}\mu\text{g}$ of LPS, 50 μg of milk + $10^{-1}\mu\text{g}$ of LPS. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.1.2.7 MILK SPECIFIC IGG1- AND IGG2A-TITERS

As expected, the titers of milk specific IgG1 were not significantly different in groups challenged with milk alone compared to groups, where $10^{-4}\mu\text{g}$, $10^{-3}\mu\text{g}$, $10^{-2}\mu\text{g}$ or $10^{-1}\mu\text{g}$ of LPS were added to milk. The titers of IgG2a showed slightly more variability, but without relevant differences.

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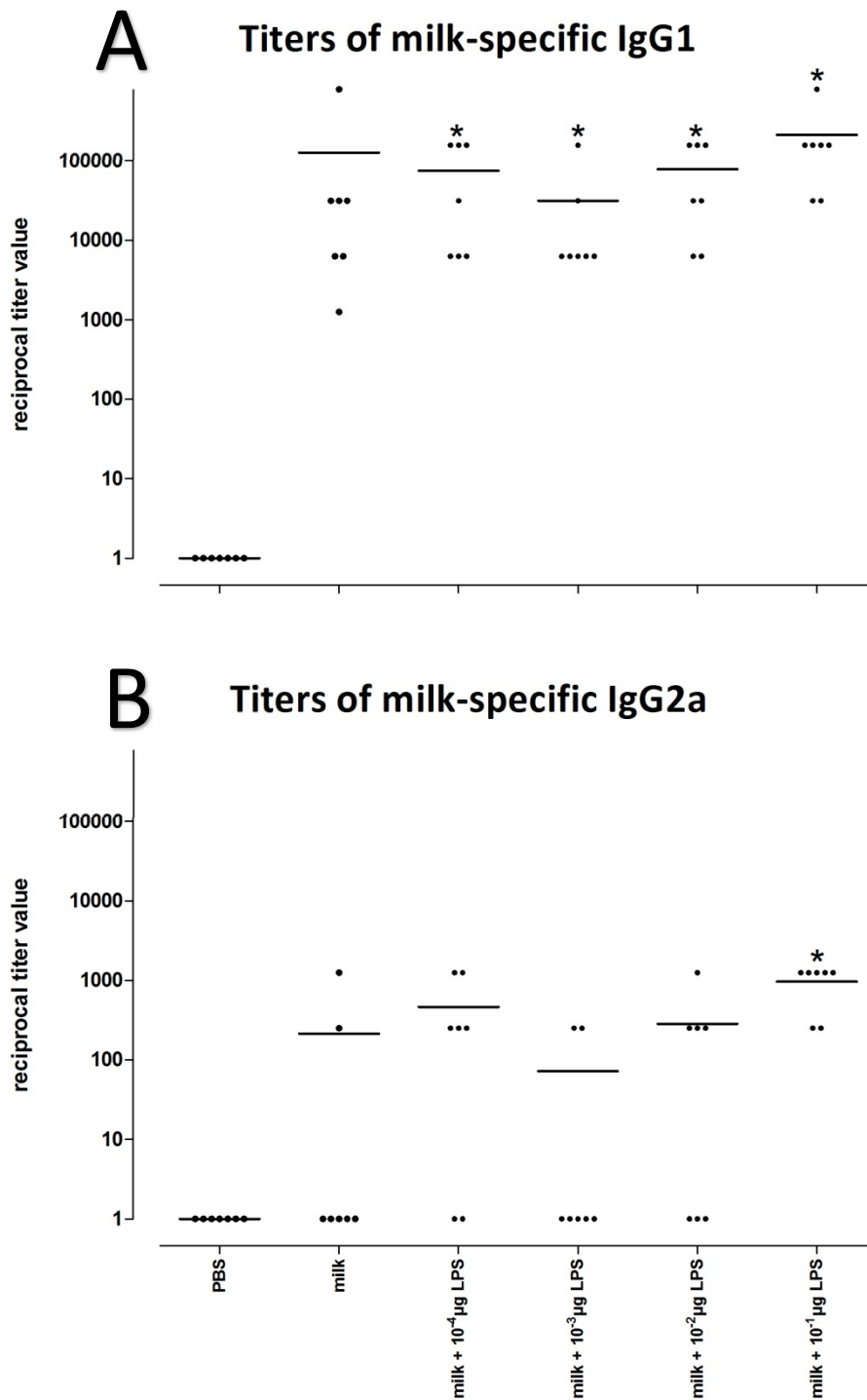


Figure 3.22. Milk-specific titers of IgG1 (A) and IgG2a (B) in BALB/c mice after 6 intranasal administrations of 50 μ l of PBS, 50 μ g of milk, 50 μ g of milk + 10^{-4} μ g of LPS, 50 μ g of milk + 10^{-3} μ g of LPS, 50 μ g of milk + 10^{-2} μ g of LPS, 50 μ g of milk + 10^{-1} μ g of LPS. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

These results indicate, that the eosinophilia in airways and lungs of BALB/c mice reacting to milk cannot be boosted to the magnitude of C57BL/6 mice reacting to milk by adding between $10^{-4}\mu\text{g}$ and $1\mu\text{g}$ of LPS to milk.

3.2.2 COMPARISON BETWEEN THE IMMUNOLOGICAL RESPONSE OF C57BL/6 MICE OF MILK, OVA, AND LPS DEPLETED OVA WITH ADDED DOSES OF LPS

To test whether C57BL/6 mice react differentially to allergens depending on the dose of LPS present within the allergen-challenge solution, we used LPS-depleted OVA as an allergen, and added incremental doses of LPS corresponding to amount of LPS found in the milk ($\sim 10^{-5}\mu\text{g}$ of LPS per administration of $50\mu\text{g}$ of milk) and OVA (between 10^{-3} and $10^{-2}\mu\text{g}$ of LPS per administration of $50\mu\text{g}$ of OVA).

3.2.2.1 TOTAL CELL NUMBER IN BAL

In BAL of PBS challenged C57BL/6 mice we found $62,857 \pm 8,854$ total cells in this experiment. When milk was administered, the total cell number was boosted to $828,571 \pm 148,100$ cells. Only $128,571 \pm 41,230$ total cells were found in C57BL/6 mice that received LPS depleted OVA (“cleaned OVA”). None of the groups that received cleaned OVA with added LPS to it showed a significant increase of total BAL cells compared to OVA or cleaned OVA.

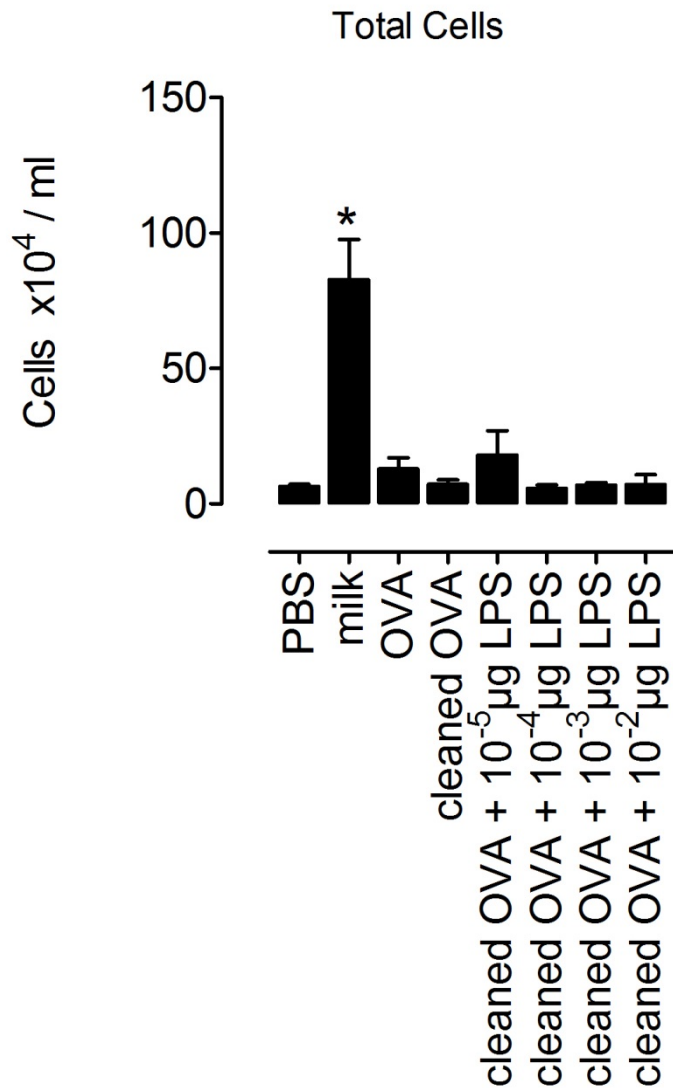


Figure 3.23. Total cell number in bronchoalveolar lavage fluid (BAL) of C57BL/6 mice after 6 intranasal administrations of 50µl of PBS, 50µg of milk, 50µg of OVA, OVA + 10⁻⁵µg of LPS 50µg of OVA + 10⁻⁴µg of LPS, 50µg of OVA + 10⁻³µg of LPS, 50µg of OVA + 10⁻²µg of LPS (n= 7). Shown as mean group value ± SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.2.2 DIFFERENTIAL CELL COUNTS IN BAL

We found $68,705 \pm 4,929$ macrophages in PBS treated, $67,277 \pm 18,830$ in milk treated and $3,577 \pm 7,448$ in OVA treated C57BL/6 mice. None of the different doses of LPS added to cleaned OVA induced a significant change in total BAL macrophages compared to PBS, milk nor OVA treated animals. PBS treated C57BL/6 mice had only $1,103 \pm 396$ eosinophils. The eosinophils in BAL increased to $672,758 \pm 135,500$, when C57BL/6 mice were challenged with milk. Only a mean of $19,835 \pm 8,026$ total eosinophils were found in C57BL/6 mice that received OVA, $24,180 \pm 7,360$ when they received cleaned OVA. Although there were a mean of $119,054 \pm 87,530$ eosinophils in the BAL of C57BL/6 mice that received cleaned OVA with added $10^{-5}\mu\text{g}$ of LPS, this increase was not nearly as high as the increase of eosinophils of milk treated C57BL/6 mice compared to PBS. C57BL/6 mice that received cleaned OVA with $10^{-4}\mu\text{g}$, $10^{-3}\mu\text{g}$ and $10^{-2}\mu\text{g}$ of LPS had a mean of $7,767 \pm 5,152$, $2,931 \pm 1,325$ and $4,914 \pm 3,550$ eosinophils in their BAL respectively. We found 149 ± 76 neutrophils in PBS treated, $20,222 \pm 6,689$ in milk treated and $2,330 \pm 1,419$ in OVA treated C57BL/6 mice. The group that received cleaned OVA had 329 ± 182 neutrophils in their BAL. None of the different doses of LPS added to cleaned OVA induced a significant change in total BAL neutrophils, but a very mild trend to a dose dependent effect is visible. PBS treated C57BL/6 mice only had $3,203 \pm 937$ lymphocytes. The lymphocytes in BAL increased to $66,885 \pm 12,570$, when C57BL/6 mice were challenged with milk. A mean of $39,128 \pm 19,740$ total lymphocytes were found in C57BL/6 mice that received OVA, $11,866 \pm 2,693$ when they received cleaned OVA. There were a mean of $13,190 \pm 5,592$ lymphocytes in the BAL of C57BL/6 mice that received cleaned OVA with added $10^{-5}\mu\text{g}$ of LPS. C57BL/6 mice that received cleaned OVA with $10^{-4}\mu\text{g}$, $10^{-3}\mu\text{g}$ and $10^{-2}\mu\text{g}$ of LPS had a mean of $9,709 \pm 5,344$, $8,700 \pm 1,584$ and $18,545 \pm 13,150$ lymphocytes in their BAL respectively.

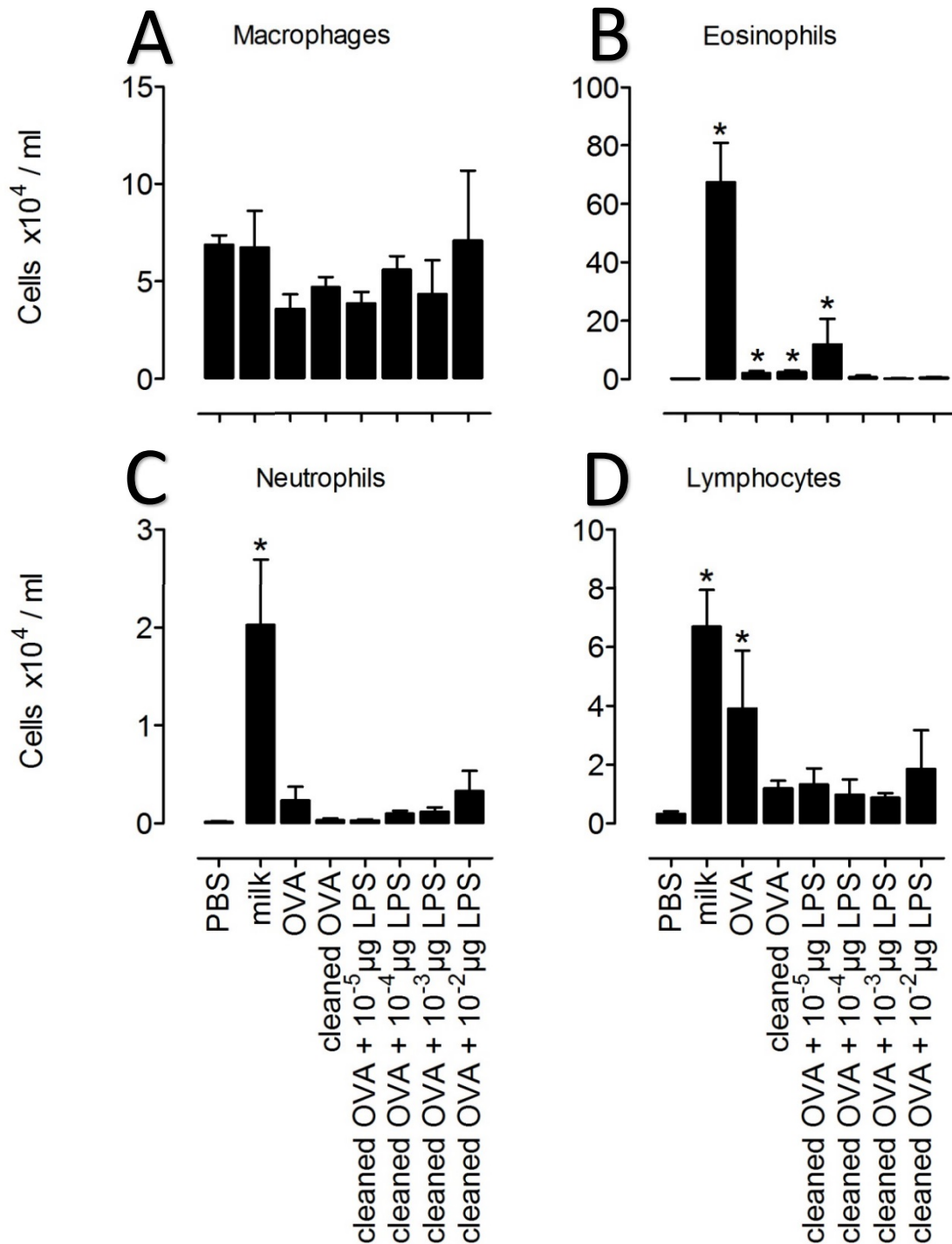


Figure 3.24. Total macrophages (A), eosinophils (B), neutrophils (C) and lymphocytes (D) in bronchoalveolar lavage fluid (BAL) of C57BL/6 mice after 6 intranasal administrations of 50ul of PBS, 50μg of milk, 50μg of OVA, OVA + 10⁻⁵μg of LPS 50μg of OVA + 10⁻⁴μg of LPS, 50μg of OVA + 10⁻³μg of LPS, 50μg of OVA + 10⁻²μg of LPS, 50μg of. (n= 7) Shown as mean group value ± SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.2.3 LUNG INFLAMMATION

Findings from previous experiments were confirmed, that C57BL/6 mice react with stronger lung inflammation to milk than to OVA. But there was no increase of lung inflammation in cleaned OVA treated mice, when LPS in doses between $10^{-5}\mu\text{g}$ to $10^{-2}\mu\text{g}$ were added to cleaned OVA.

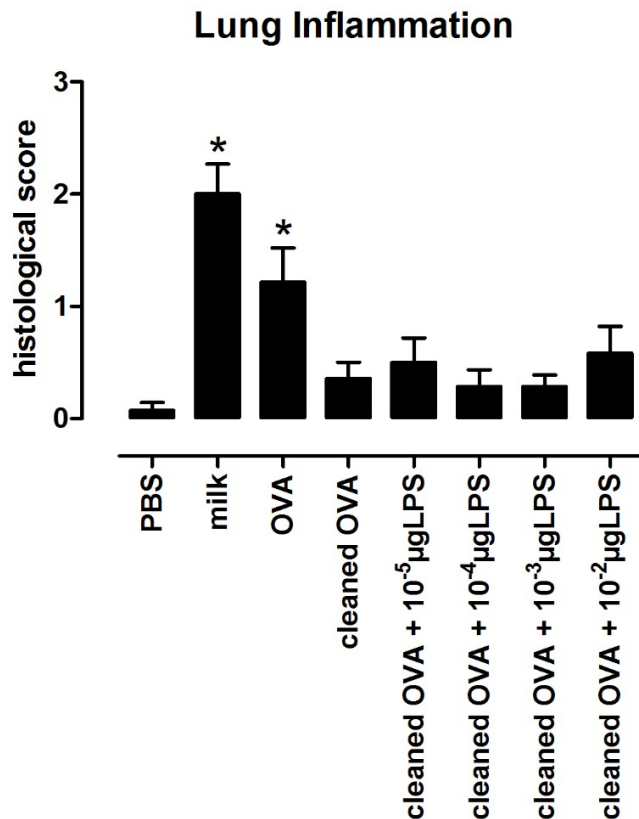


Figure 3.25. Assessment of the lung inflammation of H&E stained lung sections from C57BL/6 mice after 6 intranasal administrations of 50ul of PBS, 50 μg of milk, 50 μg of OVA, OVA + $10^{-5}\mu\text{g}$ of LPS 50 μg of OVA + $10^{-4}\mu\text{g}$ of LPS, 50 μg of OVA + $10^{-3}\mu\text{g}$ of LPS, 50 μg of OVA + $10^{-2}\mu\text{g}$ of LPS, 50 μg of. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.2.4 EOSINOPHIL ACCUMULATION IN THE LUNGS

Findings from previous experiments were confirmed, that C57BL/6 mice develop more severe lung eosinophilia when milk was administered compared to OVA-administration. But there was no increase of lung eosinophilia in cleaned OVA treated mice, when LPS in doses between $10^{-5}\mu\text{g}$ to $10^{-2}\mu\text{g}$ were added to cleaned OVA.

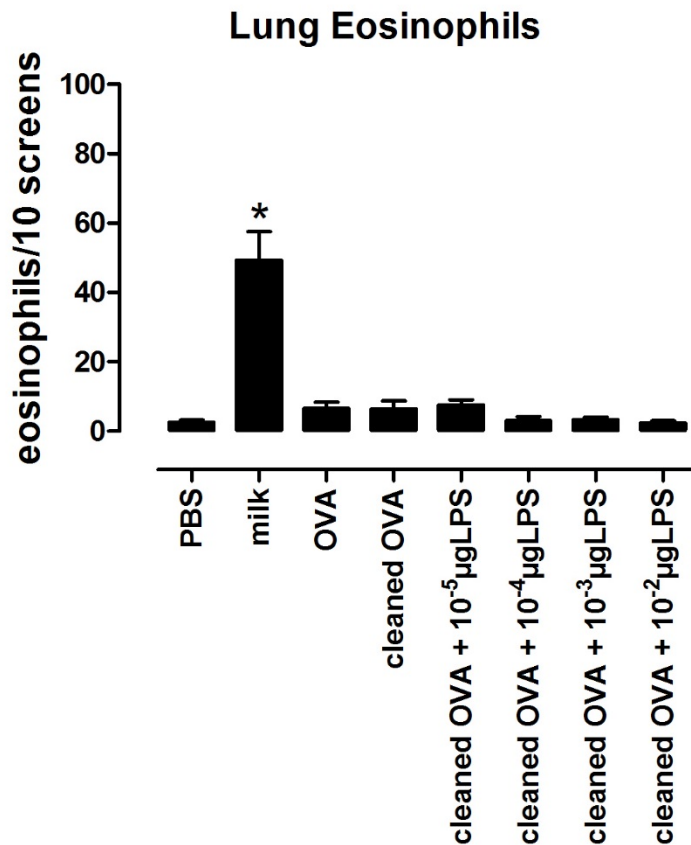


Figure 3.26. Number of eosinophils per randomly chosen 10 microscope screens of LUNA stained lung sections from C57BL/6 mice after 6 intranasal administrations of 50µl of PBS, 50µg of milk, 50µg of OVA, OVA + 10⁻⁵µg of LPS 50µg of OVA + 10⁻⁴µg of LPS, 50µg of OVA + 10⁻³µg of LPS, 50µg of OVA + 10⁻²µg of LPS, 50µg of. (n= 7) Shown as mean group value ± SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.2.5 MUCUS PRODUCTION

Findings from previous experiments were confirmed, that C57BL/6 mice develop more severe mucosal hyperplasia when milk was administered compared to OVA administration. But there was no increase of mucosal hyperplasia in cleaned OVA treated mice, when LPS in doses between 10⁻⁵µg to 10⁻²µg were added to cleaned OVA.

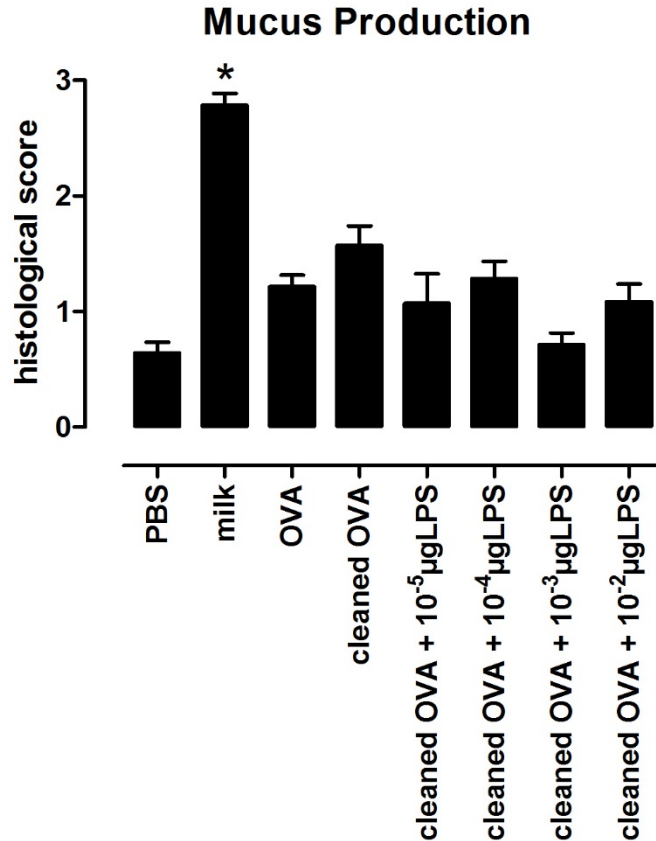


Figure 3.25. Scores of mucosal hyperplasia of PAS stained lung sections from C57BL/6 mice after 6 intranasal administrations of 50ul of PBS, 50μg of milk, 50μg of OVA, OVA + 10⁻⁵μg of LPS 50μg of OVA + 10⁻⁴μg of LPS, 50μg of OVA + 10⁻³μg of LPS, 50μg of OVA + 10⁻²μg of LPS, 50μg of. (n= 7) Shown as mean group value ± SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

3.2.2.6 OVA-SPECIFIC IGG1- AND IGG2A-TITERS IN SERA

As expected, C57BL/6 mice that received PBS did not develop any detectable IgG1 nor IgG2a in their sera. In C57BL/6 mice that received OVA we measured a mean OD-titer of $6.9 \times 10^6 \pm 0.9 \times 10^6$ of IgG1 and $2.4 \times 10^4 \pm 1.0 \times 10^6$ of IgG2a. The levels of OVA-specific IgG1 and IgG2a were not changed significantly regardless whether cleaned OVA, or cleaned OVA with 10⁻⁵μg, 10⁻⁴μg, 10⁻³μg, 10⁻²μg of added LPS were administered, although a mild upward trend was noticeable.

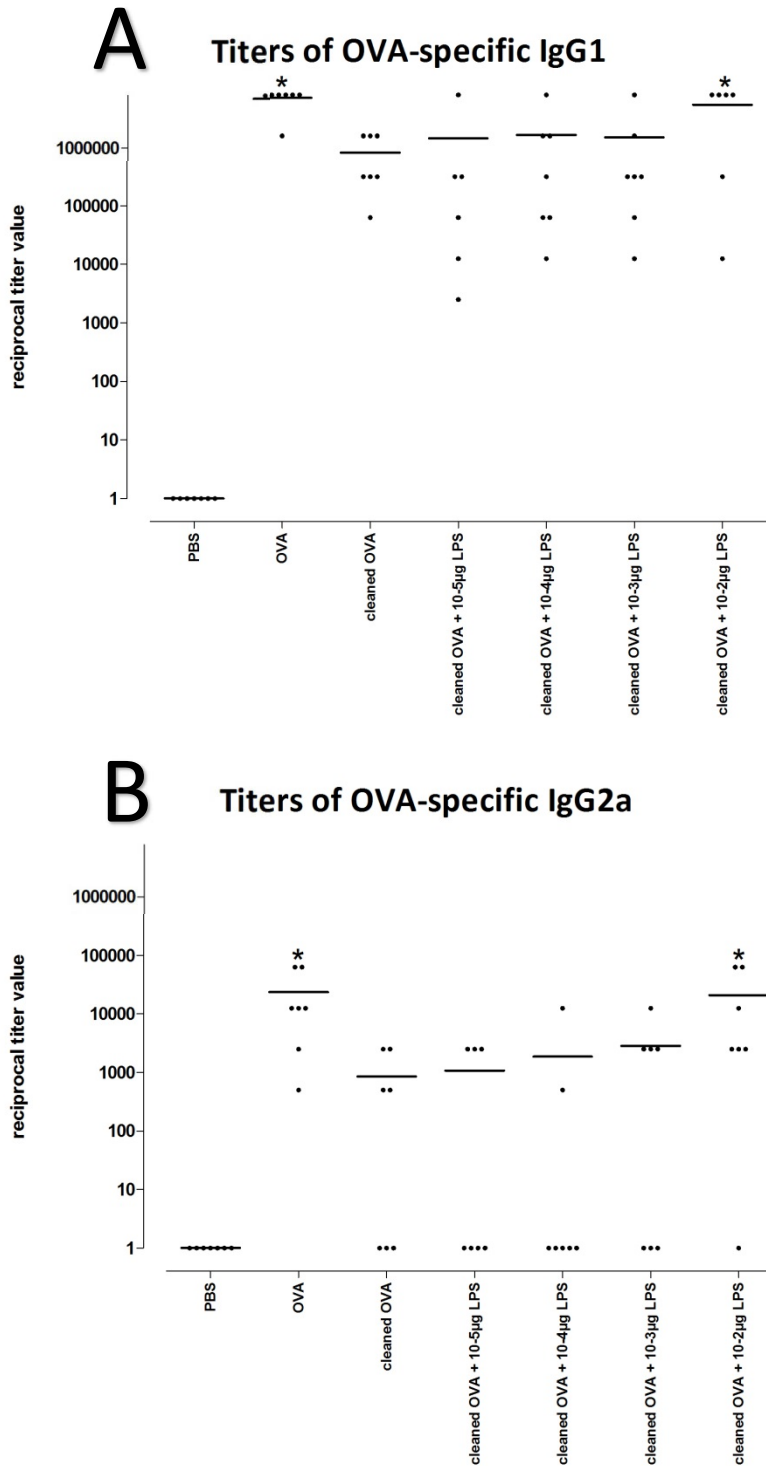


Figure 3.22. OVA-specific titers of IgG1 (A) and IgG2a (B) in C57BL/6 mice after 6 intranasal administrations of 50 μl of PBS, 50 μg of milk, 50 μg of OVA, OVA + $10^{-5}\mu\text{g}$ of LPS 50 μg of OVA + $10^{-4}\mu\text{g}$ of LPS, 50 μg of OVA + $10^{-3}\mu\text{g}$ of LPS, 50 μg of OVA + $10^{-2}\mu\text{g}$ of LPS, 50 μg of. (n= 7) Shown as mean group value $\pm$ SEM and analyzed via One-way ANOVA. * $p < 0.05$ vs. the PBS-treated BALB/c mice.

4 DISCUSSION

This study displays how differences in genetic backgrounds impact the type and magnitude of experimental allergic reactions after exclusively intranasal application of different allergens. We observed that C57Bl/6 mice, compared to BALB/c counterparts, have a stronger allergic response to intranasally administered milk, featuring eosinophilic airway and lung inflammation, extensive mucus production of the lung epithelium and the formation of milk specific IgG1- and IgG2a-antibodies. Further, in C57Bl/6 mice airway eosinophilia was 12-times higher in milk-challenged compared to OVA-challenged mice, while airway eosinophilia was of comparable magnitude in BALB/c mice that received OVA or milk.

Despite intensive research, the complex interaction of genetic and environmental factors that predispose to or protect from development of allergic asthma is still not fully understood [161]. In this work, we focused on adjuvant effects of LPS, as our data indicated that milk and OVA contain different doses of LPS. Depending on the dose, LPS has adjuvant or suppressor effects on allergic responses [94, 99, 162, 163]. Additionally, antigen-presenting cells in BALB/c mice express higher levels of the LPS-receptor TLR4 than C57Bl/6 mice [159]. Therefore, within the scope of this thesis, we tested whether the immunomodulatory properties of LPS affect allergic responses to milk and/or OVA in BALB/c and C57Bl/6 mice. In agreement with previous studies [94] we found that higher doses of LPS (more than $10^{-2}\mu\text{g}$ of LPS) suppress eosinophilic allergic responses. On the other hand, addition of low doses of LPS (less than $10^{-2}\mu\text{g}$ of LPS) did not affect eosinophilic allergic responses to milk in BALB/c mice nor eosinophilic allergic responses to OVA in C57Bl/6 mice in our asthma model. Altogether, our results suggest that the difference in response to allergens between BALB/c and C57Bl/6 mice is attributed to other mechanisms rather than adjuvant properties of LPS.

Variations in the haplotypes of MHC-molecules between inbred BALB/c and C57Bl/6 mice strains could provide such a mechanism. BALB/c express the H-2^d-allele while C57Bl/6 mice express the H-2^b-allele [164-166]. Different MHC-complexes can bind a given antigen peptide fragment with different affinities. This can result in variability of the magnitude and duration of the interaction between antigen presenting cells and other immune cells that require the presented peptide fragment of an antigen as a co-stimulatory signal to become activated to carry out their

function [34]. A peptide fragment with very weak or no affinity for a given MHC-molecule does not provide with the necessary co-stimulation via MHC-peptide-complexes and therefore might prevent the activation of effector functions that result in allergy [167]. To elucidate whether the genetic background or different MHC haplotypes have a greater impact on the development of milk-induced allergic diseases between BALB/c and C57Bl/6 mice, in future experiments allergic response to milk could be compared between wild-type C57Bl/6 and B6.C-H-2^d, a mouse strain with C57Bl/6 background and MHC allele that is expressed in BALB/c mice.

More recent work reveals variations between strains in the leukocyte composition of the respiratory tract. Surprisingly, in C57BL/6 mice more respiratory eosinophils and B-cells than BALB/c mice have been verified. On the other hand BALB/c mice seem to be richer in T-cells, of which some subsets carry out regulatory functions [168]. This can potentially alter the magnitude of an allergic response to a specific allergen by a mouse strain. It has to be noted, that the presence of eosinophils does not equal allergic disease. Neither does a higher baseline of eosinophils in a mouse strain necessarily equal to a higher affinity to allergic diseases in general. This becomes more conceivable when one is reminded of the fact, that many immunotolerant tissues such as the gut are very rich in eosinophils in general [169]. But it might still impact allergic responses to specific allergens such as milk, while there is no impact on other allergens. If that is the case, comparison of many different mice strains with gradual differences in the numbers of baseline eosinophil, T-cells and B-cells could reveal such an impact.

Furthermore, differences in timing and magnitude of cytokine release between BALB/c and C57BL/6 mice were experimentally demonstrated, e.g. INF γ , TNF α , IL-10, IL-12 and IL-13 [168, 170]. These and other findings indicate, that the classical view on C57BL/6 mice as typical Th1-responders and BALB/c mice as typical Th2-responders is oversimplified. Even in these mice strains, that are notorious for their Th1- or Th2-bias we could demonstrate, that milk induces a stronger eosinophilic response in C57BL/6 mice than BALB/c mice. Leveling out the cytokine levels of the mice strains, either by administering the cytokines or antagonizing them with antibodies should equalize the reactions of BALB/c and C57BL/6 mice.

Last but not least, OVA is only one single protein but milk features numerous different macromolecules [79, 171, 172]. One also has to bear in mind, that milk contains compounds, with proinflammatory potential, apart from LPS [79, 171, 172]. Oligosaccharides, fats and fatty acids and proinflammatory proteins could act as adjuvants. It is conceivable, that these

compounds might act as adjuvants or immunomodulatory in one strain, but not the other. To elucidate, whether any of the compounds found in the test allergens might act in this fashion, the adjuvant underlying mechanisms and effector pathways have to be established. By blocking the necessary receptors for these pathways e.g. with specific antagonistic antibodies or using knock out mice with deficiencies in crucial genes of these pathways, the effects of the adjuvants can be studied. Another approach is to deplete milk of one compound at the time and monitor the allergic response of the mice to the remaining compounds as a whole. This could be achieved via use of chromatography columns coated with antibodies with high affinity for the compound in question. If the remaining milk compounds fail to induce allergy, this could indicate an important role of this compound. This top down approach is probably more realistic than a bottom up approach, where combinations of 2 or more compounds of milk are tested one after the other, because of the chemical complexity of milk.

Altogether, in this work, we assessed the mechanisms that lead to divergent allergic responses to 1) the same allergen between 2 mice strains, and 2) different allergens in the same mouse strain. Our data suggest that mechanisms other than adjuvant effects of LPS are responsible for the observed differences. Further investigation of other plausible mechanisms for observed discrepancy in allergic responses could lead to important insights into the initiation of allergic asthma in humans.

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My life doesn't make any sense without you!

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