Immunization with live, virulence-attenuated *Listeria monocytogenes* provides newborn mice with long-term protection against asthma

by

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BSc, University of British Columbia, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

APRIL 2011

ABSTRACT

Asthma is a chronic respiratory disorder that leads to inflammation and narrowing of the airways. The global prevalence, morbidity, mortality and economic cost associated with asthma have been on the rise since the 1960's and continue to increase dramatically by 50% every decade. Today, over 300 million people across all ages, genders and ethnic backgrounds suffer from asthma. In short, asthma has reached epidemic levels. Current treatment options either alleviate symptoms only temporarily, burden the asthmatic with life-long controller medication, or rely on environmental control measures. Children are disproportionally affected by asthma, indicating that asthma most often is initiated early in life. A vaccination strategy able to prevent or cure asthma early in life is therefore urgently needed.

We have successfully developed a novel vaccine platform based on the live, attenuated, intracellular bacterium *Listeria monocytogenes*. We hypothesized that this vaccine platform would induce a sustained anti-allergic Th1 immune response after only one dose given to newborn mice, thus preventing asthma upon future challenge with the allergen.

To test our hypothesis, neonatal mice immunized intraperitoneally with different *Listeria monocytogenes* vaccine strains were compared to negative and positive controls. We examined the protective effects of the following vaccines: the live vaccine strain *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA, synthesizing ovalbumin proteins, 2) the same yet heatkilled vaccine strain HK*Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA and 3) the live vaccine strain *Lm* Δ(*trpS actA*)/pSPO expressing no specific antigens.

Subsequent sensitization and intranasal challenge with ovalbumin to induce asthma was followed by a detailed analysis of asthma severity. This analysis included the total number and types of cells in the bronchoalveolar lavage fluid, as well as histology of lung tissue identifying goblet cell metaplasia and cell infiltration of the airway epithelium. Serum antibody levels and cytokine profiles were also examined, as was airway resistance.

We found that only neonatal mice immunized with the live *Listeria monocytogenes* strains were protected from asthma. This protection did not appear to be mediated by shifts in the Th1/Th2 responses and was found even in the absence of specific antigen expression by *Listeria monocytogenes*.

PREFACE

This thesis was made possible through preliminary work of Dr. Tobias Kollmann and Dr. Daniela Loeffler who were involved in the design and optimization of the research protocol. The performance and analysis of this research project is the student's work. The outcome of this thesis benefitted greatly from collaborative work with Dr. Kelly McNagny's laboratory and technical support from Bing Cai.

The use of animals for the research of this thesis was examined and approved by the Animal Care Committee of the University of British Columbia under the protocol application number A08-0825 on December 17, 2008.

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LIST OF ABBREVIATIONS

Ag	Antigen
AHR	Airway hyper-reactivity
Alum	Alum hydroxide gel
ASMC	Airway smooth muscle cell
APC	Antigen presenting cell
BALF	Bronchoalveolar lavage fluid
BCG	Bacillus Calmette-Guerin
BCR	B cell receptor
BHI	Brain-heart infusion
Ca^{2+}	Calcium
CFU	Colony forming unit
CTL	Cytotoxic T lymphocyte
CO_2	Carbon dioxide
CTLA-4	Cytotoxic T lymphcoyte antigen 4
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoabsorbent assay
EMTU	Epithelial-mesenchymal trophic unit
ER	Endoplasmic reticulum
FcγRIIB	Low affinity IgG Fc receptor
FcεRI	High affinity IgE Fc receptor
H&E	Hematoxylin and eosin
HepB	Hepatitis B
HK*Lm*	Heat-killed *Listeria monocytogenes*
i.n.	Intranasal
i.p.	Intraperitoneal
IFN-γ	Interferon gamma

IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
LLO	Listeriolysin O
Lm	*Listeria monocytogenes*
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MBP	Major basic protein
MCh	Methacholine
MHC	Major histocompatibility complex
NaCl	Sodium chloride
NK cell	Natural killer cell
NLR	Nod-like receptor
OVA	Ovalbumin
PAF	Platelet activating factor
PAMP	Pattern-associated molecular pattern
PRR	Pattern recognition receptor
PAS	Periodic Acid Schiff
PBS	Phosphate buffered saline
RBC	Red blood cell
SEM	Standard error of the mean

SNP	Single-nucleotide polymorphism
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th1 cell	T helper cell 1
Th2 cell	T helper cell 2
Th17 cell	T helper cell 17
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
T_{reg}	T regulatory cell
trpS	Tryptophanyl-tRNA synthetase
TSLP	Thymic stromal lymphopoietin
VCAM-I	Vascular cell adhesion molecule I
WT	Wild-type

ACKNOWLEDGMENTS

I am thankful to Dr. Tobias Kollmann for the opportunity to join his laboratory, giving me guidance and counsel along the way and being a supervisor whose expertise and passion inspired me deeply. He has kept his promise to help me get to where I want to be today.

This project owes much to the insights of my committee members Dr. Kelly McNagny and Dr. Stuart Turvey and my external examiner Dr. John Dean who enriched my research work through their sharing of experiences from their fields of research and clinical work. Their questions have encouraged me to broaden my analysis and I have benefitted greatly from their advice.

I need to acknowledge with gratefulness the members of Dr. Kollmann's laboratory, in particular, Dr. Daniela Loeffler and Kinga Smolen who trained me with thoroughness and patience in my first steps as a scientist, as well as AllerGen NCE for funding this research project. Dr. Loeffler's preceding work, knowledge and experience have been key to the success of this thesis. She has shown continuous faith and confidence in my work and has been a great source of encouragement.

I am deeply grateful to Mr. Matthew Walko who has been my bridge over troubled water. He showed grace when research-related frustrations were challenging to handle and kept cheering me on during the final stretch of this project. Words will never be enough to express my gratitude.

I also owe heartfelt thanks to Ms Elizabeth Scott (soon to be Mrs Eshpeter) for enthusiastically celebrating the scientific victories with me and providing strong support and encouragement when it was time to push through difficulties. Knowing her has been a great privilege and comfort – it was being understood without needing to say anything.

Deep appreciation goes to my parents for instilling in me dedication and discipline for whatever I choose to undertake. They have shown me unconditional and incessant support and love throughout all those years and across all these miles of ocean and land.

The greatest expression of gratitude must go to my Creator.
Soli Deo Gloria.

DEDICATION

To my Maker

CHAPTER 1: INTRODUCTION

1.1) ASTHMA

1.1.1) Types of asthma

Asthma is classified based on the underlying stimuli that trigger the disease [1]. Patients suffering from extrinsic (allergic or atopic) asthma – mostly children – are generally asymptomatic until they are exposed to allergens they have previously been sensitized to. After exposure to the allergen, abnormal adaptive immune responses develop and are characterized by the production of allergen-specific immunoglobulin E (IgE) antibodies and involvement of T helper 2 (Th2) cells that identify allergen-derived antigens (Ag) [2]. Oftentimes, these patients exhibit a hereditary predisposition towards the development of allergic asthma and are also more susceptible to other allergies [3]. Intrinsic (non-allergic or non-atopic) asthma usually develops in later stages of life and is typically the result of non-allergenic triggers such as cold air or exercise. This type of asthma manifests itself in an IgE-independent fashion and appears to be non-familial [4]. For the course of my investigations, the focus will be on atopic asthma.

1.1.2) Disease pathophysiology

Asthma is a chronic respiratory disease that manifests itself as inflammation of the pulmonary airways, elevated mucus production by epithelial goblet cells, bronchial smooth muscle hyper-responsiveness triggered by environmental factors resulting in bronchoconstriction and narrowing of the airways [5]. The clinical symptoms of asthma include shortness of breath, chest tightness, wheezing, chronic cough and in severe cases can lead to complete airflow obstruction (asphyxiation) [6]. The ultimate airway obstruction and airway narrowing is brought about by 4 sequential steps: **a)** allergen sensitization, **b)** type I hypersensitivity reaction (early-phase reaction), **c)** late-phase reaction and **d)** chronic allergic inflammation [7].

Allergen sensitization. Prior to the first hypersensitivity reaction, sensitization to an allergen is required [8,9]. This starts with the activation of antigen-presenting cells (APC)

through their interaction with an allergen. The allergen is generally a protein, but may also be a lipid or carbohydrate [10]. Activated APCs migrate to a regional lymph node or the local mucosa where they present allergen-derived peptides on major histocompatibility complex II (MHC-II) molecules to T-cell receptors (TCR) on naïve T cells. This interaction encourages naïve T cells to differentiate into Th2 cells and is facilitated by both, the binding of Jagged to Notch on the surface of antigen-presenting cells and naïve T cells respectively, and an interleukin 4 (IL-4) dominated environment created primarily by basophils [11,12]. Upon their activation, Th2 cells generate further IL-4 and interleukin 13 (IL-13), which in combination with co-stimulatory molecule binding on Th2 and B cells induces B cell maturation and antibody isotype switching towards IgE. IgE spreads locally, enters the lymphatics and blood and accesses the interstitial fluid where it is bound by cell surface receptors (FcεRI) on mast cells, basophils and eosinophils [13,14]. This sensitizes these cells to immediately trigger type I hypersensitivity reactions upon later allergen exposure.

Type I hypersensitivity reaction. Exposure of FcεRI-bound IgE to allergen leads to the release of pro-inflammatory mediators from mast cells which induce a lung inflammatory response that results in acute functional organ changes characterized by vasodilation, increased vascular permeability and mucus secretion into the airways, as well as bronchoconstriction. The pro-inflammatory mediators can be released quickly through degranulation (e.g. histamines and proteases), produced via phospholipid breakdown (e.g. prostaglandins, leukotrienes and platelet-activating factors), or actively transcribed and translated anew in mast cells (e.g. cytokines, chemokines and growth factors) [14]. The latter process takes the longest and induces the transition into the late-phase hypersensitivity reaction.

Late-phase reaction. While the type I hypersensitivity reaction is mostly mediated by IgE, the late-phase reaction is characterized by the action of innate and adaptive immune cells that have been recruited to the lung tissue through pro-inflammatory mediators of the early-phase hypersensitivity reaction [7]. The recruitment of inflammatory leukocytes (e.g. T cells, neutrophils and eosinophils) from the circulation into the tissue is facilitated by increased vascular permeability, upregulated adhesion molecules on vascular airway endothelial cells and the synthesis of chemotactic molecules and chemokines [11]. Antigen-stimulated T cells, other

tissue-resident immune cells and their proinflammatory cytokines all take part at this stage of the hypersensitivity reaction [12,15].

Chronic inflammation. In the case of repetitive or chronic allergen exposure, inflammation persists and can structurally and functionally affect all layers of the airway via tissue remodeling [8]. This can be scored as thickening of the airway walls, goblet cell hyperplasia, swelling of the lamina reticularis below the epithelial basement membrane and airway smooth muscle hyperplasia [11]. Chronic inflammation is also characterized by a heavily increased density of innate and adaptive cells in the epithelial-mesenchymal trophic unit (EMTU) - the pulmonary tissue composed of the airway epithelium and underlying submucosa [7]. A complex interaction between recruited/resident cells in the EMTU and epithelial cells, structural cells, nerve cells, blood vessels and lymphatic vessels is considered to sustain inflammation, driven predominantly by Th2 cells.

1.1.3) Risk factors for asthma development

In order to halt the continuously rising tide of asthma and allergic diseases, it is crucial to identify the factors responsible for pathogenesis and to implement primary prevention strategies. However, despite many studies, the precise underlying etiology that leads to childhood asthma still remains unclear [16]. Current research findings suggest that a multi-factorial interplay of genetic pre-dispositions and lifestyle-dependent environmental factors linked to industrialization and urbanization all contribute to the development of asthma. Establishing the root cause(s) of asthma clearly is a complex undertaking, since it is difficult to differentiate between the cause and the consequence of asthma. Duffy et al. (1998) suggest that “the nature of the relationship between allergic sensitization to specific allergens and asthma can either be a direct causative one, with allergen exposure causing asthma in susceptible individuals, or an indirect one, where the genetically determined atopic diathesis causes both asthma and expression of sensitization to ubiquitous aeroallergens” [17]. The determining factors in the process of allergen sensitization include the allergen concentration, the kind of allergen, route and frequency of allergen exposure, genetic susceptibility to allergen sensitization in the host, and co-exposure to sensitization-facilitating agents such as viral products and stimulatory substances such as endotoxins [16-18]. It also appears that the time of exposure and sensitization to allergens and

endotoxins is crucial for wiring the asthmatic immune system into responding suboptimally to environmental stimuli [16,18]. The perinatal time frame during which the immune system develops – especially the time spanning *in utero* and the first year of life – has been shown to be particularly sensitive for priming the immune system to protect from, or enable the development of asthma and atopic disease. As such, events in early life that could interfere with the perinatal maturation of both, the innate and adaptive arm of the immune system (e.g. viral respiratory infections or persistent allergic sensitization), could imprint atopic tendencies into the neonate's immune system [18]. Similarly, the mother's immune system, antibody levels, nutrition and exposure to allergens and pollutants may already impact the fetal immune system development and maturation [19]. Therefore the early stages in life should form the focus of prophylactic strategies for individuals susceptible to atopy.

An individual's genetic heritage also plays a role in how the immune system responds upon exposure to specific environmental risk factors. For example, a loss-of-function mutation in the filaggrin-encoding gene *FLG* has been shown to increase the epithelial permeability to allergens in humans, compromise the protective function of the epithelium and represents a significant risk factor for peanut allergy [20]. Atopy has also been linked with genetic differences responsible for immune cell signaling efficiencies and immune balancing capabilities. Carrying single nucleotide polymorphisms (SNP) associated with the cytotoxic T-lymphocyte antigen 4 (CTLA-4) or the endotoxin receptor CD14 could therefore be unmasked by changes in the environment and threaten the immune balance [19,21]. Through genome-wide association studies, light was also shed onto other asthma susceptibility loci expressed particularly in the airway epithelium and in T-helper cells. Although the biological function of many of these genetic variants has not yet been established, significant associations between asthma and SNPs have been identified and confirmed, for example, for chromosome 2, implicating *IL1RL1/IL18R1*, on chromosome 9, flanking *IL33I* and the *ORMDL3/GSDMB* locus which was strongly associated with early-onset of asthma and mapped onto the asthma susceptibility locus of chromosome 17q21 [22].

However, more recent studies have revealed that the classical asthma risk factors – genetic predispositions towards atopy and environmentally induced epigenetic changes in gene expression – cannot fully account for the pathophysiology of clinically manifested asthma nor

the overall epidemiology of asthma. It is unlikely that the penetrance of a asthma susceptibility gene could have established itself over the course of a handful of decades. The focus has therefore shifted, suspecting that the underlying host genome directs the resident microbiome in the lung and/or at other mucosal surfaces and could provide the "missing link" in the mediation of atopic asthma [23].

1.1.4) Options for asthma treatment

Current treatment options for allergic asthma aim to relieve bronchoconstriction and reduce airway inflammation. According to the guidelines of the "Canadian Thoracic Society Asthma Management Continuum" (2010), asthma management should include environmental control measures in combination with continuous use of controller medication (e.g. inhaled corticosteroids), rather than sporadic drug therapy focused on temporary relief of asthma symptoms (e.g. short-acting β2-agonists) [24].

1) **environmental control measures**

   In order to implement environmental control measures for atopic asthma, specific asthma-triggering allergens are identified via the skin-prick test. Using environmental interventions like pillow and mattress encasements, air purifiers or HEPA filters on vacuum cleaners can then, for example, limit the exposure to asthma-triggering allergens like dust mites [25]. However, efforts to reduce or eliminate exposure to these specific allergens are not only time-consuming and costly but also do not provide complete protection [24,26].

2) **drug therapy**

   In the early days of asthma therapy, the focus was on alleviating bronchospasm with bronchodilators that act, for example, as agonists of β-adrenergic receptors. However since then, inflammation has been revealed as the main underlying mechanism driving asthmatic symptoms, including bronchospasm [27]. Anti-inflammatory drugs are therefore today's first choice in managing mucosal edema, controlling airway narrowing and preventing asthma attacks [24]. This is achieved through the reduction or neutralization of effector- and inflammatory molecules. However, these drug therapies (e.g. inhaled corticosteroids) are non-curative, only effective for acute disease and require long-term medication to confine the chronic asthma symptoms.

3) **immunotherapy**

Immunotherapy (desensitization) applies repeated injections of small allergen doses to create tolerance to those allergens that cause asthmatic symptoms in patients with atopic conditions [28]. The goal is the induction of an altered T-cell and antibody response that is skewed towards an anti-inflammatory T helper 1 (Th1)-response. Current concerns associated with immunotherapy in asthmatic patients revolve around safety issues, the time- and labour-intensive procedures required to reach tolerance, the lack of trained personnel and the specificity of this procedure in the light of allergic susceptibility to multiple allergens in most patients [29,30].

4) **anti-IgE monoclonal antibody therapy**

Anti-IgE monoclonal antibody therapy administers the drug Omalizumab [31]. It contains non-anaphylactogenic anti-IgE antibodies which neutralize serum free IgE antibodies by blocking their binding to FcεRI receptors on mast cells, thereby inhibiting degranulation and pro-inflammatory mediator release of these cells. Similar to immunotherapy, anti-IgE therapy entails a time- and labour-intensive procedure and is costly.

5) **vaccination**

The rationale behind vaccine development for allergic asthma is the targeting of the underlying causes of asthma brought about by Th2-skewed immune responses, rather than focusing on the resolution of asthma symptoms [32,33]. Although several vaccine strategies have been or are being tested in clinical trials, a successful candidate that a) does not rely on multiple booster injections over several years and b) does not include the risk of an IgE-mediated adverse event (e.g. anaphylaxis) has yet to emerge [34].

We therefore believe that the development of an immunotherapeutic vaccine, modifying the course of asthma pathology through the conversion or deviation of detrimental allergic responses toward protective immune responses, is currently the best option for effective and long-lasting asthma treatment.

1.1.5) Perinatal Th1/Th2 polarization and implications for childhood asthma

Research to date suggests that CD4+ Th2 lymphocytes play a fundamental role in the pathogenesis of asthma [8]. Especially asthma's inflammatory processes appear to be the result

of strong Th2 immune responses towards aeroallergens in genetically susceptible patients [35]. Non-allergic individuals exhibit, in contrast, a propensity for Th1 responses to allergens, characterized by synthesis of interleukin 12 (IL-12) which promotes Th1 differentiation, as well as interleukin 2 (IL-2) and interferon gamma (IFN-γ) production that downregulate Th2 responses or maintain a Th1/Th2 balance. The human body relies on these Th1 immune responses when combating pathogenic infections and mediating allergen invasion [8].

The heightened susceptibility to common infectious diseases and tendency to develop Th2-mediated allergic conditions early in life may stem from the pre-dominant Th2 allogeneic maternal environment during pregnancy, which is necessary for materno-fetal compatibility [19]. After birth, neonates may temporarily continue expressing fetal, Th2-promoting genes and therefore exhibit delayed or reduced Th1 functional capacity – seen in low production of IFN-γ [19,35]. This poses a challenge to the neonate's "immunologically inexperienced" immune system in the face of an allergen-rich, post-natal environment [36,37]. Thus, Th1 maturation towards a functional Th1/Th2 balance must immediately follow birth in order to minimize the window of vulnerability towards diseases associated with immune imbalances.

The "hygiene hypothesis" has also been postulated as a mechanistic explanation of the increase in allergies and asthma in the industrialized world. According to this view, allergy and asthma have become an "epidemic in the absence of infection" [38]. A decrease in exposure to infectious agents in early life is believed to result in the absence of a shift in the immune system's allergic Th2 bias to a non-allergic Th1 response [39].

However, accumulating evidence suggests that neither the hygiene hypothesis nor the oversimplified Th1 vs. Th2 concept adequately explain the observed rise in allergic diseases, nor do they reveal the underlying mechanisms causing the asthmatic immune pathology. More recent studies in asthmatic humans in fact found mixed Th2 *and* Th1 responses characterized by elevated levels of both Th2 type interleukin 5 (IL-5) and Th2-suppressing IFN-γ, suggesting that Th1 and Th2 responses are not mutually exclusive when considering atopic pathology [40,41]. These observations call into question the classical thought of environmental factors polarizing the immune response towards a strict Th2 response while inhibiting Th1 responses and argue more favourably for failed *immune regulation* underlying atopic responses. It should be kept in

mind that it is with caution that Th1/Th2 paradigms studied in murine models can be translated to the human immune system, as the clear separation between Th1 and Th2 seen in mice is not as prominent in humans [42,43].

T helper 17 (Th17) cells have been recently proposed as a third subset of T helper cells involved in the mediation of the pathophysiology in inflammatory lung disorders [44,45]. They act as source of interleukin 17 (IL-17), which induces the production of several pro-inflammatory cytokines from structural cells and therefore participate in the recruitment of inflammatory cells to the site of inflammation [46,47]. In a mouse model of allergic inflammation, IL-17 was seen upregulated in lung tissue after allergen challenge. It has also has been found at elevated levels in bronchoalveolar lavage fluid (BALF) and blood in asthma patients [48,49].

Lastly, a specialized subset of T cells known as regulatory T cells (T_{reg}) has also been shown to control different stages of asthma pathogenesis through mediating suppression of adaptive immune responses controlled by Th1 and Th2 cells or by interactions with APCs [50-53]. This action is thought to be inferred through T_{reg} cytokines like interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) or surface molecules like CTLA-4 and Notch-3 [52].

In summary, current data suggests that environmental changes affect several converging immune responses, including cells that regulate immunomodulatory pathways (e.g. antigen presenting cells and effector T cell signaling). APCs participate in the first stages of asthma pathophysiology through their direct interaction with the allergen. APCs such as monocytes/macrophages and dendritic cells are part of the innate immune system that functions as a sentinel at the interface between the host and the environment. APCs thus possibly initiate the entire pro-allergic Th2- and/or Th17-dominated immune responses in asthmatics. Taken together, several immunomodulatory pathways are likely to play a role in asthma pathogenesis:

- An increased production of Th2 cytokines like IL-4, IL-5 and IL-13, which act in concert to initiate and sustain the allergic asthmatic inflammatory response by enhancing the production of IgE and the growth, differentiation and recruitment of mast cells, eosinophils and basophils [42].
- An increase in allergen-specific pro-inflammatory Th17 cells [47].

- A lack of T_{reg} cells mediating immunosuppression through cytokines that generally restrain inappropriate immune responses towards environmental triggers (e.g. IL-10 and TGF-β) [51].

It already is a complex task to decipher all these interacting factors within a *fully matured* subject; however, it is even more difficult to do so in the context of a *developing* (i.e. rapidly changing) neonatal immune system [19].

1.2) VACCINE DEVELOPMENT FOR CHILDHOOD ASTHMA

1.2.1) Vaccination rationale

Immunization is a powerful tool for controlling and preventing life-threatening diseases and has been shown to avert millions of deaths each year by preventing specific microbial infections [54]. By mimicking a natural infection without causing disease, the immune system mounts an immune response against particular disease-specific immunogens. This leads to immunity and protection of the host upon later exposure [55]. The immunogen represents an antigen that is specifically targeted by the immune system and is administered in the vaccine cocktail along an adjuvant that allows for a more optimal immune response. For immunization to succeed, both arms of the adaptive immune system need to be activated which leads to a) cell-mediated immunity regulated by the two T cell subsets CD4+ and CD8+ and b) humoral immunity mediated by antibody production of B cells [54,55]. Receptors on B and T cells (BCR and TCR respectively) primed to recognize specific antigens, clonally expand upon exposure to these antigens and eradicate the pathogen. A subset of these expanded cells continues to mature into antigen-specific memory cells, which eliminate future infections with the same pathogen with greater speed and efficiency [54-56].

1.2.2) Vaccine strategies for early life administration

The underlying cause of asthma is a complex interaction between environmental, genetic and developmental components. Early life vaccination thus aims to target the latter component and direct the neonatal immune development away from an *in utero*-induced Th2-bias. Since neonates are at an increased risk for contracting allergic diseases as well as infections, vaccination early in life is both, crucial and challenging [57]. The challenge arises from the limited antigen presentation capacity in this population group, weak and short-lived antibody responses, decreased cell-mediated immune responses, apparently skewed Th2-type immune responses and the presence of inhibitory, maternal antibodies [58,59]. However, suitable vaccines administrated early in life – for example HepB (Hepatitis B) and BCG (Bacillus Calmette-Guerin) – have shown that effective and safe vaccine strategies for neonates can be implemented [60,61]. Ideal neonatal asthma vaccine candidates that result in long-lasting and robust immune responses should therefore:

a) deliver vaccine antigens directly to antigen presenting cells,

b) activate both cellular (CD4 and CD8) and humoral (B cell) effector responses and induce immunologic memory,

c) act as strong immune adjuvants to augment adaptive immune responses,

d) mount protective or curative immune responses to asthma after only one immunization dose administered early in life and

e) circumvent typically weak and short-lived antibody response of the "immature" immune system and the inhibitory influence of maternal antibodies.

1.3) *Listeria monocytogenes*

1.3.1) Characteristics of the bacterium

Listeria monocytogenes (*Lm*) is a gram-positive, facultative, intracellular bacterium that is highly motile throughout its different life stages: It relies on its flagella while residing outside host cells and propels itself through actin polymerization inside infected cells and when spreading from cell to cell [62]. *Lm* can establish systemic food-borne disease (listeriosis) in the

immuno-compromised and elderly and can infect fetuses by crossing the placenta. *Lm* is able to infect a variety of host cells and is enclosed by the cell's phagosome upon invasion (Fig.1). While residing inside the phagosome, bacterially expressed peptides are presented on MHC-II molecules to CD4 T cells. *Lm* is able to evade the phagosome by synthesizing listeriolysin O (LLO), a pore-forming protein [63]. After evading this vacuole, *Lm* hijacks the host's actin resources, which are then polymerized into actin filaments that allow the bacterium to move inside the host's cytoplasm and infect adjacent cells without entering the extracellular, antibody-patrolled milieu [64]. This process of actin nucleation relies on the bacterial surface protein ActA, which recruits host cell proteins normally involved in the construction of the host cell cytoskeleton (e.g. profilin) [64,65]. Inside the cytoplasm, *Lm* replicates rapidly and continues synthesizing bacterial proteins. These peptides are proteasomally degraded and transported to the host's endoplasmic reticulum (ER) where they are assembled onto major histocompatibility complex (MHC-I) molecules. The peptide/MHC-I complex is then shuttled via the Golgi apparatus to the host's cell membrane. Bacterial antigens are presented there via MHC-I molecules to cytotoxic T-lymphocytes (CTL) CD8 T cells, eliciting a pre-dominantly CD8 T cell-mediated immune response [62]. *Lm* infections therefore are predominantly cleared via cell-mediated immunity through both CD4 and CD8 Th1 T cells.

1.3.2) *Listeria monocytogenes* as a vaccine vehicle

Over the last decade, research efforts have focused on the development of vaccine strains based on the delivery of antigen-encoding deoxyribonucleic acid (DNA) or heterologous antigens through live virulence-attenuated or non-pathogenic bacterial strains [66]. Some groups have focused on the administration of whole, heat-killed bacteria like *Mycobacterium bovis, Propionibacterium acnes* and *Bordetella pertussis* to prevent allergic sensitization and allergic inflammation following local allergen challenge [67]. However, one of the most potent inducers of IFN-γ, the best-known anti-Th2 effector molecule, is the intracellular bacterium *Lm* [68]. Several publications have demonstrated that even heat-killed *Lm* (HK*Lm*) acts as a strong immune adjuvant [69-71]. When injected with model allergens, HK*Lm* is able to inhibit and reverse allergic diseases in adult mice and dogs by mounting antigen-specific immune responses characterized by high Ag-specific IFN-γ production and large quantities of antigen-specific

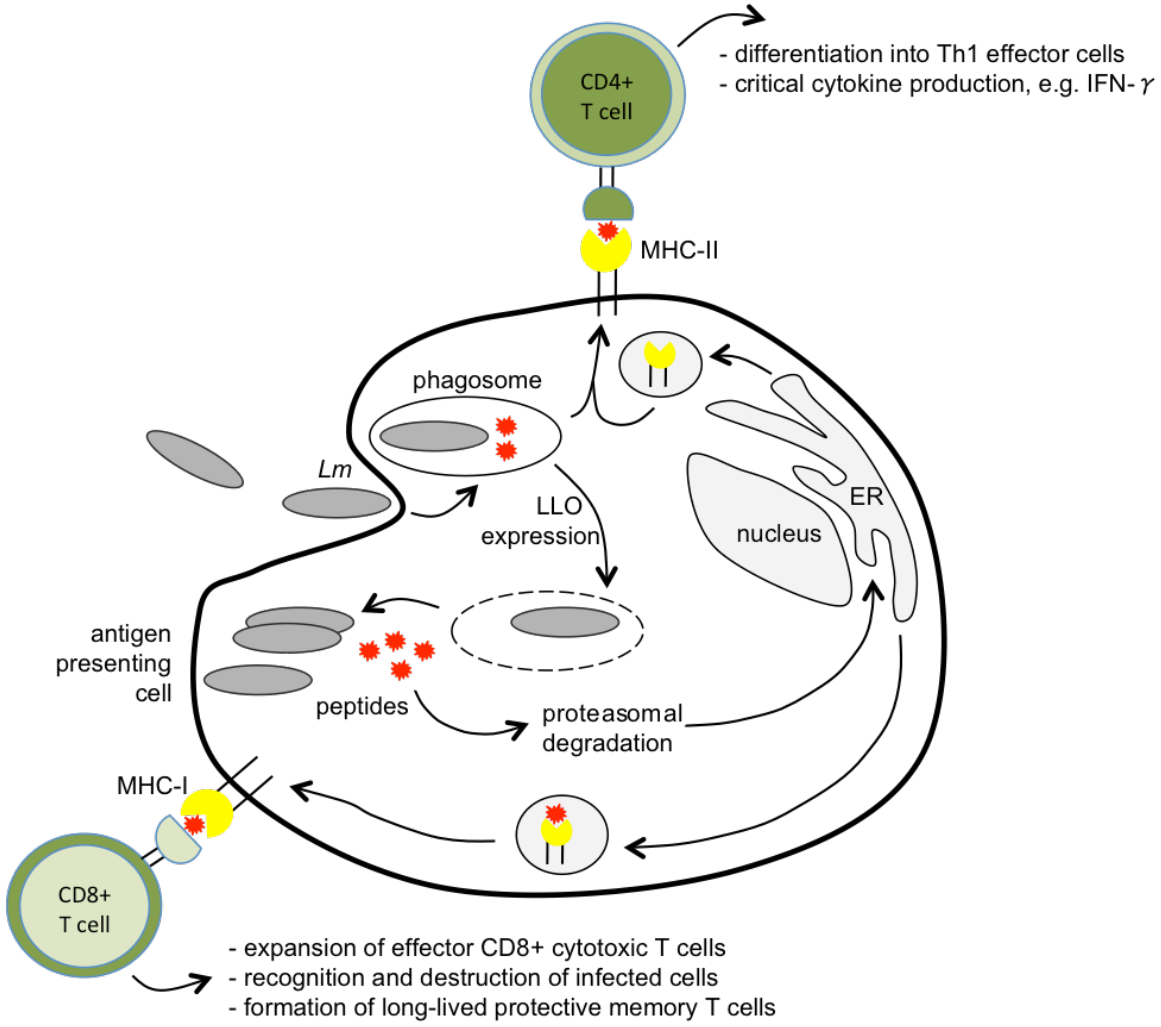

Figure 1. The intracellular lifestyle of *Listeria monocytogenes*. *Listeria monocytogenes* (*Lm*) is taken up by an antigen presenting cell and is localized to a phagosome. Bacterial proteins expressed in the phagosome (red) are presented via MHC-II molecules to CD4+ T-cells. The virulence factor listeriolysin O (LLO) allows *Lm* to escape this vacuole and replicate in the host cytoplasm. Bacterial peptides synthesized in the cytosol undergo proteasomal degradation and are transported to the host's endoplasmic reticulum (ER) where they are assembled onto MHC-I molecules. The peptide/MHC-I complex is then shuttled via the Golgi apparatus (not shown) to the host's cell membrane. Bacterial antigens are presented there via MHC-I molecules to cytotoxic T-lymphocytes (CTL) CD8+ T cells, eliciting a cellular immune response. The exogenous pathway of antigen presentation results in differentiation of CD4+ T cells into Th1 effector cells that synthesize Th1 cytokines like IFN-γ. The endogenous pathway of antigen presentation on the other hand leads to the expansion of effector CD8+ cytotoxic T cells which recognize and destroy infected cells. A population of CTL will also remain to form long-lived protective memory T cells.

IgG2a. We have observed that neonatal immunization with live *Lm* induces long-term, Th1 cell-mediated immunity against antigens expressed by *Lm* [72,73].These properties and several other factors therefore make *Lm* a particularly promising candidate for the development of a live vaccine carrier strain [74]:

- The infectious cycle of *Lm* and the host's immune response to *Lm* infections has been well studied in both, cell culture and animal models.
- The bacterium can be easily cultivated in large quantities, which is economically advantageous for generating *Lm*-based vaccines industrially.
- Access to *Lm*'s genome since 2001 has permitted genetic manipulations to investigate virulence genes and has enabled the design of multiple virulence-attenuated *Lm* strains [75]. This is important for safe administration of *Lm*.
- *Lm* is lacking lipopolysaccharide (LPS) expression as a gram-positive bacterium and therefore exhibits reduced risk of a LPS-induced sepsis-like syndrome in the host. This represents another safety advantage of vaccination with *Lm*.
- *Lm* shows tropism for antigen presenting cells such as dendritic cells (DC) and macrophages - the defense cells of the innate immune system. This places *Lm* as a recombinant antigen delivery system right at the core of immune-modulation and allows for specific priming of T cell subsets.
- *Lm* has been shown to elicit strong IFN-γ-producing CD4 and CD8 Th1 cell responses, thereby activating both arms of the cell-mediated adaptive immune system and resulting in broad immunity [75].
- Studies in mouse models have shown that prior exposure and immunity to *Lm* does not reduce *Lm*'s capacity to mount a specific and strong immune response against heterologous antigens expressed by *Lm*. This is particularly valuable in the vaccine design for neonates, who may harbour pre-existing maternal immunity.
- A single immunization with a *Lm*-based vaccine vehicle at birth induces life-long protective cellular (CD8 and CD4 Th1 cells) and humoral (non-allergic IgG type) immune responses in the recipient without the need for boosts.
- *Lm* can also be stored without refrigeration, which is logistically beneficial for transporting *Lm*-based vaccines to end users in remote locations.

- Due to *Lm*'s natural ability to invade and present antigens in the gut mucosa, it has great potential to be formulated as a needle-free, oral vaccine. This is the easiest and preferential route of immunization.

It is therefore not surprising that *Lm* has already been successfully tested *in vivo* in humans as a carrier for tumour, viral, bacterial and parasitic antigens [76]. Phase I safety trials in adults using attenuated *Lm* via mutations in *actA* (encoding ActA – the protein involved in actin polymerization and cell-to-cell spread) and *plcB* (encoding PC-PLC – an enzyme facilitating phagosomal escape after cell-to-cell spread) triggered robust mucosal, humoral and cellular immunity without serious side-effects [77]. Unfortunately, advancing *Lm*-based vaccines into human clinical trials is generally slow. This is due to challenges in the design and development of *Lm* vaccines. Once constructed, the application of *Lm*-based vaccines in clinical trials involving neonates may also be hampered by concerns revolving around toxicology and safety regulations [78]. We therefore used a virulence-attenuated *Lm* strain for the administration of our antigen.

1.3.3) *Listeria monocytogenes* virulence attenuation

The safe delivery of antigens in our experiments through live pathogenic bacteria like *Lm* requires attenuation. This is particularly important when vulnerable populations such as neonates are the vaccine target. As such, the in-frame deletion in *actA* resulted in 3-fold virulence reduction while still eliciting strong primary and secondary Th1 CD4 and CD8 T cell responses [79]. This strain administrated at high doses was safe in murine neonates, provided life-long protection from challenges with wild-type (WT) *Lm* after only one immunization at birth and therefore exhibited a suitable balance between the level of attenuation and the level of immunogenicity [75].

1.3.4) The *Listeria monocytogenes* vaccine plasmid construction

Based on the above outlined observations, we designed the novel, highly effective and promising *Lm*-vaccine platform *Lm* Δ(*trpS actA*)/pSPO which is entirely safe and extremely well-tolerated by newborns [73,75]. This pSPO plasmid-based *Lm* platform can be easily manipulated to deliver several foreign antigens, such as allergic proteins, in their full length

form. We constructed *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA (*Lm*-OVA) – a neonatal vaccine strain based on highly-attenuated, recombinant *Lm* expressing the model allergen chicken egg albumin (ovalbumin, OVA). The first step in the design of this *Lm*-based vaccine entailed the insertion of a vaccine antigen into the extra-chromosomal pSPO plasmid DNA and transforming it into virulence-attenuated *Lm* for antigen expression. Although integration of the expression cassette into *chromosomal* DNA is of greater stability, it results in lower antigen expression levels than the application of multi-copy replicating plasmids that we employed. The bacterial expression plasmid pSPO in our vaccine strains encoded the following genes (Fig. 2):

- Two origins of replication, which are necessary for plasmid assembly in Gram-negative *Escherichia coli* and subsequent plasmid propagation in Gram-positive *Lm*.
- An antibiotic resistance gene for selection during cloning processes.
- *trpS* – the essential gene tryptophanyl-tRNA synthetase. This gene has been deleted from the chromosomal DNA of *Lm* and put into trans expression though the DNA-delivery plasmid. This results in a balanced-lethal plasmid system in which only bacterial strains containing the *trpS*-encoded plasmid are rendered viable. Since this gene is under the control of its own promoter, the plasmid is completely stable in *trpS*-deficient strains and results in stable replication and maintenance of the plasmid in *Lm* without antibiotic selection. The balanced-lethal plasmid system also acts as a safety check-point for this vaccine strain.
- The antigen expression cassette (only in the *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA vaccine strain) encoding the model vaccine antigen OVA. This antigen expression cassette is under the control of the listerial *hly* promoter to which it is fused at its 5' end. OVA expression under this promoter is predominantly activated in the host's phagosome after which the synthesized OVA peptides are presented on MHC-I molecules to CD8+ T cells.

  When comparing different bacterial antigen delivery systems (e.g. protein antigen vs. antigen-encoding DNA and mRNA), OVA secretion by the *Lm* carrier bacterium resulted in the strongest immune responses involving OVA-specific CD8+ and CD4+ T cells [68]. Previous studies in our lab have also demonstrated that phagosomal-driven antigen expression led to broader and more efficient immune protection than cytosol-driven

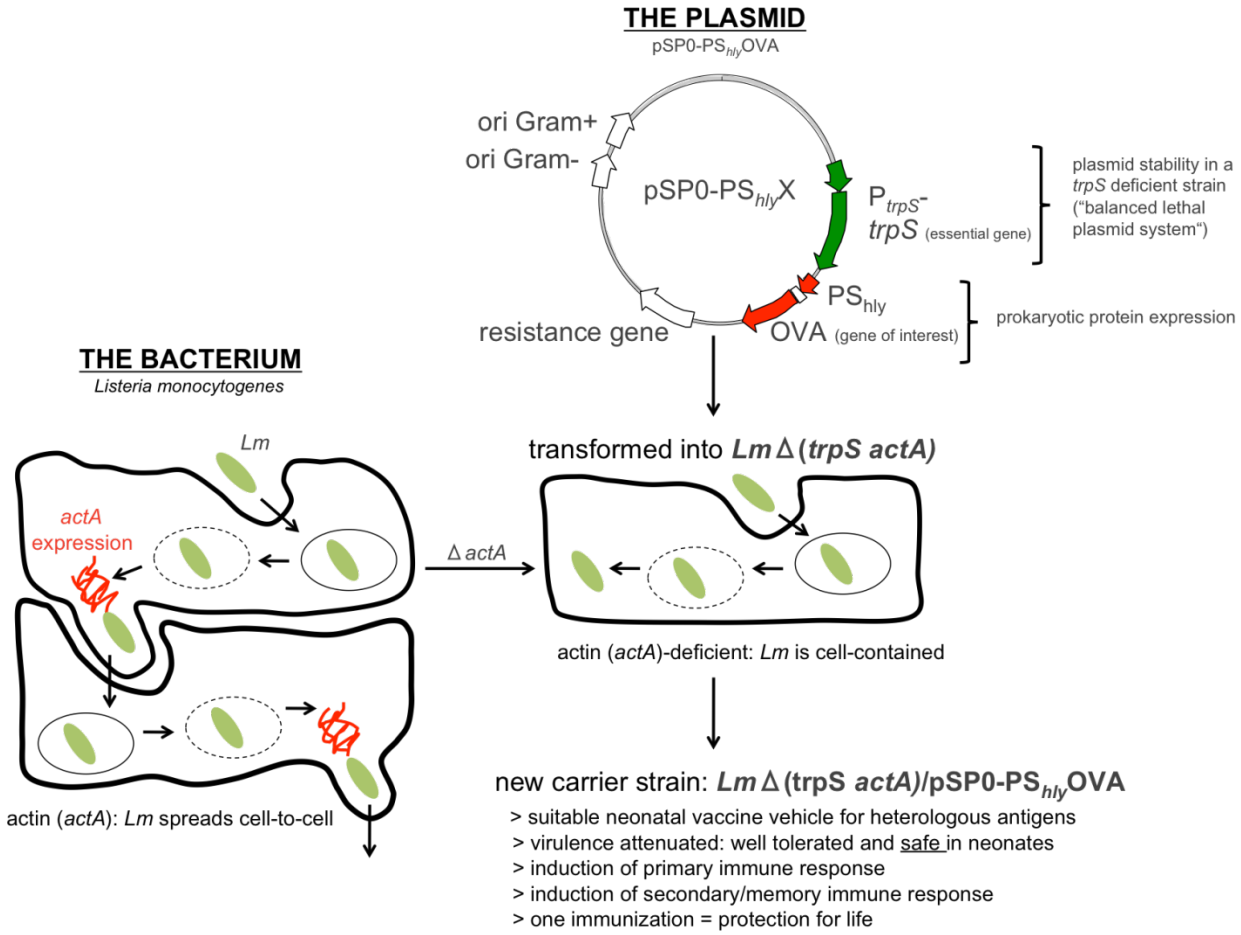

Figure 2. Construction of the *Lm*-based vaccine strain *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA. An in-frame deletion of the virulence gene *actA* responsible for cell-to-cell spread renders the intracellular bacterium *Listeria monocytogenes* safe and well-tolerated in neonates. The pSPO vaccine vector transformed into this *Lm* strain carries the expression cassette for the delivery of OVA antigens (red). After deletion of the essential *trpS* gene from *Lm*'s chromosomal DNA, *trpS* is put into trans expression on the pSPO plasmid (green) where it accounts for the balanced-lethal plasmid system. The subsequent vaccine strain *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA induces primary and secondary immune responses and results in protection from asthma after only one immunization given early in life.

synthesis. Antigen localization of the bacterial carrier and time of expression are crucial variables in determining the efficiency of CD4 and CD8 immune responses [74].

This outlines the construction of the two vaccine strains *Lm* Δ(*trpS actA*)/pSPO and *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA. The third vaccine strain used in these experiments was HK*Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA which differed from *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA only in that it had been heat-killed.

1.4) HYPOTHESIS AND OBJECTIVES

I hypothesized that **prophylactic, neonatal immunization with the live, attenuated *Lm* strain *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA expressing OVA, triggers an antigen-specific and protective immune response in the adult and averts Th2-driven asthma pathology by promoting a Th1-type immune defense mediated by CD4 and CD8 T cells**. I also examined if the same vaccination strategy employing *Lm* Δ(*trpS actA*)/pSPO, lacking specific antigen expression, could confer protection from asthma after allergen challenge by solely relying on live *Lm* as a strong immune modulator. Lastly, I hypothesized that prophylactic immunization with heat-killed *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA would confer less protection from asthma than both live-attenuated *Lm* strains.

CHAPTER 2: RESEARCH CHAPTERS

2.1) INTRODUCTION

Asthma is a chronic respiratory disorder that leads to inflammation and narrowing of the airways [8]. The global prevalence, morbidity, mortality and economic cost associated with asthma have been on the rise since the 1960's and continue to increase by 50% every decade [80]. Today, over 300 million people of all ages, genders and ethnic backgrounds suffer from asthma, thereby reaching epidemic levels [81]. Current treatment options alleviate the symptoms only temporarily or rely on environmental control measures with the combination of life-long controller medication [23-25]. Since children are disproportionally affected by asthma, vaccination strategies to prevent or cure asthma early in life are urgently needed.

With that end in view, we have successfully developed a novel, neonatal vaccine platform based on live, highly attenuated *Lm*. We hypothesized that neonatal mice prophylactically immunized with *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA, a live *Lm* vaccine strain producing the model allergen OVA, would be protected from allergic OVA sensitization after just one immunization given around birth. Furthermore, our goal was to determine if vaccination with the live bacterial strain *Lm* Δ(*trpS actA*)/pSPO, lacking specific antigen expression, would also confer protection against allergic immune responses by solely relying on *Lm*'s strong immune adjuvant properties. We predicted that both live-attenuated *Lm* strains would confer better protection from asthma than immunization with the dead, heat-killed bacterial strain HK*Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA. After immunization, subsequent sensitization with OVA and intranasal OVA challenge to induce asthma was followed by analysis of asthma severity.

2.2) METHODS

The goal of these experiments was to assess protection from OVA-induced allergic reactions in adult mice that were prophylactically immunized as newborns with one of two live *Lm* strains or heat-killed *Lm*.

2.2.1 Experimental setup

Vaccine groups: As illustrated in Fig. 3, neonatal mice 6 days of age were immunized intraperitoneally (*i.p.*) with the following attenuated *Lm* vaccine strains:

- ***Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA** ("*Lm*-OVA Asthma" group)
  delivering OVA antigens (live)
- ***Lm* Δ(*trpS actA*)/pSPO** ("*Lm* Asthma" group)
  delivering no specific antigens (live)
- **HK*Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA** ("HK*Lm*-OVA Asthma" group)
  incapable of protein expression (heat-killed, dead).

Six weeks after immunization with these vaccine strains, mice were sensitized *i.p.* twice with one week between each event (day 1 counted as the first administration). The injected sensitizing agent was 100µg OVA, dissolved in phosphate buffered saline (PBS) and adsorbed onto Alum hydroxide gel (= OVA-PBS/Alum). This protocol has been shown previously to induce B cells to produce IgE. Aero-allergen exposure was then mimicked by intranasally (*i.n.*) challenging anesthetized mice on days 22, 23, 24, 26 and 28 with 200µg OVA dissolved in 100µl PBS (OVA-PBS). Subsequent analysis (day 29) included examination of the BALF in order to determine the total number of live cells residing in the BALF and to quantify the different cell types via hematoxylin and eosin stain (H&E), namely eosinophils, macrophages, neutrophils and lymphocytes. The increase in total cells and the specific cell subsets in the BALF is used in the allergy field as an indicator of the severity of an allergic reaction in the lung resembling asthma. Supernatants of BALF were harvested to determine cytokines via enzyme-linked immunoabsorbent assays (ELISA) or Luminex-based assays, and histological analysis of lung tissue itself was conducted to evaluate airway inflammation and pathological changes

within the tissue. Goblet cells in lung tissue were stained with Alcian Blue/Periodic Acid Schiff (PAS) and the histological mucus index was quantified for goblet cell hyperplasia. Homogenized spleen and lung tissue was restimulated with OVA and heat-killed *Lm* in order to quantify IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, TGF-β and IFN-γ cytokine production by tissue-resident cells via Luminex and ELISA. The concentration of IgE/IgG1 and IgG2a in the serum was quantified via OVA-specific ELISA to determine Th2 or Th1 lymphocyte dominance respectively. Mice were also examined for airway resistance after allergen challenge, as measured by the flexiVent approach.

Controls: The positive control, termed "asthma group", was predicted to develop allergic/asthma-like reactions to the above protocol. Mice in this group followed the same experimental schedules outlined above but had received Sodium chloride (NaCl) *i.p.* immunizations as neonates, followed by subsequent asthma induction via *i.p*. sensitization with OVA-PBS/Alum and exposure to OVA-PBS during *i.n.* challenges. The negative control, termed "naïve group", was predicted to show no signs of allergy or asthma. Mice in this group followed the same experimental schedules outlined above but encountered no OVA antigens in their treatment. They received *i.p.* immunizations with NaCl as newborns, were "sensitized" *i.p.* with PBS-Alum and exposed to PBS during *i.n.* challenges.

2.2.2) Mice

Lm class I immunodominant peptides have been characterized only in the murine H-2^d haplotype, while class II immunodominant peptides have been described only in the H-2^b haplotype [75]. We therefore used F_1 neonates (H-2^d x H-2^b) from crosses between C57B10.D2 (H-2^d) and C57BL/6 (H-2^b) mice provided from The Jackson Laboratories. F_1 neonates were vaccinated as 5-7 days old pups since this time period resembles human neonates the closest in terms of the immune system's development and maturation. Mice reached the adult stage between week 6 and week 12. All animals were cultivated under pathogen-free conditions at the Child and Family Research Institute of the University of British Columbia according to animal experiment protocols approved by the Institutional Animal Care and Use Committee (Preface).

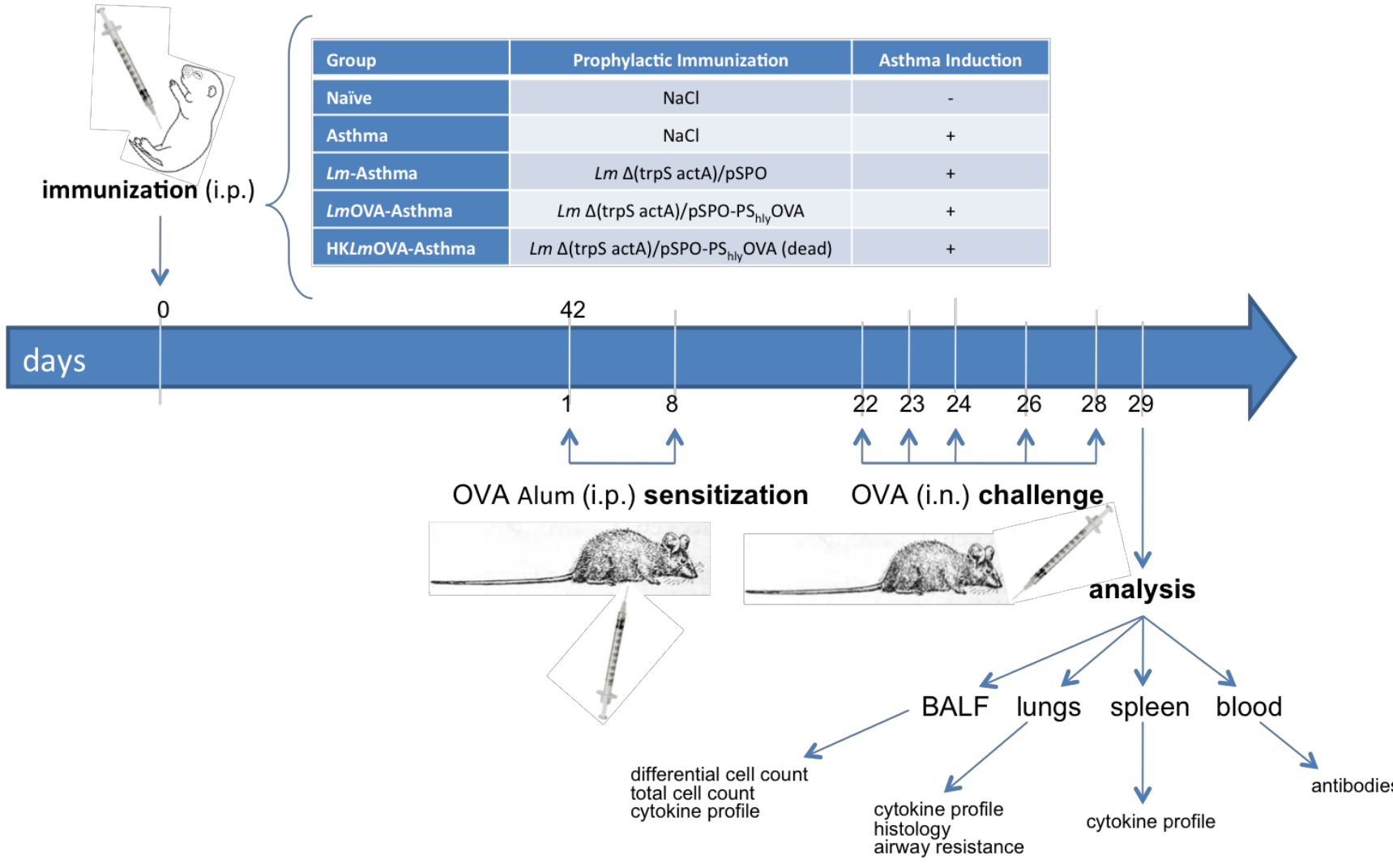

Figure 3. Experimental setup for prophylactic vaccine. Neonatal mice were immunized *i.p.* with the live-attenuated *Listeria monocytogenes* vaccine strains *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (*Lm*OVA), *Lm* Δ(*trpS actA*)/pSPO and HK*Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA. Mice group in the naïve and asthma group were injected with NaCl (day 0). 42 days after immunization, mice were sensitized *i.p.* with OVA adsorbed onto Alum. The naïve group was "sensitized" without OVA (day 1 and 8). Anesthetised mice were then challenged *i.n.* with OVA, while the naïve group was "challenged" without OVA (days 22, 23, 24, 26 and 28). Subsequent analysis on day 29 included examination of the bronchoalveolar lavage fluid (BALF), lung, spleen and blood in order to evaluate the multi-factorial contributors to asthma pathology.

2.2.3) Immunization *i.p.* with virulence-attenuated *Lm* vaccine strains

Our experiments utilized three different *Lm* vaccine strains: 1) The live vaccine strain *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA, synthesizing ovalbumin proteins, 2) the same yet heat-killed vaccine strain HK*Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA and 3) the live vaccine strain *Lm* Δ(*trpS actA*)/pSPO expressing no specific antigens. All strains were grown in brain-heart infusion (BHI) in a bacterial shaker at 190rpm and 37°C to mid-log phase (OD_{600}=1) and were washed and resuspended in endotoxin-free isotonic 0.9% saline solution. All bacterial strains were injected *i.p.* into mice at a volume of 100μL and a final concentration of 10^7 colony-forming units (CFU). This concentration was confirmed by serially diluting the infection aliquots to 10^2 and 10^1 CFU and plating these dilutions on BHI plates immediately upon immunization. Immunization with correct HK*Lm* concentrations was verified by plating 10^2 and 10^1 CFU dilutions prior to heat-killing and spreading the entire heat-killed infection aliquot on BHI plates after infection and observing no bacterial growth upon incubation at 37°C. Heat-killing of bacteria took place in a heat-block at 110°C for 20min.

2.2.4) OVA-sensitization through *i.p.* OVA-PBS/Alum injections

Since the generation of a strong immune response with an immunogen can be slow and inefficient, the addition of an adjuvant to the antigen results in an improved immune response compared to the sole administration of antigen [82]. Adjuvants such as Alum can enhance an immune response by localizing antigens for longer time periods and facilitating the interaction between appropriate immune cells (e.g. T and B cells, antigen presenting cells) and the immunogen. In order to prevent catabolism, adjuvants and immunogens are mixed and injected in suspension. As such, 5mg of Chicken OVA Albumin (Worthington P6M9094) was dissolved fresh on the day of injection in 5mL of commercial, sterile 1x PBS to a final concentration of 1μg/μL. The OVA solution was then filtered through a 0.45μm filter into a sterile, endotoxin-free Erlenmeyer flask with a stir bar. The OVA solution was stirred at medium speed while Alum (Sigma A8222) was added in a drop-wise fashion in a 1:1 ratio. The OVA-PBS/Alum solution was stirred for 30min during which Alum effectively adsorbed OVA antigens. 200μL of this solution, containing 100μg of OVA, was injected into the peritoneal cavity of mice in the

"asthma group" and vaccine groups. "Naïve mice" were injected with 200μL of filter-sterilized PBS and Alum in a 1:1 ratio.

2.2.5) OVA-challenge through *i.n.* OVA-PBS instillations

10mg OVA was dissolved fresh on the day of challenge in 5mL of commercial, sterile 1x PBS to a final concentration of 2μg/μL. The OVA solution was then filtered through a 0.45μm filter into a sterile tube. Mice were anesthetized with Isofluorane and 100μL of the OVA solution was pipetted into the nostrils of mice in the "asthma group" and *Lm*-vaccinated mice. "Naïve mice" received 100μL of sterile PBS *i.n.*

2.2.6) Sequence of cell and tissue extraction

On the day of analysis (day 29), mice were sacrificed by halothane over-exposure. The sequence of cell and tissue extraction was as follows: 1) extract BALF from lungs, 2) obtain blood through cardiac puncture, 3) remove lungs for histology and restimulation assay, 4) remove spleen for restimulation assay. Mice tested for airway resistance with the flexiVent were raised solely for this purpose and were transferred for flexiVent analysis on the day of analysis (day 29) from the to Child and Family Research Institute to the Biomedical Research Centre at the University of British Columbia.

2.2.7) Bronchoalveolar lavages – total cell count

After mice were sacrificed by halothane over-exposure on day 29, the trachea was dissected and a micro-incision made to allow access to the lungs via a catheter. This catheter was fixed to the trachea with surgical thread. Three microsyringes were filled with 1mL of 1 x PBS and successively plugged into the catheter to gently wash the lungs. All three washes were pooled, stored on ice and the volume of this extracted BALF was recorded. It was centrifuged at 1200rpm and 4°C for 5min. 1mL of the supernatant was frozen down to -20°C for later analysis of *in vivo* cytokine production of cells in the BALF. The cell pellet was resuspended in 1mL red blood cell (RBC) lysis buffer and incubated at room temperature for 2-5min. The reaction was stopped by adding the same volume of PBS and centrifuging the cell suspension again at

1200rpm and 4°C for 5min. The cell pellet was then resuspended in 1mL of PBS and 10µL of this cell suspension was mixed with 90µL trypan blue. Living cells were then counted on a Neubauer chamber, using light microscopy (Carl Zeiss, West Germany, 47 34 15-9901).

2.2.8) Bronchoalveolar lavages – differential cell count

In order to determine the different types of cells constituting the total cell count, 100µL of the above outlined cell-PBS suspension containing the total cell count was fixed onto glass slides using a cytocentrifuge (cytospin3, Shandon), rotating at 500rpm for 3min. The glass slides were allowed to dry overnight and stained with H&E the next day (Hema 3® Stain Set, Fisher Scientific). Macrophages, lymphocytes, neutrophils and eosinophils were then counted manually under the light microscope at a magnification of 400x.

2.2.9) Blood

Blood was collected from euthanized mice through cardiac puncture in a tube for capillary blood collection (Microtainer ®, BD). After it was centrifuged at 14,000rpm for 5min, the serum supernatant was transferred into small tubes and frozen down to -20°C until antibody analysis via ELISA.

2.2.10) Lung histology - tissue infiltration of cells

On the day of analysis, half the lung of one mouser per experimental group was removed and stored in PBS on ice. It was transferred to 10% formalin the same day and incubated overnight at 4°C. The next day, the lung was transferred to 70% ethanol and given to the histology lab at the Child and Family Research Institute for paraffin embedding, sectioning of 5µL thickness and in-house lung tissue H&E staining. The stained lung sections from four different experiments were examined and one representative airway per experimental group was captured under the Olympus BX51 light microscope. It was analyzed with the Image-Pro MDA 5.1 Analyzer software by counting the number of nuclei on a fixed surface area centered around six different airway cross section. Since no cells were residing inside the airway lumen (flushed out by

BALF), the surface area of the airway lumen was deducted from the total surface area when determining the ratio of tissue-infiltrating cells per mm^2:

$$\frac{\text{cell number}}{mm^2} = \frac{\text{number of nuclei in total surface area}}{(\text{total surface area } [mm^2] - \text{surface area of airway lumen } [mm^2])}.$$

2.2.11) Lung histology – goblet cell metaplasia

The staining agent Alcian Blue/PAS dyes mucus in the airways and mucins of goblet cells with blue colour. The mucus is a combination of cellular debris from necrotic airway epithelial cells, inflammatory cells, plasma protein exudate and mucins produced by goblet cells [6]. In order to prepare the 5µL-thick lung cross sections for this dye, the tissue was deparaffinized, fixed in 2% paraformaldehyde/PBS for 30min, then stained with microwaved 1% Alcian blue in 3% acetic acid (pH=2.5), incubated in microwaved 1% periodic aqueous acid and incubated in microwaved Schiff's reagent for 15min. Microwaving the respective staining components resulted in better dye penetration into the tissue and better colour contrasts. Between each staining step, the slides were rinsed with deionized water and finally mounted with Euklitt. The stained lung sections were examined and captured under the Olympus BX51 light microscope and then analyzed with the Image-Pro MDA 5.1 Analyzer software. Goblet cell metaplasia and mucus hypersecretion was quantified by determining the percentage of blue dye localized to the airway epithelium. Mucus secreted into the airway lumen was not included in this calculation:

$$\text{\% of epithelial tissue occupied by goblet cells} = \frac{\text{surface area of blue dye (goblet cells)}}{\text{surface area of pink/white dye (epithelial cells)}} \times 100$$

2.2.12) Preparation of restimulation plates for lung and spleen cells

One day prior to analysis (day 28), 96-well restimulation plates were prepared with OVA, HK*Lm* and medium R10 stimuli and plated with lung and spleen cells on the following day of analysis (day 29). To prepare the OVA stimulant, 2mg OVA was dissolved in 4mL R10 medium to a final concentration of 500µg/mL. Of this OVA solution, 2 x 100µL were plated per mouse in order to analyze both, lung and spleen cell responses. To prepare the HK*Lm* stimulant, a *Lm* Δ(*trpS actA*)/pSPO infection aliquot was thawed and serially diluted in NaCl to 10^2 and 10^1

CFU. These concentrations were plated on BHI plates to confirm the correct bacterial concentration in the infection aliquot prior to heat-killing. This infection aliquot was then heat-killed in a heat block at 110°C for 20min and diluted in R10 to a final concentration of 1 x 10^7 dead cells/mL. 100µL of this bacterial suspension was plated twice in the 96-well plate. Afterwards, the entire heat-killed infection aliquot was plated on BHI and no bacterial growth was observed after overnight incubation at 37°C, confirming the absence of live bacteria in the restimulation plates. In order to subtract medium-induced background responses from the OVA and HK*Lm* stimuli, 2 x 100µL of R10 were plated in each well per mouse to be analyzed. These 96-well plates coated with 2 x 100µL of each OVA, HK*Lm* and R10 per mouse were then incubated at 4°C for 24h.

2.2.13) Preparation of lung cells for restimulation assay

The goal of this experiment was to analyze the *ex vivo* cytokine production of cells of lung tissues after being restimulated with OVA, HK*Lm* (and R10) for 48h. After mice were sacrificed on day 29, their lungs were extracted, transferred into RPMI medium and stored on ice. The lung tissue was then cut into small pieces and digested at 37°C for 1h under constant horizontal shaking (300rmp) in collagenase type II (invitrogen, concentration: 300U/mL) and DNAse I (invitrogen, concentration: 150 µg/mL). The digested tissue suspension was filtered through a 40µm cell strainer and centrifuged with 300g at 4°C for 5min. The cell pellet was resuspended in 5mL RBC lysis buffer and incubated at room temperature for 2-5min. 5mL of R10 medium was added to stop the reaction and centrifuged again with 300g at 4°C for 5min. After resuspending the cell pellet in 5mL R10, 10µL of the sample was added to 90µL trypan blue in order to determine the total live cell number of each sample with the Countess™ (invitrogen™). The remaining cell suspension was centrifuged again with 300g at 4°C for 5min and resuspended in R10 medium to a final concentration of 1 x 10^7 cells/mL. 100µL of this cell suspension was plated in the 96-well plate, which already contained 100µL of stimulant per well (OVA, HKLm or R10). The plate was sealed with a gas permeable sealing membrane ("Breathe Easy", Diversified Biotech, USA Scientific) and incubated in a pathogen-free 37°C tissue culture incubator (5% CO_2) for 48h. After that, the plate was centrifuged at 1200rpm for 5min at

room temperature and 160µL of the supernatant was transferred into a new 96-well plate. The new plate was sealed with an aluminum membrane (Nalgene Nunc International) and frozen at -80°C until cytokine analysis.

2.2.14) Preparation of spleen cells for restimulation assay

In this experiment, we aimed to analyze splenocytes for their *ex vivo* cytokine production after being restimulated for 48h with OVA, HK*Lm* (and R10). Mice were sacrificed on the day of analysis and their spleen was extracted and stored on ice. The spleen was crushed between two ethanol-sterilized, frosted glass slides and 7mL RBC lysis buffer. 7mL RPMI medium was added to stop the reaction and the 14mL splenocyte solution was then centrifuged at 1200rpm and 4°C for 5min. The cell pellet was resuspended in 4mL R10 and run through a 70µm cell strainer. 10µL of this cell suspension was diluted in 90µL PBS (1:10 dilution) and mixed in a 1:1 ratio with trypan blue. Subsequently, the total live cell number of each sample was determined with the Countess™ (invitrogen™). The remaining cell suspension was centrifuged again with 1200rpm at 4°C for 5min and resuspended in R10 medium to a final concentration of 1 x 10^7 cells/mL. 100µL of this cell suspension was plated in the 96-well plate that already contained 100µL of stimulant per well (OVA, HK*Lm* or R10).

2.2.15) Cytokine profiling (Luminex multiplex assay)

As described above, the supernatants of the BALF as well as supernatants from restimulated lung cells and splenocytes were frozen for later analysis with the Millipore MILLIPLEX ™ MAP Kit for mouse cytokines. While BALF supernatants were analyzed undiluted, lung and splenocyte supernatants were diluted by a factor of 1.5 in R10 before Luminex analysis. Calibration microspheres and sheath fluid were purchased from Luminex xMAP Technology. All supernatants were analyzed for IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-12 (p70) cytokines. All analyses were performed according to the manufacturer's protocol. Acquired fluorescence data were analyzed with the MasterPlex™ QT software.

2.2.16) Total and antigen-specific antibody level (ELISA)

The following ELISA kits and sample dilutions were used to quantify antibody and cytokine levels in serum, BALF supernatants and supernatants of lung cells restimulated with OVA: The mouse anti-OVA IgG1 ELISA kit from BioVendor R&D was used at serum dilutions of 1:100. The mouse anti-OVA IgG2a ELISA kit supplied by Alpha Diagnostic International examined serum at dilutions of 1:20. The mouse anti-OVA IgE ELISA kit ordered from Shibayagi was used at serum dilutions of 1:20. Total IgE levels were determined with the ELISA MAX^{TM} Deluxe Set provided by BioLegend at serum dilutions of 1:600 and total IgG1 levels were checked on serum dilutions of 1:50,000 using anti-IgG1 ELISA kits provided by Bethyl Laboratories. The mouse IL-13 and TGF-β levels were measured with the ELISA Ready-SET-Go kits from eBioscience, applying undiluted BALF supernatant samples and supernatants of OVA-restimulated lung cells.

2.2.17) Determination of airway reactivity (flexiVent)

Airway hyper-responsiveness in asthma manifests itself in narrowing of the airways in response to environmental triggers. Since the asthmatic lung mucosa exhibits increased bronchial responsiveness to external stimuli, asthma severity can be estimated by measuring the airway responsiveness to inhaled parasympathomimetic agents such as methacholine [83,84]. Airway responsiveness to methacholine (MCh) was determined using a constant flow method employed by the flexiVent (SCIREQ Scientific Respiratory Equipment Inc., Montreal). Mice were anesthetized with Avertin ® (150mg/kg, i.p.) and tracheotomized with a cannula, which was attached to a ventilator that delivered a constant flow of air. Mice were given pancuronium bromide (0.8 mg/kg, i.m.) to block spontaneous breathing. After baseline airway resistance stabilized (R_{base}), increasing doses of aerosolized MCh (6.25mg/mL, 12.5mg/mL, 25 mg/mL, 50mg/mL, 100mg/mL) were infused via a nebulizer. Total respiratory system resistance was determined immediately before MCh infusion (R_{min}), and then every 2 seconds following infusion until the maximum response was reached (R_{max}). One experiment (n=1) was conducted and examined 3-10 mice per group. MCh-induced increases in resistance were calculated as follows:

$$\text{\% increase in airway resistance} = \frac{(R_{max}-R_{min})}{(R_{base})} \times 100$$

2.2.18) Statistics

We analyzed our data with the t-test. We thus based our power calculations on the following: Our desired t-test α (significance level) shall be 0.05, and our desired power 0.90 (this is 1 minus β, where β is the probability of making a type II error, i.e. false negative). Therefore we relied on 8-12 mice per group for the experimental setup to detect an anticipated 50% difference in outcome with a 90% power to have a significant level of $p \leq 0.05$. Each experiment had to be repeated at least twice to ensure reproducibility. Unless indicated otherwise, the displayed results below represent pooled data from four separate experiments. The results are expressed as mean ± standard error mean (SEM) deviation. Statistically significant differences in comparison to the asthma group were marked with asterisks (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.002$).

2.3) RESULTS

2.3.1) Adult mice neonatally immunized with the live-attenuated *Lm* vaccine strains are protected against airspace inflammation in comparison to the HK*Lm*-treated mouse group

Mice were immunized *i.p.* as neonates with 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (*Lm*-OVA Asthma) and *Lm* Δ(*trpS actA*)/pSPO (*Lm* Asthma) as well as HK*Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (HK*Lm*-OVA Asthma). Six weeks later, they were sensitized with OVA/Alum and intranasally challenged five days in a row with OVA. To determine if mice immunized with our live-attenuated and heat-killed *Lm* vaccines were protected from asthmatic cell infiltration of the alveolar and bronchial airspaces upon allergen sensitization and allergen challenge, we extracted and quantified cell samples from the airways on day 29 post-sensitization. This was achieved by flushing the lungs with PBS and withdrawing the BALF. We recorded the obtained lavage volume in order to account for different cell concentrations found in different lavage volumes of different samples. Cell quantification of the BALF showed that adult mice in the groups "*Lm*-OVA Asthma" and "*Lm* Asthma" which received neonatal vaccinations with live-attenuated *Lm*

strains were protected from asthmatic cell infiltration of the airways since the total cell count in BALF in both groups was significantly reduced when compared to the asthmatic group (Fig. 4). In particular, vaccination with the antigen-expressing *Lm*-OVA strain achieved slightly more protective results than vaccination with the *Lm* strain expressing no specific antigens. In contrast, mice in the "HK*Lm*-OVA Asthma" group that received the neonatal HK*Lm*-OVA vaccination did not exhibit a reduction in airway-infiltrating cells, but showed similar results as the asthma group. The negative and positive groups are shown as "naïve" and "asthma" group respectively (Fig. 4).

2.3.2) Adult mice neonatally immunized with the live-attenuated *Lm* vaccine strains show significantly fewer infiltrating eosinophils in the lungs after asthma induction than the HK*Lm*-treated mouse group

Next, we investigated the kind of inflammatory cell types that compose the BALF and reside in the bronchial airways. We were particularly interested in the type of cells that
produce the wide array of pro-inflammatory mediators that are involved in the induction of asthma pathogenesis [85]. The central role of eosinophils in asthmatic responses has been recognized since the 1960's [86]. Elevated levels of other inflammatory cells such as antigen-presenting cells (e.g. macrophages), neutrophils and lymphocytes have also been associated with asthmatic inflammation [87]. After staining the BALF with H&E dye, we found no difference in the number of airway-residing macrophages between mice from the vaccine groups and mice from the negative or positive control. The quantification of lymphocytes in the BALF showed only elevated levels for groups that had been subject to asthma induction (the positive control and all vaccine groups). While this increase in lymphocyte count for the "*Lm*-OVA Asthma" and "HK*Lm*-OVA Asthma" group resembled the positive control, mice in the "*Lm* Asthma" group interestingly showed a drastic increase when compared to the positive control. However, this did not apply consistently for all mice in this group, making it subject to considerable variance, and thus statistically not significant.Elevated neutrophil levels were also seen only in groups that were exposed to OVA allergens during asthma induction. For this cell type, mice in the "*Lm* Asthma" and "HK*Lm*-OVA Asthma" group resembled the positive control, while mice from the "*Lm*-OVA Asthma" group exhibited slightly higher levels of neutrophils.

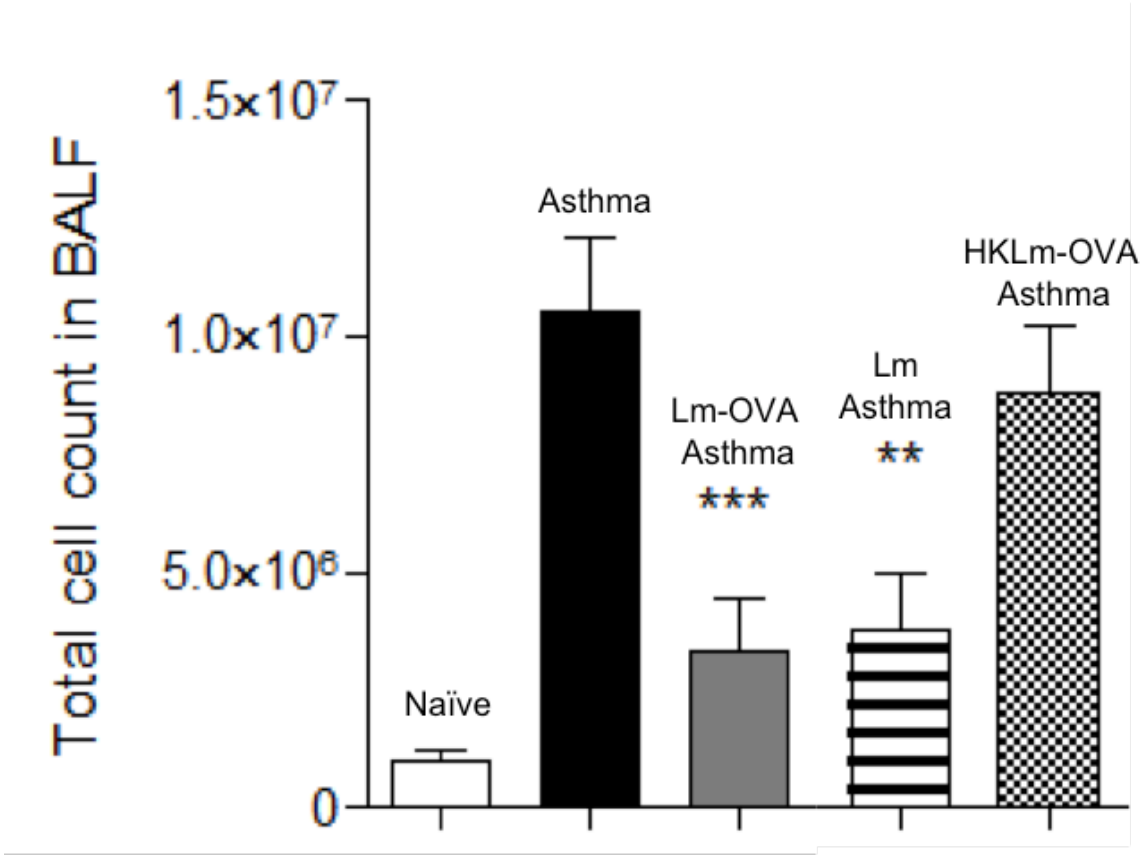

Figure 4. Adult mice neonatally immunized with the live-attenuated *Lm* strains are protected against asthma in comparison to HK*Lm*-treated mice. Mice were immunized *i.p.* as neonates with 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (*Lm*-OVA Asthma) and *Lm* Δ(*trpS actA*)/pSPO (*Lm* Asthma) as well as HK*Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (HK*Lm*-OVA Asthma). Six weeks later, they were sensitized with OVA/Alum and intranasally challenged five days in a row with OVA. On day 29 post-sensitization, the bronchoalveolar lavage fluid (BALF) was extracted from the lungs to determine the total amount of airway-infiltrated cells. Mice in the groups "*Lm*-OVA Asthma" and "*Lm* Asthma" which received neonatal vaccinations with live-attenuated *Lm* strains were protected from asthmatic cell infiltration of the airways, while mice in the "HK*Lm*-OVA Asthma" group were not. Values are expressed as mean ± SEM.

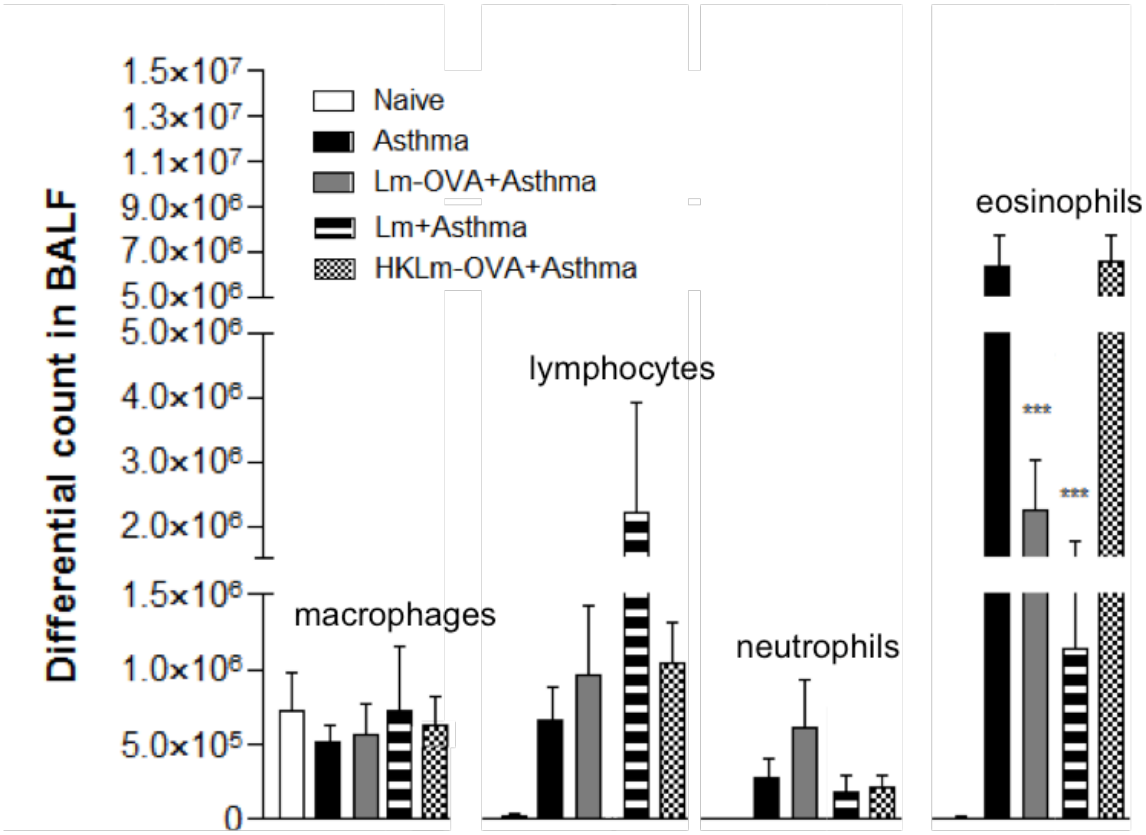

Figure 5. Adult mice neonatally immunized with the live-attenuated *Lm* strains show fewer infiltrating eosinophils into the lungs than HK*Lm*-immunized mice. Mice were immunized *i.p.* as neonates with 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA (*Lm*-OVA+Asthma) and *Lm* Δ(*trpS actA*)/pSPO (*Lm*+Asthma) as well as HK*Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA (HK*Lm*-OVA+Asthma). Six weeks later, they were sensitized with OVA/Alum and intranasally challenged five days in a row with OVA. On day 29 post-sensitization, the bronchoalveolar lavage fluid (BALF) was extracted from the lungs and stained with H&E dye to determine the different type of proinflammatory cells. The only statistically significant change between the asthma control and vaccine groups was seen on the level of eosinophils. Eosinophils infiltrated into the bronchial airways prevailed in greatest abundance in both the positive control and the "HK*Lm*-OVA Asthma" group (eosinophilia). On the other hand, mice from the "*Lm*-OVA Asthma" and "*Lm* Asthma" group, which shared neonatal immunization with a live-attenuated *Lm* vaccine strain, exhibited significantly lower eosinophil levels. Values are expressed as mean ± SEM.

Overall, the only statistically significant change between the asthma control and vaccine groups was seen at the level of eosinophils. Eosinophils prevailed in greatest abundance in both the positive control and the "HK*Lm*-OVA Asthma" group. However, mice from the "*Lm*-OVA Asthma" and "*Lm* Asthma" group, which shared neonatal immunization with a live-attenuated *Lm* vaccine strain, showed significantly fewer eosinophils infiltrating into the bronchial airways than the HK*Lm*-treated mice. While the degree of reduced eosinophil burden in the "*Lm*-OVA Asthma" group was relatively consistent among the different mouse samples, eosinophil levels in the "*Lm* Asthma" group deviated from the mean with greater variance (Fig. 5).

2.3.3) Immunization of newborn mice with live-attenuated *Lm* prohibits allergic airway disease in comparison to HK*Lm* immunizations

After examining the degree of inflammatory cell infiltration into the alveolar and bronchial airspaces, we set out to investigate the extent of cell infiltration into the airway *tissue* – in particular: the airway submucosa between the epithelium and the smooth muscle cell lining. On the day of analysis, i.e. after asthma had been induced, the murine lung was removed, cut into cross sections of 5μL thickness and stained with H&E. The stained lung sections from four different experiments were examined; one representative airway per experimental group is displayed in Fig. 6. The sections were analyzed for the degree of cell infiltration into the airway epithelium and lung submucosa by counting the number of nuclei on a fixed surface area centered around six different airway cross sections per slide. The number of tissue-infiltrating cells was the highest for lung samples from the asthmatic and HK*Lm*-OVA-immunized group. They showed a similar extent of
tissue-localized cellular infiltration and airway remodeling, including substantial thickening of the epithelium and submucosa.
These are characteristics that are typical of allergic airway disease. In contrast, mice from the "*Lm*-OVA Asthma" and "*Lm* Asthma" group exhibited ordinary airway morphologies and low tissue-infiltrated cell densities that closely mirrored the naïve group. When contrasted with the

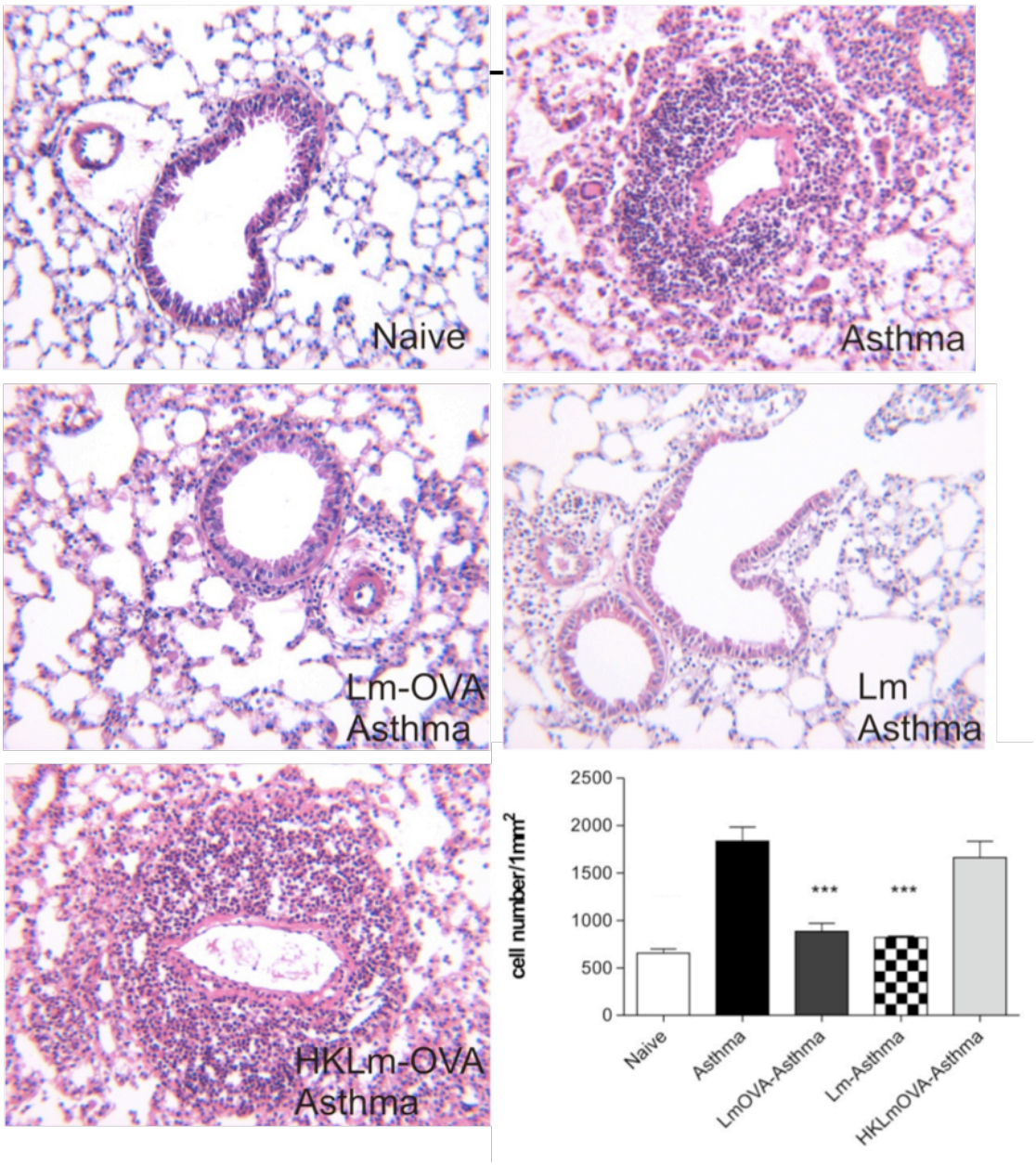

Figure 6. Immunization of newborn mice with live-attenuated *Lm* prohibits allergic airway disease in comparison to HK*Lm* immunizations. Mice were immunized *i.p.* as neonates with 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (*Lm*OVA-Asthma) and *Lm* Δ(*trpS actA*)/pSPO (*Lm*-Asthma) as well as HK*Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (HK*Lm*OVA-Asthma). Six weeks later, they were sensitized with OVA/Alum and intranasally challenged five days in a row with OVA. The above pictures show cross sections of H&E-stained murine lung airways from day 29 post-sensitization. The stained lung sections from four different experiments were examined and one representative airway per experimental group was captured. It was analyzed for its degree of cell infiltration into the airway epithelium and lung submucosa by counting the number of nuclei on a fixed surface area centered around six different airway cross sections. In comparison to the asthma control and HK*Lm*-immunized group, mice in the "*Lm*-OVA Asthma" and "*Lm* Asthma" group that received neonatal live-attenuated *Lm* vaccines, showed significantly less cell infiltration into pulmonary tissue upon asthma induction and did not display allergic airway disease characterized by airway remodeling. Values are expressed as mean ± SEM.

asthma group, these two vaccine groups therefore displayed a significant reduction in tissue-invaded cells per mm^2 (Fig. 6).

2.3.4) Immunization of newborn mice with live-attenuated *Lm* prevents goblet cell metaplasia in comparison to HK*Lm* immunizations

In order to further examine airway remodeling in more detail, we quantified the extent of goblet cell metaplasia in the different experimental groups. Goblet cell metaplasia is one of the hallmarks of chronic airway inflammation and linked to increased mucus production. Mucus in combination with cellular debris from necrotic airway epithelial cells, inflammatory cells and plasma protein exudate may lead to clogging of the airway lumen and has been found heavily in airway biopsies of asthma fatalities [6,88]. The staining agent Alcian Blue/PAS was used to dye murine lung cross sections from day 29 post-sensitization. This dye binds mucins – a component of the mucous goblet cell secretion – resulting in blue colour. We therefore determined the percentage of mucin-producing goblet cells (blue) intermingled with pseudostratified ciliated columnar cells of the respiratory epithelium (pink). In the positive control and "HK*Lm*-OVA Asthma" group, approximately half of the epithelial cells were occupied by mucin-producing goblet cells. In contrast, the goblet cell density in mice neonatally immunized with live-attenuated *Lm* vaccine strains was only between 10-30%. This represents significantly less disease burden from airway remodeling in mice from both the "*Lm*-OVA Asthma" and "*Lm* Asthma" group. Of those two groups, mice in the "*Lm*-OVA Asthma" group achieved the best protective effects from airway remodeling (Fig. 7).

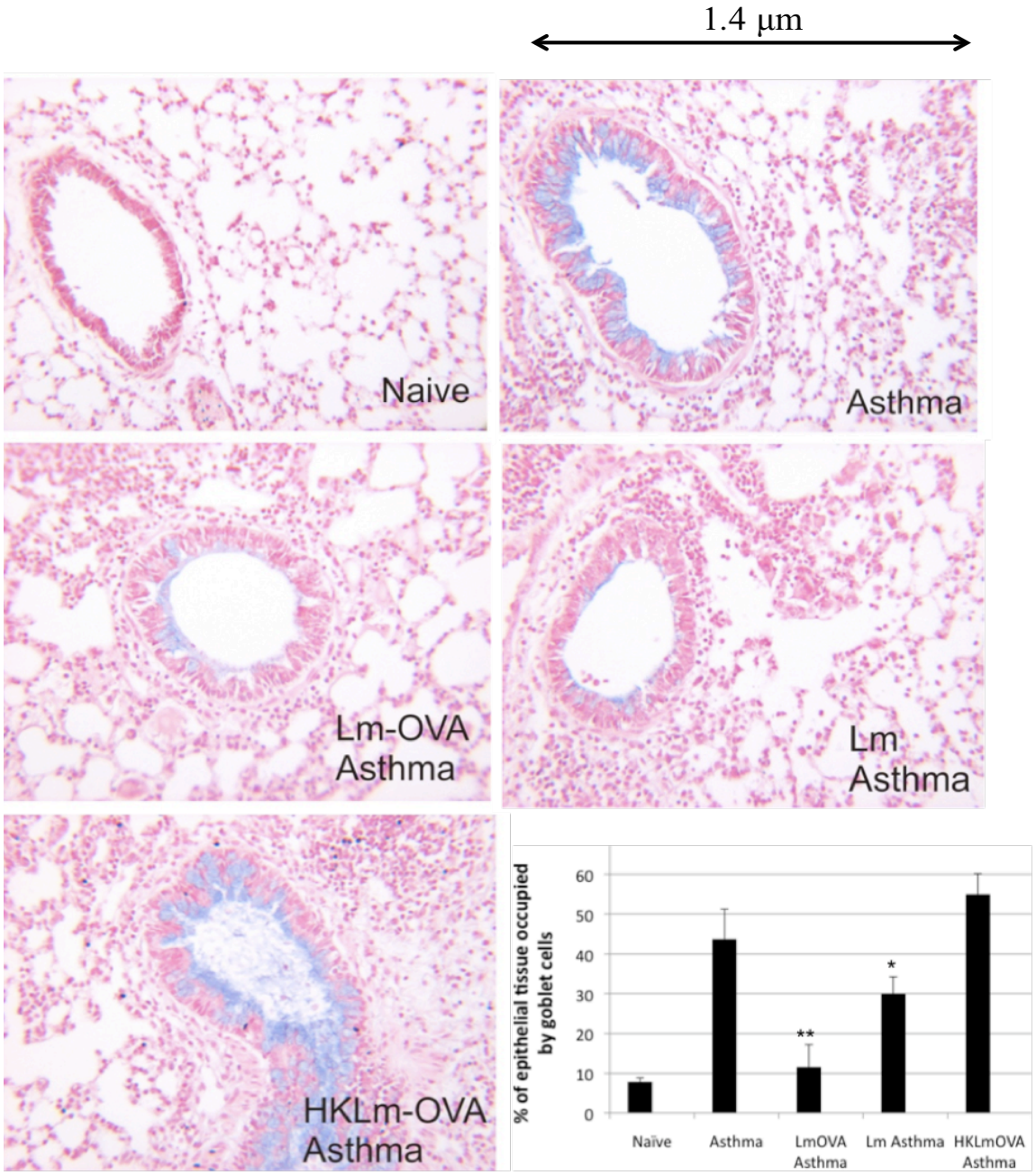

Figure 7. Immunization of newborn mice with live-attenuated *Lm* prevents goblet cell metaplasia in comparison to HK*Lm* immunizations. Mice were immunized *i.p.* as neonates with 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (*Lm*OVA Asthma) and *Lm* Δ(*trpS actA*)/pSPO (*Lm* Asthma) as well as HK*Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (HK*Lm*OVA Asthma). Six weeks later, they were sensitized with OVA/Alum and intranasally challenged five days in a row with OVA. The above pictures show cross sections of Alcian Blue/PAS-stained murine lung airways from day 29 post-sensitization. This dye stains mucins produced by goblet cells with blue colour. Lung sections from four different experiments were examined and one representative airway per experimental group was captured. It was analyzed for goblet cell metaplasia by quantifying the area of blue dye localized to the airway epithelium in three different airways per experimental group. In comparison to the positive control and HK*Lm*-immunized group, mice in the "*Lm*-OVA Asthma" and "*Lm* Asthma" group that received neonatal live-attenuated *Lm* vaccines, showed significantly lower percentages of mucin-producing goblet cells in the airway epithelium. Values are expressed as mean ± SEM.

2.3.5) Immunization of newborn mice with live-attenuated *Lm* results in less airway hyper-responsiveness and airway resistance when compared to asthmatic mice

The flexiVent is an invasive tool measuring pulmonary function and respiratory impedance in mice and is the clinical gold standard for measuring reactive airway disease [83,84]. Contrary to other analytic tools of asthma pathology, the flexiVent permits insights into the phenotypic characteristics of asthma in a live (but anesthetized) mouse. It investigates airway hyper-responsiveness and -reactivity (AHR) in terms of airway resistance and its principle resembles the spirometer used to measure asthma in human patients. Since asthmatic mice exhibit bronchoconstriction, decreased alveolar spaces and increased bronchial responsiveness, their airways present with greater airway resistance than naïve mice when stimulated with a pharmacologic agent [89]. As such, the flexiVent system measures the extent of bronchial responsiveness to the inhaled parasympathomimetic MCh as an estimate for asthma severity. Aerosolized MCh stimulates muscarinic receptors on smooth muscle cells and submucosal glands. This induces airway contractions, resulting in narrowed airways and increased airway resistance. In our flexiVent experiment (n=1), we aimed to investigate whether mice in the "*Lm*-OVA Asthma" and "*Lm* Asthma" group – the two experimental groups that received neonatal immunizations with live-attenuated *Lm* – were indeed functionally protected from asthmatic airway hyper-responsiveness. We examined 3-10 mice per experimental group. Our dose-response curve to increased MCh challenges (6.25, 12.5, 25, 50 and 100mg/mL) showed that of all the experimental groups, asthmatic mice (positive control) presented the greatest airway resistance in response all MCh doses, except for the lowest dose (6.25mg/mL). Both vaccine groups followed trends that indicated lower levels of airway resistance than seen in the asthma group, however, this was only of statistical significance when airways of "*Lm* Asthma" mice were stimulated with the highest MCh concentration (100mg/mL). Oddly enough, the increase in airway resistance in the naive group over the course of the flexiVent experiment was partially greater than the vaccine groups. We are currently in the process of repeating this experiment. This oddity may be explained by the fact that in this particular experimental setup, cages of the naïve group were housed in a different room than cages of the other experimental groups (Fig. 8).

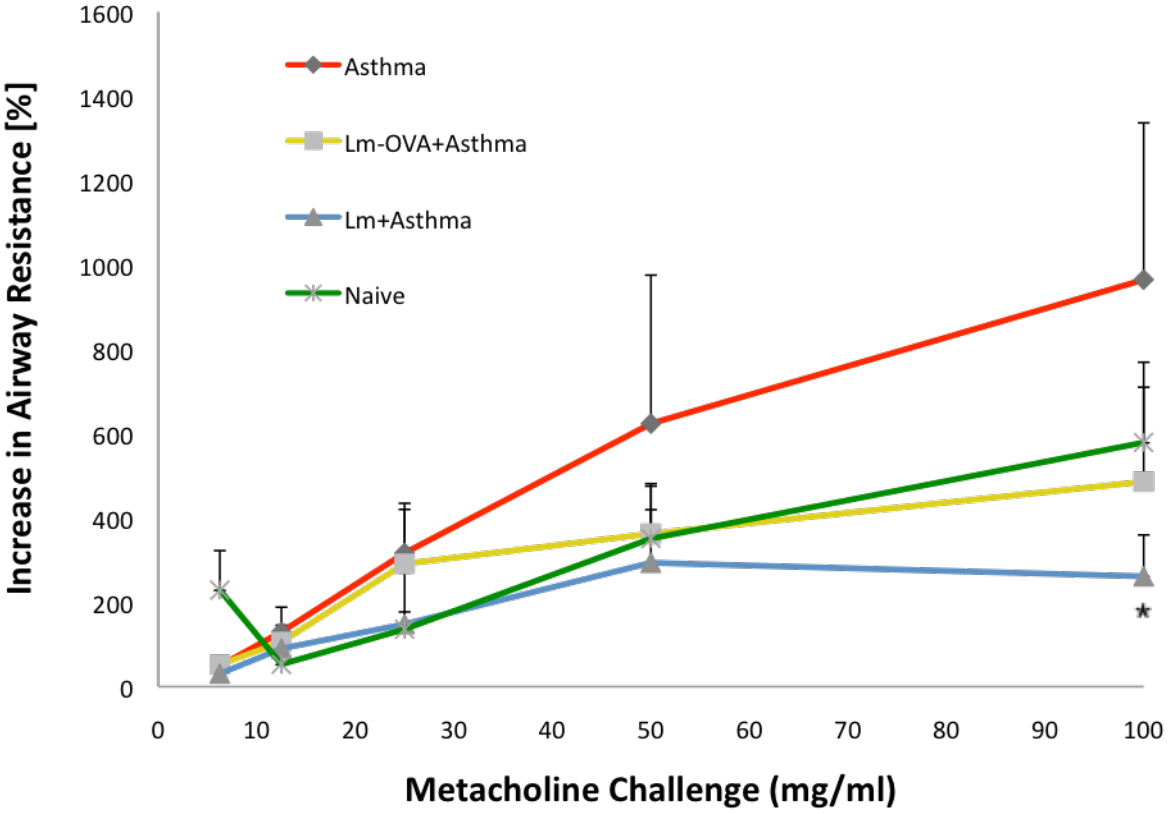

Figure 8. Immunization of newborn mice with live-attenuated *Lm* results in less airway hyper-responsiveness and airway resistance when compared to asthmatic mice. Mice were immunized *i.p.* as neonates with 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA (*Lm*-OVA+Asthma) and *Lm* Δ(*trpS actA*)/pSPO (*Lm*+Asthma). Six weeks later, they were sensitized with OVA/Alum and intranasally challenged five days in a row with OVA. This graph shows the increase in airway resistance experienced by mice on day 29 post-sensitization in response to increasing doses of aerosolized methacholine (6.25, 12.5, 25, 50 and 100mg/mL). The flexiVent analysis system was used to analyze one experiment (n=1) with 3-10 mice per experimental group. Our dose-response curve to increased MCh challenges shows that of all the experimental groups, asthmatic mice presented the greatest airway resistance in response all MCh doses except the lowest dose (6.25mg/mL). Both vaccine groups followed trends that indicated lower levels of airway resistance than the asthma group, however, this was only of statistical significance when airways of "*Lm* Asthma" mice were stimulated with the highest MCh concentration (100mg/mL). The relatively high level of airway resistance seen in the naïve group did not match our expectations and be explained by the fact that in this particular experimental setup, cages of the naïve group were housed in a different room than cages of the other experimental groups. Values are expressed as mean ± SEM.

2.3.6) The cytokine profile of lung-residing cells

Asthma pathophysiology is driven, orchestrated and exacerbated by a variety of inflammatory cells and a complex interaction of their pro-inflammatory mediators [90-93]. We investigated the T cell cytokines IL-2, IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ, as well as TGF-β. *Ex vivo* production of these mediators by lung and spleen cells was examined after a 48h restimulation assay using OVA or HK*Lm* as stimuli. Cells of the lung and spleen were chosen for analysis due to their relevance in asthma pathology (lung) and in establishing effective anti-*Lm* immunity (spleen) [94,95]. In order to sample the cytokine profile of lung cells, we examined the *ex vivo* production of cytokines from cells resident in the lung tissue as well as the *in vivo* cytokine production found in BALF.

In order to keep the scope of this thesis limited, only cytokine profiles produced by *ex vivo* stimulated lung cells are shown and discussed here. The rationale behind this decision is rooted in my research question, which is primarily concerned with whether *lung* cells could be prevented from Th2-skewed cytokine responses through the prophylactic administration of *Lm*. Since cytokines exert their effect predominantly in a paracrine fashion, *ex vivo* stimulated lung cells were expected to present the most relevant and most prominent cytokine response to evaluate asthma severity [93].

In the "HK*Lm*-OVA Asthma" group, there was no difference in cytokine levels when compared to the asthma group. The "*Lm*-OVA Asthma" group, in contrast, showed elevated levels in IFN-γ, IL-12 and IL-4 when stimulated with OVA – although these augmentations were not significant and a matter of relatively low concentrations. IL-10, IL-13 and IL-5 on the other hand were reduced in this group under the same stimulus (significant for IL-10 and IL-5). The situation looked different in the "*Lm*-OVA Asthma" group. Only reduced IL-4 levels distinguished OVA-stimulated lung cells from the asthmatic cytokine profile. In response to HK*Lm* stimulation, both "*Lm*-OVA Asthma" and "*Lm* Asthma" lung cells secreted drastically lower levels of IL-10 and IL-5 (Fig. 9). All experimental groups showed no considerable difference in TGF-β or IL-2 production in the presence of an OVA-stimulus (data not shown).

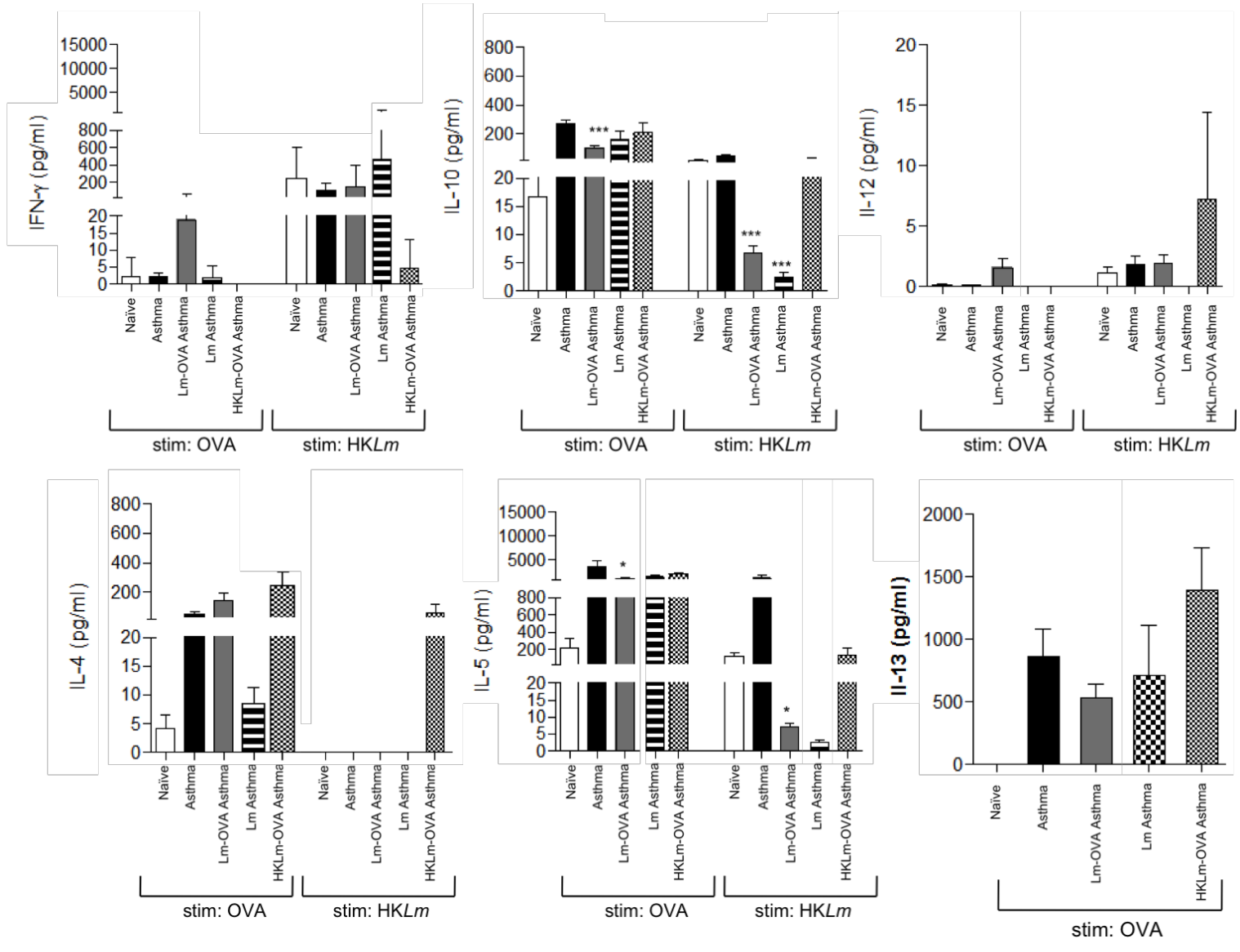

Figure 9. The cytokine profile of lung-residing cells. Mice were immunized *i.p.* as neonates with 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA (*Lm*OVA), *Lm* Δ(*trpS actA*)/pSPO and HK*Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA. Six weeks later, they were sensitized with OVA/Alum and intranasally challenged five days in a row with OVA. This graph shows the *ex vivo* lung cell production of IL-4, IL-5, IL-12, IL-13, IL-10 and IFN-γ cytokines after a 48h restimulation assay using OVA and HK*Lm* as stimulants. In the "HK*Lm*-OVA Asthma" group there was no difference in cytokines levels when compared to the asthma group. The "*Lm*-OVA Asthma" group, in contrast, showed elevated levels in IFN-γ, IL-12 and IL-4 when simulated with OVA. IL-10, IL-13 and IL-5 on the other hand were reduced in this group under the same stimulus (significant for IL-10 and IL-5). The situation looked different in the "*Lm* Asthma" group: only reduced IL-4 levels distinguished OVA-stimulated lung cells from the asthmatic cytokine profile. Values are expressed as mean ± SEM.

2.3.7) When compared to HK*Lm*-treated mice, neonatal mice immunized with the live-attenuated *Lm* vaccine strains showed less anti-OVA IgE but similar anti-OVA IgG1 and anti-OVA IgG2a antibody levels in serum after asthma induction

In order to further clarify the participation of inducers or inhibitors of allergic inflammatory reactions in response in response to our neonatal *Lm*-based vaccines, we investigated the levels of serum antibodies. Since specific T lymphocyte-synthesized cytokines direct the production of a specific immunoglobulin class (IgE vs IgG) and subclass (IgG1 vs IgG2a) in B cells, serum antibodies can be used as an indicator of possible Th lymphocyte dominance [96,97]. Therefore, on day 29 post-sensitization we examined serum concentrations of antigen-specific and total immunoglobulin IgG1/IgE and IgG2a as markers for Th2 and Th1-mediated immune responses respectively (only antigen-specific antibody levels are shown). ELISA analysis of the serum revealed that all experimental groups that were subject to asthma induction (OVA *i.p.* and *i.n.*), exhibited elevated circulating anti-OVA IgE and anti-OVA IgG1 antibodies. Both groups neonatally immunized with the live-attenuated *Lm* vaccine strains showed less anti-OVA IgE levels than mice in the "HK*Lm*-OVA Asthma" group. However, when contrasted with asthma-typical antibody levels, anti-OVA IgE was only significantly reduced in mice that were neonatally immunized with the OVA-expressing live-attenuated *Lm* strain ("*Lm*-OVA Asthma" group). There was no reduction in anti-OVA IgG1 for any of the experimental vaccine groups – on the contrary, mice in the "*Lm* Asthma" group presented augmented anti-OVA IgG1 levels when compared to the asthma group (Fig. 10). Analysis of the serum with an anti-OVA IgG2a ELISA resulted in no detectable antibody levels in any of the experimental groups (data not shown).

2.3.8) Summary of findings

Table 1 displays a summary of the experimental outcomes. While trends that show upregulation in comparison to the asthma group are indicated with an upwards-pointing arrow in a green box, downregulation is shown with a downwards-pointing arrow in a red box. All trends have taken the SEM into consideration. It needs to be kept in mind that downregulation of certain cytokines (i.e. inhibitory cytokines) may not automatically translate into disease-suppression.

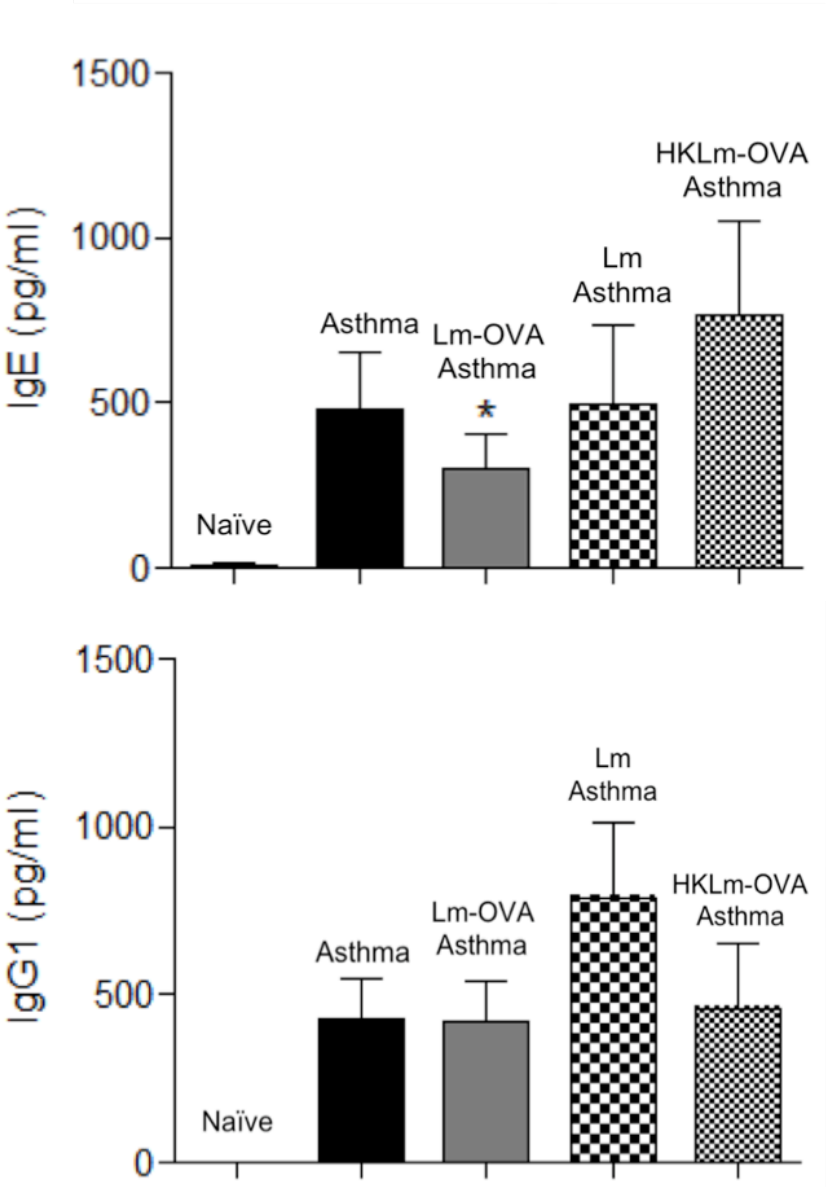

Figure 10. When compared to the HK*Lm*-treated mouse group, mice neonatally immunized with the live-attenuated *Lm* vaccine strains show less IgE but similar IgG1 antibody levels in serum after asthma induction. Mice were immunized *i.p.* as neonates with 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA (*Lm*-OVA Asthma) and *Lm* Δ(*trpS actA*)/pSPO (*Lm* Asthma) as well as HK*Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA (HK*Lm*-OVA Asthma). Six weeks later, they were sensitized with OVA/Alum and intranasally challenged five days in a row with OVA. On day 29 post-sensitization, blood serum was collected through cardiac puncture and analyzed with ELISA for anti-OVA antibody concentrations. All experimental groups that were subject to OVA during the asthma induction exhibited elevated circulating anti-OVA IgE and anti-OVA IgG1 antibodies. Both groups neonatally immunized with the live-attenuated *Lm* vaccine strains showed less anti-OVA IgE levels than mice in the "HK*Lm*-OVA Asthma" group. However, when contrasted with asthma-typical antibody levels, anti-OVA IgE was only significantly reduced in mice that were neonatally immunized with the OVA-expressing live-attenuated *Lm* strain ("*Lm*-OVA Asthma" group). There was no reduction in anti-OVA IgG1 for any of the experimental vaccine groups – in contrary, mice of the "*Lm* Asthma" presented augmented anti-OVA IgG1 levels when compared to the asthma group. Values are expressed as mean ± SEM.

⇓ = decreased level relative to asthma ⇑ = increased level relative to asthma ⇒ = no change relative to asthma * = changed level with significance			EXPERIMENTAL GROUP		
			LmOVA Asthma	Lm Asthma	HKLm-OVA Asthma
total cell count: BALF			⇓***	⇓**	⇒
differential cell count: BALF	eosinophils		⇓***	⇓***	⇒
	neutrophils		⇒	⇒	⇒
	lymphocytes		⇒	⇒	⇒
	macrophages		⇒	⇒	⇒
lung tissue: cell infiltration (H&E)			⇓***	⇓***	⇒
lung epithelium: goblet cell metaplasia			⇓	⇓	⇒
airway hyper-responsiveness: flexiVent			⇓	⇓	n/a
lung cells	OVA stim	IL-5	⇓*	⇒	⇒
		IL-4	⇑	⇓	⇒
		IL-2	⇒	⇒	⇒
		IL-12	⇑	⇒	⇒
		IL-10	⇓***	⇒	⇒
		IFN-γ	⇑	⇒	⇒
		IL-13	⇓	⇒	⇒
		TGF-β	⇒	⇒	⇒
	HKLm stim	IL-5	⇓*	⇓	⇒
		IL-4	⇒	⇒	⇒
		IL-2	⇒	⇒	⇒
		IL-12	⇒	⇒	⇒
		IL-10	⇓***	⇓***	⇒
		IFN-γ	⇒	⇒	⇒
serum	anti-OVA IgE		⇓*	⇒	⇑
	anti-OVA IgG1		⇒	⇑	⇒

Table 1. Summary of findings. Trends that show upregulation in comparison to the asthma group are indicated with an upwards-pointing arrow in a green box, downregulation is shown with a downwards-pointing arrow in a red box. Statistically significant different changes in comparison to the asthma group were marked with asterisks (*p≤0.05, **p≤0.005, ***p≤0.002).

2.4) DISCUSSION

Inflammation is generally a valuable repair mechanism of the body in response to tissue damage. However, in allergic inflammatory conditions like asthma, the factors initiating and sustaining inflammation persist, thereby exacerbating inflammatory symptoms and preventing resolution. It is very likely that no single cell type or mediator drives the complex pathologenic

mechanisms behind asthma. In order to decipher the role of cells and mediators that play a multifactorial role in preventing or enabling protection from asthma pathogenesis, we therefore examined each experimental group individually. We first focused on establishing the mechanisms of how asthma pathophysiology was established (asthma group) in order to deduce how this phenotype was abolished in our experimental groups prophylactically vaccinated with live-attenuated *Lm*.

2.4.1) The asthma and dead "HK*Lm*-OVA Asthma" group

When comparing the macromolecular structures of asthmatic and naïve lungs, it seems that disease in our chronic asthma model was very likely brought about primarily by heavy infiltration of inflammatory cells into the airways (BALF, Fig. 4) and bronchial tissue (histology, Fig. 6) – in particular, eosinophils (Fig 5). The same phenotype was seen in mice that were immunized with HK*Lm*-OVA and underwent asthma induction. This kind of drastic upregulation of tissue-resident and recruited cells is the hallmark of the late phase and chronic stage of allergen-induced airway inflammation [98]. It is enabled by the preceding release of pro-inflammatory mediators from mast cells which are released upon binding of allergens to cross-linked FcεRI-IgE, and results in vasodilation, increased vascular permeability and leukocyte recruitment from the periphery to the airways [99]. While vasodilation is controlled by mediators acting on local nerve cells, the influx of inflammatory cells is facilitated by cytokines like TNF-α which encourage the upregulation of adhesion molecules on endothelial cells. Expressed chemokines like interleukin 8 (IL-8) and chemotactic mediators like LTB_4 also attract leukocytes from the blood vessels to the inflammatory lung tissue [100].

The dense infiltration of eosinophils into the lungs in particular, is strongly associated with the pathogenesis of asthma [98]. Eosinophil-derived mediators like the major basic protein (MBP) damage the mucosal epithelium, while the platelet activating factor (PAF) has been linked to the induction of vasodilation, increased microvascular permeability and contractile effects on the smooth muscle cells [101,87]. PAF has also been reported to further exacerbate eosinophilia by mediating eosinophil chemotaxis and adherence to endothelial cells. Eosinophilic PAF may also account for the increased mucus hypersecretion of goblet cells seen in our asthmatic mice (Fig. 7). Furthermore, increased amounts of eosinophils and eosinophil-

derived products (e.g. MBP and PAF) are correlated with airway hyperreactivity in response to aerosolized MCh [101-103]. This trend was mirrored in our flexiVent data (Fig. 8), which detected the greatest airway hyper-responsiveness in asthmatic mice that – as seen in the differential cell count of the BALF – suffered from severe eosinophilia (Fig. 5). At the base of this association is thought to be the action of eosinophilic mediators causing damage to the mucosal epithelium and subsequently affecting the barrier function of these cells [41,87,98,101].

When identifying the cause-and-effect between heavy tissue infiltration (Fig. 6) and airway remodeling (Fig. 7+8), it is difficult to determine which event comes first. Do the infiltrated cells induce airway remodeling or does airway remodeling and damaged structural integrity of the respiratory tissue allow for heavy cell infiltration? Perhaps there is a positive feedback mechanism at play in which increased vascular permeability, vasodilation and damaged airways allow for increased influx of pro-inflammatory cells, which in turn exacerbate tissue damage by their inflammatory mediators acting on the protective structural components of the airways. It appears however that TGF-β, an immunomodulatory growth factor shown to participate in tissue remodeling, does not contribute to this process in our asthma model [104].

Which factors exactly induced eosinophilia and heavy cell infiltration into the airways and airway tissue? IL-4, IL-13 and IL-5, which were all produced at elevated levels by our asthmatic, OVA-stimulated lung cells in comparison to naïve mice (Fig. 9), have withal been implicated in this phenotype [90,93]. IL-4 increases expression of the vascular cell adhesion molecule I (VCAM-I) on endothelial and airway epithelial cells. VCAM-I is an integrin receptor that enables eosinophil and lymphocyte trafficking by offering docking sites for their membrane-bound integrins (e.g. VLA-4). IL-4 is also involved in Th2 cell differentiation and suppression of IFN-γ synthesized by Th1 cells, which further amplifies Th2 cell-driven inflammatory responses [90,93]. Although IL-13 resembles IL-4 in its impacts on B cells, it does not affect T lymphocytes. But it has been shown to also upregulate VCAM-I and extend the cellular viability of eosinophils in humans via expression of the surface antigen CD69. Beyond cell recruitment, IL-13 contributes to asthma pathology by triggering contractions of the airway smooth muscles and inducing heightened mucus production by goblet cells [90,93]. Both of these effects were observed in our asthma groups analyzed for airway hyper-responsiveness and goblet cell

hyperplasia (Fig. 7+8). IL-5 participates in eosinophilia by facilitating eosinophil maturation, acting as a chemoattractant for eosinophils and supporting eosinophil survival in lung tissue.

Since all three cytokines IL-4, IL-13 and IL-5 are mostly T lymphocyte-derived, perhaps the elevated population of lymphocytes seen in the differential cell count of asthma-induced mice was primarily a Th2 lymphocytes population, capable of activating large amounts of eosinophil-enabling cytokines (Fig. 5) [90,93]. Alternatively, mast cells could have acted as a source of the same set of eosinophil-activating cytokines [99,100]. Recruitment of eosinophils and other inflammatory cells may also have occurred independent from cytokines studied in our experiments. For example, the upregulation of previously mentioned adhesion molecules like e.g. VCAM-I could have been induced by increased chemokine synthesis of airway smooth muscle cells, epithelial and endothelial cells [105-107]. Especially eotaxin, synthesized after FcεRI-IgE-dependent activation of the airway smooth muscle cells (ASMCs), has been suggested to enhance the chemokine signal in the airways, thereby amplifying the recruitment of more inflammatory cells [108-110]. Eotaxin not only contributes to the recruitment of eosinophils but also controls their maturation, differentiation and extends its effects to mast cell growth and the invitation for basophils and lymphocytes to infiltrate the airways. Furthermore, the involvement of FcεRI has been demonstrated in IgE-mediated muscle contraction through intracellular Ca^{2+} spikes, which was hypothesized to play a critical role in airway responsiveness [110-112]. Regardless of the pathway, the subsequent asthma-eliciting effects have been clearly illustrated in the data for our positive control and "HK*Lm*-OVA Asthma" group: heavy airway inflammation (Fig. 4-6), tissue remodeling (Fig. 7) and hyper-responsiveness (Fig. 8).

Neutrophils accumulated in the airways have the potential to change airway function via the release of proteases, lipid mediators and other pro-inflammatory molecules that degrade structural lung tissue components (e.g. type III collagen). Previous animal experiments have demonstrated such airway-altering function of neutrophils in asthma [87]. It is questionable however whether asthma pathogenesis in our animal model involved the influence of the relatively insignificant elevation in neutrophils.

The most common type of extracted APC within the BALF was quite likely the pulmonary macrophage residing on the respiratory surface of the lung alveoli. Pulmonary macrophages have been shown to clear the airways of infectious or allergenic agents [87]. Since

all experimental groups exhibited the same macrophage density as the naïve group, yet manifested different degrees of allergenic responses, inflammation was either modulated at stages *after* the sensitizing allergen-APC interaction, occurred *independent* from alveolar macrophages and/or at sites other than the pulmonary airways. Since we chose *systemic* sensitization for our experimental model (*i.p.* injections with OVA-PBS/Alum), subsequent antigen presentation after our allergen challenges probably occurred via recruited dendritic cells and macrophages, and not through resident alveolar macrophages that would have encountered and presented antigens in a local *i.n.* sensitization protocol. These speculations receive support from the two findings that airway macrophages are relatively poor antigen presenters and that APCs are generally found to be more densely infiltrated *into* asthmatic tissue [47]. It therefore seems more likely that tissue-resident macrophages or other APCs in the tissue (e.g. dendritic cells), excluded from BALF samples, were the primary modulators of inflammatory responses.

When investigating cytokines other than IL-4, IL-5 and IL-13 – which are undoubtedly important players in asthma – IFN-γ, IL-10 and IL-12 become relevant. As indicated earlier, IFN-γ synthesized by Th1 cells acts as an opponent of IL-4 and Th2 responses [92,93]. Inadequate IFN-γ production can therefore create immunological disequilibrium and skew the immune balance towards Th2-mediated responses, which facilitate atopy [113]. Perhaps in our asthma model, the overwhelming IL-4 production of OVA-stimulated lung cells suppressed the anti-inflammatory potential of IFN-γ cytokines to naïve levels, allowing for no allergy-inhibiting function of these mediators (Fig. 9). In clinical studies it has been shown that reduced IFN-γ production in neonates is linked with the subsequent development of atopy [114]. Additional support for this notion comes from the observation that patients afflicted with immune disorders associated with heightened Th1 cytokine production exhibit lower predisposition toward the development of atopy [115,116].

Of particular interest to us was also cytokine IL-10. Although it can mediate immunosuppressive or immunostimulatory behaviour, it has mostly been associated with decreased asthma burden through the suppression of IL-4-induced IgE synthesis, inhibition of eosinophil and lymphocyte influx after allergen challenge and reduced airway hyperresponsiveness [92]. In our experiment however, asthmatic lung cells stimulated with

OVA showed *increased* IL-10 levels, suggesting that IL-10 may have exerted pro-inflammatory effects on asthma pathophysiology (Fig. 9). This is a plausible explanation since IL-10 has been described earlier as a factor inhibiting synthesis of Th1-derived cytokines like IFN-γ [117].

Analysis of OVA-specific IgE and IgG1 antibodies in asthmatic mice (Fig. 10) confirmed the observation of the Austrian immunologist Clemens von Pirquet who noted in 1906 that "the conception that antibodies, which should protect against disease, are also responsible for disease, sounds at first absurd" [118]. IgE and IgG1 antibodies are indeed participants in asthma pathophysiology by binding allergens on effector-cell receptors FcεRI and FcγRIIB respectively, thereby priming the host towards antigen hypersensitivity [119-121]. Synthesis of these antibodies is initiated by the interaction of Th2 cells with B cells in an IL-4 and IL-13-rich environment. IL-4 acts on B cells to initiate immunoglobulin class switching towards IgE synthesis and upregulates expression of the high-affinity IgE receptor FcεRI on mast cells, macrophages and eosinophils. Synthesized IgE and IgG1 antibodies bind to cell surface receptors on mast cells, basophils and eosinophils, sensitizing them to future allergen exposure and setting the stage for acute and chronic inflammatory responses. Production of both, IgE and IgG1, is inhibited by the Th1 cytokine IFN-γ, which on the other hand, encourages IgG2a synthesis. Due to the antagonistic relationship between IFN-γ and IL-4, it is not surprising that IL-4 has also been reported to block IgG2a production. Antigen-specific immunoglobulin isotope production of IgG1/IgE and IgG2a can therefore act as markers for Th2 and Th1-mediated immune responses respectively. In that light, our asthma mouse model showed a clear trend towards a Th2-mediated response since we detected elevated anti-OVA IgE and IgG1 antibodies in comparison to the naïve group, but no IgG2a synthesis. Since IgE and IgG1 were present at similar concentrations, the inducers of IgE and IgG1 production were perhaps identical, or of a different nature, yet acting on the same promoter (Fig. 10). The two cytokines known to upregulate production of these immunoglobulins are IL-13 and IL-4, which are primarily generated by Th2 cells and mast cells. This was reflected in our cytokine analysis, which showed IL-13 and IL-4 to be elevated in OVA-stimulated lung cells of asthma-induced mice (Fig. 9). The complete lack of anti-OVA IgG2a synthesis however does not automatically

indicate the absence of Th1-mediated immune responses. It could also mean that our mouse strain was incapable of IgG2a synthesis.

Recent studies have shown that in atopic patients, FcεRI is not only expressed on mast cells, eosinophils, basophils and dendritic cells but also on human airway smooth muscle cells (ASMCs) [110,111,122]. This group's research revealed that ASMC-bound FcεRI is involved in the IgE-mediated release of the Th2 cytokines IL-4, IL-5, IL-13 and chemokines likes eotaxin, but no IFN-γ. Since this exact cytokine pattern was also seen in our cytokine analysis of asthmatic lung cells (Fig. 9), ASMCs and their mediators (including eotaxin) may have contributed to the observed allergen-induced eosinophil accumulation in the lungs and the elevated IgE and IgG1 levels.

It has also been shown that antibody-bound FcεRI and FcγRIIB receptors cross-linked to the same antigen may actually inhibit FcεRI signaling cascades, thereby blocking mast cell degranulation and the release of pro-inflammatory mediators [121,123]. Assuming that in the positive control of our experiment FcεRI and FcγRIIB receptors were sufficiently expressed on effector cells, IgE and IgG1 antibodies of similarly elevated concentrations must have been bound to FcεRI and FcγRIIB receptors in close vicinity, thereby cross-linked to the same antigen. However, such a degranulation-inhibitory complex strongly contrasts the pathophysiology seen in our positive control. Perhaps in our experimental setup, allergens were provided in such abundance, that FcεRI and FcγRIIB receptors had no need to compete for and co-link to antigens. In that scenario, each antibody-linked surface receptor could have bound an antigen separately, triggering more "individual" degranulation cascades that lead to the kind of severe bronchial inflammation seen in this group.

2.4.2) The live "*Lm*-OVA Asthma" and "*Lm* Asthma" group

In contrast to the positive control and mice neonatally immunized with HK*Lm*-OVA, newborn mice that received one of the two live-attenuated *Lm* vaccine strains were immune to symptoms conferred by asthma induction, thereby suggesting our hypothesis not to be incorrect. Mice in the "*Lm*-OVA Asthma" and "*Lm* Asthma" group displayed a significant reduction in the total number of cells infiltrating the bronchial airways and surrounding pulmonary tissue (Fig. 4+6),

lower degrees of eosinophilia (Fig. 5) and also exhibited significantly less goblet cell metaplasia and mucus secretion (Fig. 7). The drastic reduction of tissue- and airway-infiltrated inflammatory cells possibly also led to less airway reactivity when analyzing these groups with the flexiVent system, suggesting a healthier airway epithelium and airway muscles (Fig. 8). The different pro-inflammatory cell profiles, cytokine patterns and antibody responses between these two groups suggest that this protective effect was achieved by two different mechanisms.

Since there is a direct correlation between serum IgE levels and asthma, mice in the "*Lm*-OVA Asthma" group may have benefitted from a lower disease burden through significantly reduced anti-OVA IgE antibodies (Fig. 10). To confirm this assumption, total IgE levels in serum still need to be analyzed. If it holds true, reduced IgE antibody synthesis could have been achieved through higher levels of IFN-γ expression (Fig. 9), which is known to block IgE antibody production from activated Th2 cells [107,121]. The downregulation of asthma-related antibodies may also have occurred on another level. This points to the first effector cell after antibody synthesis: mast cells. In the chronic stage of asthma, a positive feedback mechanism exists between B cells and mast cells: B cells secrete IgE antibodies which bind to mast cell-expressed FcεRI. IgE-FcεRI complexes on mast cells that encounter allergens will then induce mast cells to produce IL-4 and IL-13. These cytokines act back on B cells to increase IgE synthesis [124]. Since we observed significantly reduced IgE levels in our "*Lm*-OVA Asthma" group, but not a complete abolition of this antibody class, the positive feedback mechanism between mast cells and B cells may have been interrupted. Our data suggests that this could have been due to reduced levels of IL-13 synthesized by OVA-stimulated lung cells in this group (Fig. 9). Alternatively, impaired crosslinking of IgE with the high-affinity IgE receptor FcεRI or reduced FcεRI expression altogether would have also interrupted the positive feedback mechanism between B cells and mast cells and lead to reduced IgE synthesis [111].

One of our introductory assumptions stated that asthma pathology results from an immune imbalance in which anti-inflammatory Th1 cells and their mediators are either not yet fully developed, lost, or overwhelmed and suppressed by Th2 inflammatory cytokines. The cytokine data from our asthmatic mice suggested that the latter was the case for our experimental model: An overbearing Th2 cytokine mix of IL-4, IL-5 and IL-13 seemed to have suppressed IFN-γ production and imprinted mice with severe asthmatic symptoms (Fig. 9). But

in the light of the cytokine profile seen in the "*Lm*-OVA Asthma" group, this statement may need to be redefined. Here, in the absence of asthma pathology, IL-4 levels are even higher than those observed in the asthma group. So perhaps, IL-5 and IL-13 are more significant contributors to asthma pathology than IL-4 and sufficient to cause an asthmatic immune profile. In that scenario it is likely that IL-13 carries out many of the pro-inflammatory functions that are also shared by IL-4. IL-13 has been shown to be capable of driving Th2 development and IgE synthesis in an IL-4-independent manner [125]. This observation also argues that immune balance can be achieved through mechanisms that are independent of equilibrium between the classical Th1 and Th2 players IFN-γ and IL-4.

The spike in lymphocyte count in the BALF of "*Lm* Asthma" mice immediately offers the suggestion that the protective effects seen in this experimental group may have been caused by an increased population of anti-inflammatory Th1 lymphocytes and their mediators (Fig. 4). Although a Th1-polarized response would have been indeed expected after neonatal immunization with an intracellular pathogen like *Lm* [75], the same increase in lymphocytes was not seen in mice immunized with "*Lm*-OVA Asthma" which applied the same bacterial delivery form for OVA antigens and therefore should have induced a similar rise in T lymphocytes. Furthermore, we observed no increase in Th1-synthesized cytokines such as IFN-γ that could have been triggered by greater Th1 populations and signaled an anti-inflammatory response. In order to further elucidate the participating types of lymphocytes and deduce their possible effects on the protection from asthma, future experiments should classify this lymphocyte population in terms of their activation status and lymphocyte subset (e.g. via flow cytometry).

Neonatal vaccination with "*Lm* Asthma" resulted in anti-OVA IgE levels that were of the same elevated concentration as that seen in the asthma group and anti-OVA IgG1 levels that even exceeded those of the asthma group – however "*Lm* Asthma" mice showed no asthmatic phenotypes (Fig. 10). It is not clear at which stage after antibody synthesis these antibodies were prevented from executing their pro-allergenic functions. Again, total IgE and IgG1 antibody levels need to be determined to paint a fuller picture. Perhaps on mast cells, cross-linking of anti-OVA IgG1 to FcγRIIB was impaired. Or FcγRIIB expression was inhibited altogether. Both scenarios would have resulted in less FcγRIIB-bound antibodies, less inflammatory downstream

signaling through degranulating mast cells and increased amounts of circulating antibodies in serum (Fig. 9). Alternatively it may be argued, that IgG1 in our experimental model did not participate in disease pathogenesis at all. In terms of IgE, it is difficult to unite the previous idea that reduced anti-OVA IgE antibody levels in the "*Lm*-OVA Asthma" group conferred protection with the observation that no reduction in anti-OVA IgE levels in the "*Lm* Asthma" group did confer protection. This argues in favor of earlier suggestions that propose different pathways for both groups towards a protective phenotype. Different mechanisms for these two groups are not surprising given the fact that OVA expression by an intracellular pathogen in the "*Lm*-OVA Asthma" group must have resulted in antigen-specific downstream effects, which were lacking in the *Lm* construct without OVA.

When looking for explanations why the "*Lm* Asthma" group could have been slightly less protected from mucus hypersecretion of goblet cells than "*Lm*-OVA Asthma" mice, the answer probably does not lie in eosinophils (Fig. 7). Eosinophils – which could have potentially damaged mucosal epithelium and increased airway reactivity via PAF – were slightly more elevated in "*Lm*-OVA Asthma" than in "*Lm*-Asthma" (Fig. 5). Instead, the larger pool of lymphocytes seen in the "*Lm* Asthma" group may have been a mix of both, increased numbers in Th1 and Th2 lymphocytes. The greater numbers of Th2 cells, capable of heightened IL-13 production (Fig. 9), could have induced goblet cell metaplasia and augmented mucus secretion in this group. This in turn could have contributed to greater airway resistance and reactivity that was also mirrored in the relationship between "*Lm* Asthma" and "*Lm*-OVA Asthma" in the flexiVent analysis (Fig. 8).

2.4.3) Conclusion

In asthma, cytokines act as cell-to-cell communicators that deliver pro-inflammatory messages for airway remodeling, bronchoconstriction and airway hyper-responsiveness through the means of increased cell activation, growth and proliferation, chemotaxis and positive feedback mechanisms leading to even greater cytokine release [93]. In chronic asthma, cytokines are responsible not only for the initiation of these inflammatory processes, but also for the persistence of these symptoms [41]. Since one cytokine may have multiple, overlapping functions and each function may in turn be modulated by a variety of cytokines, the effect of

specific cytokines in the context of our experiment was very difficult to decipher. Besides their propensity for pleiotropy and redundancy, cytokines may also exert their effects through synergistic or inhibitory interactions with each other or other cells and mediators [93]. Our investigation of only eight cytokines therefore limited our ability to capture the full picture of the molecular interactions at hand. We were also unable to make conclusions on whether priming and polarization of Th1 and Th2 cells in an *ex vivo* setting mirrors the actual cytokine production of these cells *in vivo*.

Although we can turn to the literature for guidance in terms of narrowing down the kind of cytokines associated with chronic asthma (Th2) *or Lm* infection (Th1), we need to keep in mind that the phenotype of "no asthma" in our experimental vaccine groups was the additive effect of a bacterial infection *and* subsequent asthma induction. It is therefore not surprising that our two live *Lm*-immunized groups that showed protective phenotypes in the face of asthma induction, lacked a clear-cut Th2 or Th1 cytokine response. This observation suggests that *Lm* acted as a strong, yet "gentle" and "guiding" immune response modulator that is capable of establishing a balanced immune response that counter-steers a Th2-biased response but prevents extreme Th1-polarization. Since a heavily Th1-weighed immune response could have predisposed mice to other undesirable health outcomes (e.g. autoimmunity), the nature of this modulatory effect opens up the door for *Lm* to mediate other dysfunctional immune diseases.

In conclusion, this thesis fails to disprove our original hypothesis that prophylactic immunization with the live, attenuated *Lm* strain *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA expressing OVA triggers an antigen-specific and protective immune response. However, we were not able to show that Th2-driven asthma pathology is averted by a clear-cut Th1-type immune defense. In fact, the data appears to show that protection from asthma may be regulated independently from Th1/Th2 cells. We also confirmed our hypothesis that the same vaccination strategy employing the live, attenuated vaccine strain *Lm* Δ(*trpS actA*)/pSPO expressing no specific antigens can still confer significant protection from asthma after allergen challenge. This relevant protective mechanism(s) appeared to differ from the antigen-specific immune response, yet also seemed to rely on pathways others than a classical Th1 immune response. Importantly, we conclude that protection from asthma was brought about by live, replicating *Lm* since

prophylactic immunization with heat-killed *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA conferred no protection at all.

CHAPTER 3: CONCLUSION

3.1) THE BIG PICTURE

3.1.1) The Th1/Th2 paradigm – revisited

There is more and more evidence arguing that the prevention and downregulation of asthmatic symptoms cannot be solely based on Th1 cells counterbalancing Th2 cells [126-130]. If this dichotomous Th1/Th2 paradigm were true, there should be a "baseline presence" of Th1 cells circulating or localized to naïve airways – however, there were almost no T lymphocytes in our differential cell counts of naïve mouse BALF (Fig. 5). This has also been reported for airways of non-asthmatic individuals [131]. In fact, large amounts of Th1-effector cells in the airways can lead to tissue-damaging, neutrophilic airway inflammation rather than inhibition of Th2-cell induced airway hyperresponsiveness [127]. As such, Th1 and Th2 cells have both been demonstrated to elicit pulmonary inflammation and airway hyperresponsiveness in animal models [132,133]. Although adoptive transfer studies have indeed shown a participatory role of Th2 cells in antigen-induced airway hyper-responsiveness, this experiment also suggested that the contribution of Th1 cells may depend on the conditions and timing of antigen exposure [134]. All these observations call into question the dogmatic notion that environmental or genetic factors polarize the immune response in asthmatics towards a strict Th2 response while inhibiting Th1 responses. They in fact argue more strongly for failed *immune regulation* underlying atopic responses. With the discovery of more and more cells acting as sources for cytokine production (e.g. structural cells such as epithelial or endothelial cells), an observed cytokine profile no longer needs to be matched up with either Th1- or Th2-type responses only. Furthermore, transcription factors have been suggested to be at the core of immunoregulation. For example, mice lacking the transcriptional regulator *SMAD3* exhibit elevated production of proinflammatory cytokines in pulmonary tissue [22].

In conclusion, it is quite likely that we still lurk in the dark with our current knowledge of potential mechanisms underlying the hypersensitivity reactions leading to asthma. Perhaps

the missing link in asthma regulation is a (are) molecular factor(s) that has (have) not yet been identified. Or alternatively, the kind of changes that impact or "tip over" an immune response may depend on exiguous shifts in mediator concentrations which remain unnoticed because statistical tests do not deem them significant or are impossible to be recognized with our current analysis tools.

3.1.2) The hygiene hypothesis

The hygiene hypothesis has also been postulated as a mechanistic explanation of asthma regulation – especially in the industrialized world. According to this view, allergy and asthma have become an "epidemic in the absence of infection" [38]. A decrease in exposure to infectious agents in early life is believed to result in the absence of a shift in the immune system's allergic Th2 bias to a non-allergic Th1 response. Although it is doubtful that, again, only Th1 and Th2-regulatory mechanisms are at the core for mediating allergic responses, there is overwhelming evidence for the impact of environmental factors on asthma onset [135-139]. Even in an unintentional side-story of our own experiments we have seen that mice raised under different environmental conditions may have been predisposed to develop different degrees of hyperreactive airways: The experimental setup for our flexiVent analysis included two rooms. All mice were bred and born in a pathogen-free room. Newborn mice that received live-attenuated *Lm* immunizations and mice used for the positive control were transferred at six days of age from this "clean room" to the procedure room in which other virally- and bacterially-infected mice were housed and treated. The only group that remained in the clean breeding room was the naïve mouse group. By the time of flexiVent analysis, these mice had spent more than ten weeks in a clean environment without pathogen exposure. When analyzed for airway hyperreactivity, these mice however exhibited airway resistance that was greater than those recorded for the *Lm*-vaccinated mice that were housed in the "dirty room". Although we missed to control certain variables in this "experiment" to draw clear conclusions, these observations resound with other clinical findings that propose immunoprotective mechanisms mediated by early life exposure to infectious agents [139]. Children who were raised on farms and therefore had more frequent and diverse encounters with microbial products, both indoor and outdoor, exhibited lower prevalences of asthma and atopy [140,141]. In fact, a very recent study reported

an inverse relationship between diversity of environmental microbial exposure and asthma, independent of farming lifestyle [140]. Interestingly enough, it specifically found significance in an inverse association between *Lm* load in farm dust and asthma. In more controlled studies, respiratory health was also found to be positively correlated with markers for microbial exposure such as endotoxins and the bacterial peptidogylan component muramic acid [140,142].

The mechanism through which microbially diverse stimuli confer protection against asthma in children is still unknown. But since the innate immune system acts as one of the first lines of defense against foreign allergic (and infectious) particles, it is likely to have played a crucial role in mediating immune responses towards allergenic triggers agents in our mouse model. The innate immune system is not fully matured at birth and may therefore be a crucial immunological window in time to "imprint" non-protective, non-Th2 immune responses in the newborn. Thus, the early-life interactions between microbial ligands and host innate immune cell pattern recognition receptors (PRRs) could have played an immune-modulatory role. Since the prophylactic administration of our *Lm* strain expressing OVA resulted in the same protection after OVA sensitization/challenge as the administration of a *Lm* strain without specific OVA expression, protection may have been conferred through *Listeria* pathogen-associated molecular patterns (PAMPs) interacting with specific host PRRs. One subclass of PRRs are Toll-like receptors (TLRs) on surfaces and intracellular compartments of APCs, T_{reg} and epithelial cells. They are known to interact with PAMPs, trigger signaling cascades that lead to the activation of innate and adaptive immune responses and have been found to suppress experimental allergic asthma [143,144]. Other PRRs, like Nod-like receptors (NLRs), are present in the cytosol and recognize intracellular bacterial components. Since the aforementioned protection against asthma through *Listeria* immunization was not seen in heatkilled *Lm* (which is unable to access the host cytosol), an interaction between *Lm* PAMPs and cytosolic NLRs, or an interplay between TLRs and NLRs, may be necessary for the induction of protective non-asthmatic immune responses. Specific *Listeria* PAMPs conferring this protection through PRR interactions may be highly-conserved motifs like lipoteichoic acid, lipoproteins, peptidoglycan subunits, CpG DNA and flagellin. They act as TLR ligands for host cell TLR2 and TLR5, which may have initiated downstream signals that prevented asthma development [145]. The *Listeria*-targeted TLR2 has indeed been found to be upregulated in school-aged children protected from asthma when living

on farms [146]. This hypothesis may then be extended to more general terms: Certain microorganism carry specific motifs that act as TLR and/or NLR ligands in the host to non-specifically inhibit allergic responses upon allergen challenge. These protective downstream signals triggered by microbial exposure may increase the number and functionality of T_{reg} cells for example, thereby influencing the maturation and balance of T-cell polarization [147]. Since mice in our experiments were only exposed to one type of bacterium and not fully protected from asthma upon allergen challenge, it may be that a combination of several different TLR and NLR ligand motifs is necessary to confer "complete" protection from asthma. This may explain why children who grew up on farms – and were thereby subject to a wide array of microbes – developed no asthma at all.

Alternatively, exposure of children to diverse microbes in farming communities early in life may result in the establishment of a beneficial microbiome in the lungs or on other mucosal surfaces (e.g. intestines), thereby preventing the colonization of detrimental pathogens and respiratory viruses that have been associated with higher asthma prevalences (e.g. *Haemophilus influenzae, Chlamydia pneumoniae, Moraxella catarrhalis,* or *Streptococcus pneumoniae*) [148-150]. Undesired bacteria and viruses can reduce the epithelial barrier function via the expression of proteolytic enzymes and secrete mediators that encourage the development of Th2 cell responses (e.g. CD23, CD25, CD40) [151]. The secretion of bacterial proteases and other proteolytic enzymes encourages basophils to produce thymic stromal lymphopoietin (TSLP), which is capable of promoting allergic inflammation via early IL-4 synthesis, thereby facilitating allergen sensitization, Th2 differentiation and IgE-mediated immune responses [152]. This suggestion is supported by emerging data that has shown an association between asthmatic airway hyperresponsiveness and heavy colonization of diverse bacteria in the lower airways [148,153]. Early colonization with symbiotic or commensal microbes of the upper bronchial tree may therefore protect from later colonization of harmful microbes that exert destructive effects on the airway epithelium, breaking down the barrier that protects immune cells from overexposure to allergen e.

Due to similarities between the local immune system in the lung and other mucosal surfaces (e.g. skin and gut) though shared mucosa associated lymphoid tissue (MALT), cross-talk between distal mucosal sites is possible to a certain extent [154-156]. A healthy lung

microbiome could therefore translate into beneficial effects for other related tissues, while the absence of such a protective lung microbiome in the presence of asthma could predispose patients to an increased risk of disease. As such, adult asthma patients exhibit higher risks of developing cancer and are twice as susceptible to other chronic diseases [157,158]. This may also explain why the repeated exposure to antibiotics in the first year of life, which is known to disrupt the gastrointestinal microbial balance, is associated with greater incidences of childhood asthma [159,160]: Commensal microbial exposure to TLRs and NLRs in the gut mucosa may result in the establishment of a gut microflora that also promotes or supports a healthy lung microflora. Indicative to this notion is also research data that has shown individuals with loss-of-function mutation in filaggrin – the protein that contributes to structural stability of the dermis and to an intact epithelial barrier – to be highly susceptible to atopic dermatitis [161]. Although filaggrin protein expression has not been confirmed in pulmonary epithelium, these patients are also at higher risk to develop asthma.

Furthermore, patients who initially suffer from a single allergic disorder have been reported to also develop other allergic conditions. This is known as "atopic march" and suggests that a functionally impaired epithelium barrier will increase the likelihood of more allergen sensitization in the skin, airways and other epithelial membranes and result in the development of systemic immune responses that lead to immune-deregulatory diseases at sites removed from the original location of allergen exposure. In order to gain better insights into this matter, it needs to be determined how specific patterns and variables of microbial exposure (route of exposure, time of exposure, frequency of exposure, microbial density per exposure) affect colonization of the airways, skin and gut. Some infectious antigens have been shown to demonstrate bidirectional capacities on asthma modulation. Endotoxins for example, which are ubiquitous in the environment, are able to both exacerbate and prevent asthma development [162]. This suggests that the impact of specific microorganisms as "harmful" or "beneficial" may depend on other factors, too. The timing and duration of exposure to allergens seem to be determining variables in the disease outcome – *in utero* and continuous postnatal exposure appear to be most beneficial and protective [139].

In the light of the above observations the question remains why immunization with HK*Lm*-OVA did not confer protection in our experimental model even though this has been

observed in other research groups [69-71]. One explanation could be found in the different protocols to prepare the HK*Lm*. Yeung et al. (1998) incubated their infection aliquots at 80°C for 1 h, while we heat-killed our *Lm* strains for 20min at 110°C. Perhaps higher temperatures, damaged certain essential *Lm* TLR ligand motifs.

3.2) OUTLOOKS

The goal for of our follow-up projects is:

1) to elucidate the molecular protective mechanisms of neonatal, live-attenuated *Lm* vaccination that prevents asthma onset in OVA-sensitized and OVA-challenged mice.
2) to extend the applications of this live-attenuated *Lm*-based vaccines to the delivery of more allergens, to more recipient groups, through more effective routes.

We have already realized that there is cross-talk between these two aims. For example, elucidating a new route of *Lm* vaccination that can effectively confer protection will also teach us about the mechanistic path *Lm* strikes to prevent disease.

3.2.1) How does neonatal, live-attenuated *Lm* immunization lead to protection in our asthma model?

Dissecting the relevant molecular *Lm*-mediated interactions that confer protection from Th2 hypersensitivity will identify high-priority targets for therapeutic interventions and broader applications for this vaccine vehicle. We therefore started investigating other potential players that have been shown to participate in the up- or downregulation of inflammatory processes. We are currently in the process of investigating spleen and lung cells for their *ex vivo* synthesis of IL-17, IL-1β, IL-6, TNF-α, VEGF and IL-9. We also hope to gain a fuller picture of how antigen-specific antibodies relate to overall antibody levels, and thus started investigating blood serum for total IgE and IgG1 levels. More detailed interactions and signaling mechanisms of *Lm* still take time to decipher. In the meantime, however, we are able to apply *Lm* in various controlled settings, observe the outcome, and draw conclusions on how this outcome was brought about. We and others have observed that only one immunization with live-attenuated

Lm induces long-term, Th1 cell-mediated immunity against antigens expressed by *Lm*. Over the course of my thesis, it has been established that this property may have protected the host from asthma and balanced the host immune system after allergen challenge, thereby offsetting an otherwise Th2-biased, inflammatory immune response. However, the question remains whether this prophylactic Th1-skewing through *Lm* administration may actually be harmful to the body in the absence of Th2-cell induction or in response to triggers that bring about additional Th1-skewing. To answer this question, we now investigate prophylactic *Lm* immunization in the context of Farmer's Lung – a type III hypersensitivity reaction that leads to asthma-like symptoms via Th1 immune responses. We will analyze these symptoms relative to positive and negative controls after neonatal mice have been vaccinated with our *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA strain, and sensitized and subsequently challenged with moldy hay allergen – the causative agent of Farmer's Lung. Based on these outcomes, we will be able to draw conclusions about *Lm*'s immunomodulatory capacity in the light of both Th1 and Th2-skewed immune responses.

3.2.2) Can we extend the application of a live-attenuated *Lm*-based allergy vaccines?

Extension to other allergens: The mechanisms involved in the findings of this thesis are likely to be highly relevant not just to ovalbumin, but other allergens. To this end, we have already turned our focus to food allergies of which peanut allergies are most commonly associated with fatal anaphylaxis and would therefore highly benefit from a curative treatment option. Our rationale for an *Lm*-based peanut allergy vaccine is based on recombinant protein immunotherapy that utilizes mutated forms of the highly allergenic peanut seed storage proteins Arah 1, Arah 2 and Arah 3. There is a reduced risk of an allergic reaction being elicited by these mutated peanut proteins since they feature an altered critical amino acid that eliminates IgE binding but maintains T-cell activation capacities. I have already cloned this allergen construct into our live-attenuated *Lm* vaccine platform. Similar to our *Lm*-OVA construct, *Lm* will act as a bacterial adjuvant that we hope will modulate immune responses in an antigen-specific manner towards tolerance after peanut allergen challenge.

The testing of such antigen-specific immune protection mechanisms involves the cloning of specific allergens into the *Lm* vaccine plasmid – which is a very time consuming process. Due

to the protective, anti-allergic, antigen non-specific immune responses we observed in our asthma model through neonatal immunization with *Lm* Δ(*trpS actA*)/pSPO, we therefore would like to extend our efforts to testing *Lm* Δ(*trpS actA*)/pSPO as an "all-purpose immune modulator" for allergies or other Th2-skewed diseases. This is especially valuable in view of most allergic patients suffering from hypersensitivity disorders induced by multiple allergens. To test *Lm*'s capacity for this purpose, we plan to prophylactically immunize neonatal, disease-susceptible mice with *Lm* and follow up with co-sensitization and co-challenge using multiple allergens.

Extension to other recipient groups: Previous data from our lab has demonstrated that murine neonates vaccinated with attenuated *Lm* strains mount robust, protective and antigen-specific immune responses. Similarly, the research project of this thesis has focused on the protection of *neonatally* immunized mice from the development of allergic asthma. Our next question is whether such protective immune responses can *only* be achieved through neonatal immunization. To answer this question, we repeated the same experimental schedule of this thesis with *adult* mice. They were prophylactically immunized with *Lm* Δ(*trpS actA*)/pSPO-PS_{hly}OVA (*Lm*-OVA) and *Lm* Δ(*trpS actA*)/pSPO at around six weeks of age, sensitized and then challenged with OVA. Preliminary data suggests that adult mice were also significantly protected from asthma when prophylactically immunized with *Lm*-OVA. However, the antigen non-specific immune response towards immunization with *Lm* Δ(*trpS actA*)/pSPO did not appear to be as obvious in adult mice as we have seen in neonatally immunized mice. We will repeat this experiment to confirm these observations that suggest that immunization early in life represents a crucial immunological window in time.

These promising data also lead us to hypothesize that our *Lm* vaccine platform may be able to reverse ongoing, already established pro-allergic Th2 dominated responses and therefore prevent recurrent allergic inflammation following local allergen challenge.

In order to assess the therapeutic efficacy of such a live *Lm*-based approach, we first established an asthma model for early life in our mouse model system. To that end, 6-day old newborn mice were immunized with 100µg OVA absorbed onto Alum hydroxide gel via the *i.p.* route. On days 22, 23, 24, 26 and 28 after the first OVA immunization, mice were challenged

with daily *i.n*. injections of 200µg OVA in PBS under light anesthesia with isofluorane. One day after the last *i.n*. injection, allergic conditions were assessed in one subgroup to verify the successful induction of an allergic response. The remaining mice were immunized *i.p.* with the attenuated *Lm* strains expressing or lacking OVA expression as well as heatkilled *Lm*-OVA. Six weeks after that, mice were exposed again to daily *i.n.* challenges of 200µg OVA in PBS under light anesthesia with isoflurane for five consecutive days. One day after the last *i.n.* challenge, mice were euthanize (Fig. 11). We used the same readout of severity of allergic conditions against OVA as previously outlined for the prophylactic vaccine experimental setup (Fig.3). To date, the experimental design to test this hypothesis was unsuccessful in our attempts to induce asthma in newborns. We are currently optimizing the protocol for early life asthma induction and also set out to examine the therapeutic potential of *Lm* in adult mice. This is currently an easier undertaking, since asthma induction in adult mice has already been established in the protocol for the prophylactic vaccine schedule.

Extension to other routes of vaccination: Although the protective effect of neonatal *i.p.* immunization with live-attenuated *Lm* is evident, it is still unclear as to where and how the *Lm* "action potential" travels so that *i.p.* localized bacteria confer protection to lung cells – clearly a distal site from the peritoneum. Given the findings of this thesis, we hypothesized that neonatal immunization with live-attenuated *Lm* via the intranasal (*i.n.*) route would carry immune-modulating, mobility-restricted (Δ*act*) *Lm* directly to the locale to which allergens will be directed during *i.n.* challenges and could therefore confer even better protection than *i.p.* immunization. To test this hypothesis, neonatal mice were placed on ice and then immunized *i.n.* with 10uL of 10^7 CFU of *Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA, *Lm* Δ(*trpS actA*)/pSPO as well as HK*Lm* Δ(*trpS actA*)/pSPO-PS$_{hly}$OVA. The subsequent protocol was identical to the one outlined for the prophylactic *i.p.* immunization schedule. Although both live-attenuated *Lm* strains showed again lower total cell counts in the BALF than the asthma group, this was only significant for the "*Lm*-OVA Asthma". The most striking finding in this preliminary experiment was that the "HK*Lm*-OVA Asthma" group exhibited even lower cell counts in the BALF than the "*Lm*-OVA Asthma" group.

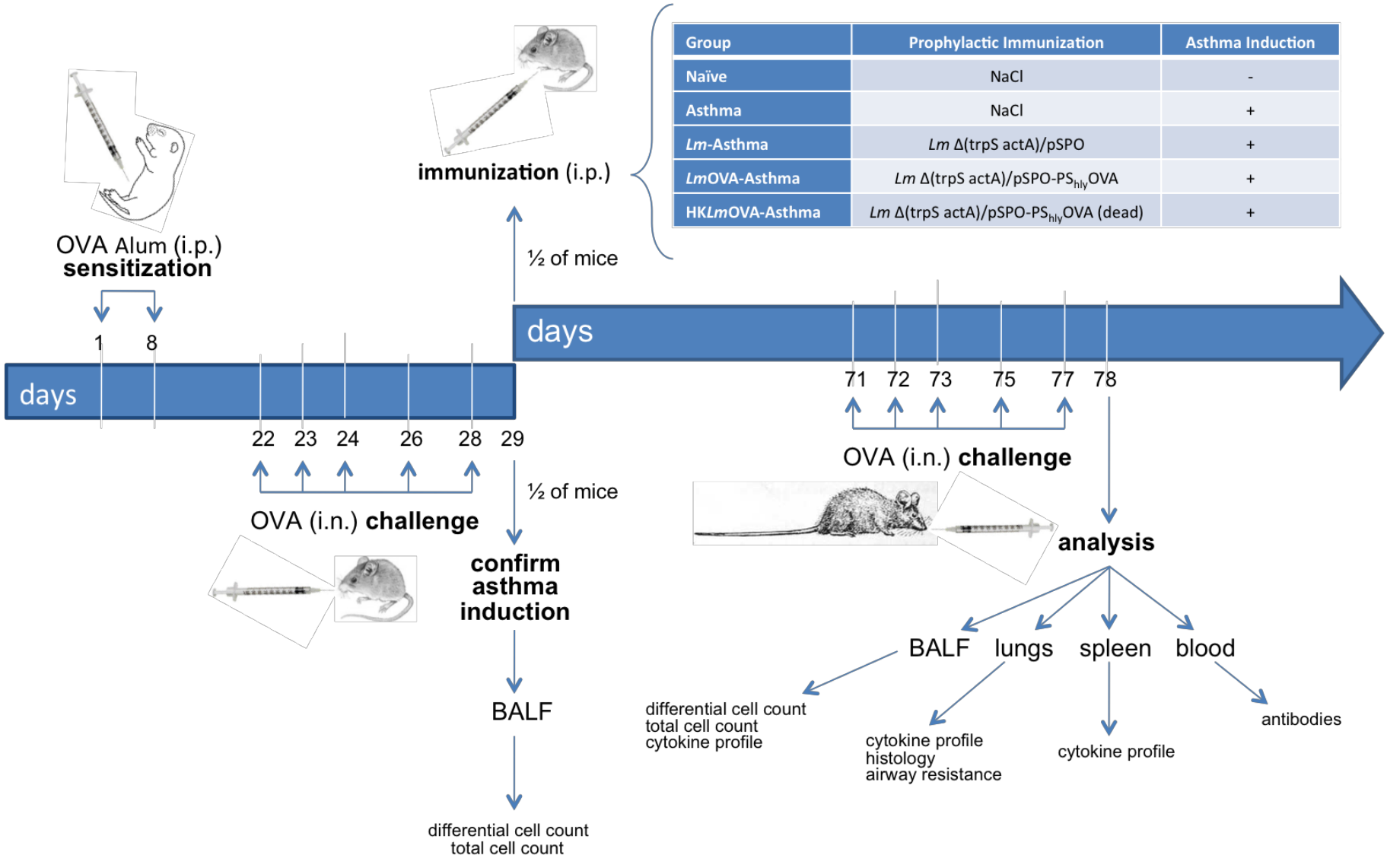

Figure 11. Experimental setup for therapeutic vaccine. 6-day old newborn mice were immunized with 100µg OVA absorbed onto Alum hydroxide gel via the *i.p.* route. On days 22, 23, 24, 26 and 28 post-OVA sensitization, mice were challenged with daily *i.n.* injections of 200µg OVA in PBS under light anesthesia with isofluorane. One day after the last *i.n.* injection, allergic conditions were assessed in one subgroup to verify the successful induction of an allergic response. The remaining mice were immunized *i.p.* with the attenuated *Lm* strains expressing or lacking expressed OVA peptides, as well as heatkilled *Lm*-OVA. Six weeks later, mice were challenged again with daily *i.n.* injections of 200µg OVA in PBS under light anesthesia with isofluorane for five consecutive days. One day after the last *i.n.* challenge, mice were euthanized an analyzed for asthmatic markers.

BIBLIOGRAPHY

1 Handoyo S, Rosenwasser LJ. Asthma phenotypes. *Curr Allergy Asthma Rep*. 2009;9:439-445.

2 Thunberg S, Gafvelin G, Nord M, et al. Allergen provocation increases TH2-cytokines and FOXP3 expression in the asthmatic lung. *Allergy*. 2010;65:311-318.

3 Duffy DL, Mitchell CA, Martin NG. Genetic and environmental risk factors for asthma: A cotwin-control study. *Am J Respir Crit Care Med*. 1998;157:840-845.

4 Holt PG, Sly PD. Non-atopic intrinsic asthma and the 'family tree' of chronic respiratory disease syndromes. *Clin Exp Allergy*. 2009;39:807-811.

5 Bara I, Ozier A, Tunon de Lara JM, Marthan R, Berger P. Pathophysiology of bronchial smooth muscle remodelling in asthma. *Eur Respir J*. 2010;36:1174-1184.

6 Barnes PJ. Pathophysiology of asthma. *Br J Clin Pharmacol*. 1996;42:3-10.

7 Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature*. 2008;454:445-454.

8 Kay AB. Allergy and allergic diseases. first of two parts. *N Engl J Med*. 2001;344:30-37.

9 Kay AB. Allergy and allergic diseases. second of two parts. *N Engl J Med*. 2001;344:109-113.

10 Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: Linking innate and adaptive immunity in asthma. *Nat Rev Immunol*. 2008;8:193-204.

11 Hegele RG. The pathology of asthma: Brief review. *Immunopharmacology*. 2000;48:257-262.

12 Sokol CL, Medzhitov R. Emerging functions of basophils in protective and allergic immune responses. *Mucosal Immunol*. 2010;3:129-137.

13 Gleich GJ, Kita H. Bronchial asthma: Lessons from murine models. *Proc Natl Acad Sci U S A*. 1997;94:2101-2102.

14 Anderson GP. The immunobiology of early asthma. *Med J Aust*. 2002;177 Suppl:S47-9.

15 33. Holt PG, Strickland DH. Interactions between innate and adaptive immunity in asthma pathogenesis: New perspectives from studies on acute exacerbations. *J Allergy Clin Immunol*. 2010;125:963-72; quiz 973-4.

16 Salam MT, Li YF, Langholz B, Gilliland FD, Children's Health Study. Early-life environmental risk factors for asthma: Findings from the children's health study. *Environ Health Perspect*. 2004;112:760-765.

17 Duffy DL, Mitchell CA, Martin NG. Genetic and environmental risk factors for asthma: A cotwin-control study. *Am J Respir Crit Care Med*. 1998;157:840-845.

18 Holt PG. Developmental factors as determinants of risk for infections and atopy in childhood. *Eur Respir Rev*. 2005;14:69-73.

19 Dietert RR, Piepenbrink MS. The managed immune system: Protecting the womb to delay the tomb. *Hum Exp Toxicol*. 2008;27:129-134.

20 Brown SJ, Asai Y, Cordell HJ, et al. Loss-of-function variants in the filaggrin gene are a significant risk factor for peanut allergy. *J Allergy Clin Immunol*. 2011;127:661-667.

21 Palmer LJ, Cookson WO. Using single nucleotide polymorphisms as a means to understanding the pathophysiology of asthma. *Respir Res*. 2001;2:102-112.

22 Moffatt MF, Gut IG, Demenais F, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med*. 2010;363:1211-1221.

23 Huffnagle GB. The microbiota and allergies/asthma. *PLoS Pathog*. 2010;6:e1000549.

24 Lougheed MD, Lemiere C, Dell SD, et al. Canadian thoracic society asthma management continuum--2010 consensus summary for children six years of age and over, and adults. *Can Respir J*. 2010;17:15-24.

25 Institute of Medicine. Committee on the assessment of asthma and indoor air, division of health promotion and disease prevention. Clearing the air: asthma and indoor air exposures. Washington DC, *National Academy Press,* 2000.

26 Finkelstein JA, Fuhlbrigge A, Lozano P, et al. Parent-reported environmental exposures and environmental control measures for children with asthma. *Arch Pediatr Adolesc Med*. 2002;156:258-264.

27 Corry DB. Emerging immune targets for the therapy of allergic asthma. *Nat Rev Drug Discov*. 2002;1:55-64.

28 Frew AJ. Allergen immunotherapy. *J Allergy Clin Immunol*. 2010;125:S306-13.

29 Mondoulet L, Dioszeghy V, Ligouis M, Dhelft V, Dupont C, Benhamou PH. Epicutaneous immunotherapy on intact skin using a new delivery system in a murine model of allergy. *Clin Exp Allergy*. 2010;40:659-667.

30 Frew AJ. Immunotherapy: How safe is safe? *Clin Exp Allergy*. 2006;36:251-253

31 Ayres JG, Higgins B, Chilvers ER, et al. 2004. Efficacy and tolerability of anti-immunoglobulin E therapy with omalizumab in patients with poorly controlled (moderate-to-severe) allergic asthma. *Allergy,* 59:7018.

32 Camporota L, Corkhill A, Long H, et al. The effects of mycobacterium vaccae on allergen-induced airway responses in atopic asthma. *Eur Respir J*. 2003;21:287-293.

33 Shi H, Wang S, Roland KL, Gunn BM, Curtiss R,3rd. Immunogenicity of a live recombinant salmonella enterica serovar typhimurium vaccine expressing pspA in neonates and infant mice born from naive and immunized mothers. *Clin Vaccine Immunol*. 2010;17:363-371.

34 Senti G, Johansen P, Haug S, et al. Use of A-type CpG oligodeoxynucleotides as an adjuvant in allergen-specific immunotherapy in humans: A phase I/IIa clinical trial. *Clin Exp Allergy*. 2009;39:562-570.

35 Bochner BS, Undem BJ, Lichtenstein LM. Immunological aspects of allergic asthma. *Annu Rev Immunol*. 1994;12:295-335.

36 Siegrist CA. The challenges of vaccine responses in early life: Selected examples. *J Comp Pathol*. 2007;137 Suppl 1:S4-9.

37 Luebke RW, Chen DH, Dietert R, et al. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J Toxicol Environ Health B Crit Rev*. 2006;9:1-26.

38 Cookson WO, Moffatt MF. Asthma: An epidemic in the absence of infection? *Science*. 1997;275:41-42.

39 Kemp A, Bjorksten B. Immune deviation and the hygiene hypothesis: A review of the epidemiological evidence. *Pediatr Allergy Immunol*. 2003;14:74-80.

40 Salvi SS, Babu KS, Holgate ST. Is asthma really due to a polarized T cell response toward a helper T cell type 2 phenotype? *Am J Respir Crit Care Med*. 2001;164:1343-1346.

41 Sung S, Rose CE, Fu SM. Intratracheal priming with ovalbumin- and ovalbumin 323-339 peptide-pulsed dendritic cells induces airway hyperresponsiveness, lung eosinophilia, goblet cell hyperplasia, and inflammation. *J Immunol*. 2001;166:1261-1271.

42 Barnes PJ. Th2 cytokines and asthma: An introduction. *Respir Res*. 2001;2:64-65.

43 Mazzarella G, Bianco A, Catena E, De Palma R, Abbate GF. Th1/Th2 lymphocyte polarization in asthma. *Allergy*. 2000;55 Suppl 61:6-9.

44 Wynn TA. T(H)-17: A giant step from T(H)1 and T(H)2. *Nat Immunol*. 2005;6:1069-1070.

45 Ivanov S, Linden A. Th-17 cells in the lungs? *Expert Rev Respir Med*. 2007;1:279-293.

46 Linden A, Laan M, Anderson GP. Neutrophils, interleukin-17A and lung disease. *Eur Respir J*. 2005;25:159-172.

47 Song C, Luo L, Lei Z, et al. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol*. 2008;181:6117-6124

48 Chakir J, Shannon J, Molet S, et al. Airway remodeling-associated mediators in moderate to severe asthma: Effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol*. 2003;111:1293-1298.

49 Schnyder-Candrian S, Togbe D, Couillin I, et al. Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med*. 2006;203:2715-2725.

50 Strickland DH, Judd S, Thomas JA, Larcombe AN, Sly PD, Holt PG. Boosting airway T-regulatory cells by gastrointestinal stimulation as a strategy for asthma control. *Mucosal Immunol*. 2011;4:43-52.

51 Akbari O, Stock P, DeKruyff RH, Umetsu DT. Role of regulatory T cells in allergy and asthma. *Curr Opin Immunol*. 2003;15:627-633.

52 van Oosterhout AJ, Bloksma N. Regulatory T-lymphocytes in asthma. *Eur Respir J*. 2005;26:918-932

53 Smyth LJ, Eustace A, Kolsum U, Blaikely J, Singh D. Increased airway T regulatory cells in asthmatic subjects. *Chest*. 2010;138:905-912.

54 WHO, *The World Health Report*. 2004, World Health Organization: Geneva, Switzerland.

55 Hanson LA, Silfverdal SA. Vaccination immunology. *Scand J Infect Dis*. 2008;40:696-701.

56 Castellino F, Galli G, Del Giudice G, Rappuoli R. Generating memory with vaccination. *Eur J Immunol*. 2009;39:2100-2105.

57 Ronchetti R, Jesenak M, Rennerova Z, Barreto M, Ronchetti F, Villa MP. Relationship between atopic asthma and the population prevalence rates for asthma or atopy in children: Atopic and nonatopic asthma in epidemiology. *Allergy Asthma Proc*. 2009;30:55-63.

58 Demirjian A, Levy O. Safety and efficacy of neonatal vaccination. *Eur J Immunol*. 2009;39:36-46.

59 Siegrist CA. Neonatal and early life vaccinology. *Vaccine*. 2001;19:3331-3346.

60 Prozesky OW, Stevens CE, Szmuness W, et al. Immune response to hepatitis B vaccine in newborns. *J Infect*. 1983;7 Suppl 1:53-55.

61 Marchant A, Goetghebuer T, Ota MO, et al. Newborns develop a Th1-type immune response to mycobacterium bovis bacillus calmette-guerin vaccination. *J Immunol*. 1999;163:2249-2255.

62 Pamer EG. Immune responses to listeria monocytogenes. *Nat Rev Immunol*. 2004;4:812-823.

63 Bielecki J, Youngman P, Connelly P, Portnoy DA. Bacillus subtilis expressing a haemolysin gene from listeria monocytogenes can grow in mammalian cells. *Nature*. 1990;345:175-176.

64 Domann E, Wehland J, Rohde M, et al. A novel bacterial virulence gene in listeria monocytogenes required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *EMBO J*. 1992;11:1981-1990.

65 Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, Cossart P. L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. *Cell*. 1992;68:521-531.

66 Fan MW, Bian Z, Peng ZX, et al. A DNA vaccine encoding a cell-surface protein antigen of streptococcus mutans protects gnotobiotic rats from caries. *J Dent Res*. 2002;81:784-787.

67 Nomoto K, Harada T, Koyanagi R, Hosoi M, Takeya K. Immune response against hamster erythrocytes in the low-responder mouse strains. XI. strain difference in the effects of various microbial adjuvants. *Jpn J Microbiol*. 1976;20:375-384.

68 Mizuki D, Miura T, Sasaki S, Mizuki M, Madarame H, Nakane A. Interference between host resistance to listeria monocytogenes infection and ovalbumin-induced allergic responses in mice. *Infect Immun*. 2001;69:1883-1888.

69 Yeung VP, Gieni RS, Umetsu DT, DeKruyff RH. Heat-killed listeria monocytogenes as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses. *J Immunol*. 1998;161:4146-4152.

70 Frick OL, Teuber SS, Buchanan BB, Morigasaki S, Umetsu DT. Allergen immunotherapy with heat-killed listeria monocytogenes alleviates peanut and food-induced anaphylaxis in dogs. *Allergy*. 2005;60:243-250.

71 Hansen G, Yeung VP, Berry G, Umetsu DT, DeKruyff RH. Vaccination with heat-killed listeria as adjuvant reverses established allergen-induced airway hyperreactivity and inflammation: Role of CD8+ T cells and IL-18. *J Immunol*. 2000;164:223-230.

72 Kollmann TR, Reikie B, Blimkie D, et al. Induction of protective immunity to listeria monocytogenes in neonates. *J Immunol*. 2007;178:3695-3701.

73 Reikie BA, Smolen KK, Fortuno ES,3rd, et al. A single immunization near birth elicits immediate and lifelong protective immunity. *Vaccine*. 2010;29:83-90.

74 Schoen C, Loeffler DI, Frentzen A, Pilgrim S, Goebel W, Stritzker J. Listeria monocytogenes as novel carrier system for the development of live vaccines. *Int J Med Microbiol*. 2008;298:45-58.

75 Loeffler DI, Smolen K, Aplin L, Cai B, Kollmann TR. Fine-tuning the safety and immunogenicity of listeria monocytogenes-based neonatal vaccine platforms. *Vaccine*. 2009;27:919-927.

76 Wallecha A, Maciag PC, Rivera S, Paterson Y, Shahabi V. Construction and characterization of an attenuated listeria monocytogenes strain for clinical use in cancer immunotherapy. *Clin Vaccine Immunol*. 2009;16:96-103.

77 Maciag PC, Radulovic S, Rothman J. The first clinical use of a live-attenuated listeria monocytogenes vaccine: A phase I safety study of lm-LLO-E7 in patients with advanced carcinoma of the cervix. *Vaccine*. 2009;27:3975-3983.

78 Brockstedt DG, Dubensky TW. Promises and challenges for the development of *Listeria monocytogenes*-based immunotherapies. *Expert Rev Vaccines*. 2008;7:1069-1084.

79 Loeffler DI, Schoen CU, Goebel W, Pilgrim S. Comparison of different live vaccine strategies in vivo for delivery of protein antigen or antigen-encoding DNA and mRNA by virulence-attenuated listeria monocytogenes. *Infect Immun*. 2006;74:3946-3957.

80 Braman SS. The global burden of asthma. *Chest*. 2006;130:4S-12S.

81 Masoli M, Fabian D, Holt S, Beasley R, Global Initiative for Asthma (GINA) Program. The global burden of asthma: Executive summary of the GINA dissemination committee report. *Allergy*. 2004;59:469-478.

82 Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J Immunol*. 1999;163:6448-6454.

83 Tomioka S, Bates JH, Irvin CG. Airway and tissue mechanics in a murine model of asthma: Alveolar capsule vs. forced oscillations. *J Appl Physiol*. 2002;93:263-270.

84 Vanoirbeek JA, Rinaldi M, De Vooght V, et al. Noninvasive and invasive pulmonary function in mouse models of obstructive and restrictive respiratory diseases. *Am J Respir Cell Mol Biol*. 2010;42:96-104.

85 Lazarus SC. Inflammation, inflammatory mediators, and mediator antagonists in asthma. *J Clin Pharmacol*. 1998;38:577-582.

86 Dunnill MS. The pathology of asthma, with special reference to changes in the bronchial mucosa. *J Clin Pathol*. 1960;13:27-33.

87 Kay AB, Corrigan CJ. Asthma. Eosinophils and neutrophils. *Br Med Bull*. 1992;48:51-64.

88 Tomita M, Kobayashi T, Itoh H, Onitsuka T, Nawa Y. Goblet cell hyperplasia in the airway of nippostrongylus brasiliensis-infected rats. *Respiration*. 2000;67:565-569.

89 Hirota JA, Ellis R, Inman MD. Regional differences in the pattern of airway remodeling following chronic allergen exposure in mice. *Respir Res*. 2006;7:120.

90 Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest*. 2008;118:3546-3556.

91 Blackburn S. Cytokines in the perinatal and neonatal periods: Selected aspects. *J Perinat Neonatal Nurs*. 2008;22:187-190.

92 Chung F. Anti-inflammatory cytokines in asthma and allergy: Interleukin-10, interleukin-12, interferon-gamma. *Mediators Inflamm*. 2001;10:51-59.

93 Chung KF, Barnes PJ. Cytokines in asthma. *Thorax*. 1999;54:825-857.

94 Conlan JW. Early pathogenesis of Listeria monocytogenes infection in the mouse spleen. *J Med Microbiol*. 1996;44:295-302.

95 Flesch IE, Wandersee A, Kaufmann SH. Effects of IL-13 on murine listeriosis. *Int Immunol*. 1997;9:467-474.

96 Ormstad H, Groeng EC, Duffort O, Lovik M. The effect of endotoxin on the production of IgE, IgG1 and IgG2a antibodies against the cat allergen fel d 1 in mice. *Toxicology*. 2003;188:309-318.

97 Mountford AP, Fisher A, Wilson RA. The profile of IgG1 and IgG2a antibody responses in mice exposed to schistosoma mansoni. *Parasite Immunol*. 1994;16:521-527.

98 Bousquet J, Chanez P, Lacoste JY, et al. Eosinophilic inflammation in asthma. *N Engl J Med*. 1990;323:1033-1039.

99 Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: Recent advances. *Annu Rev Immunol*. 2005;23:749-786.

100 Williams CM, Galli SJ. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. *J Allergy Clin Immunol*. 2000;105:847-859.

101 Xue A, Wang J, Sieck GC, Wylam ME. Distribution of major basic protein on human airway following in vitro eosinophil incubation. *Mediators Inflamm*. 2010;2010:824362.

102 Uhlig S, Goggel R, Engel S. Mechanisms of platelet-activating factor (PAF)-mediated responses in the lung. *Pharmacol Rep*. 2005;57 Suppl:206-221.

103 Lou YP, Takeyama K, Grattan KM, et al. Platelet-activating factor induces goblet cell hyperplasia and mucin gene expression in airways. *Am J Respir Crit Care Med*. 1998;157:1927-1934.

104 Duvernelle C, Freund V, Frossard N. Transforming growth factor-beta and its role in asthma. *Pulm Pharmacol Ther*. 2003;16:181-196.

105 Takeda N, Sumi Y, Prefontaine D, et al. Epithelium-derived chemokines induce airway smooth muscle cell migration. *Clin Exp Allergy*. 2009;39:1018-1026.

106 Lukacs NW, Oliveira SH, Hogaboam CM. Chemokines and asthma: Redundancy of function or a coordinated effort? *J Clin Invest*. 1999;104:995-999.

107 Riffo-Vasquez Y, Spina D. Role of cytokines and chemokines in bronchial hyperresponsiveness and airway inflammation. *Pharmacol Ther*. 2002;94:185-211.

108 Ghaffar O, Hamid Q, Renzi PM, et al. Constitutive and cytokine-stimulated expression of eotaxin by human airway smooth muscle cells. *Am J Respir Crit Care Med*. 1999;159:1933-1942.

109 Chan V, Burgess JK, Ratoff JC, et al. Extracellular matrix regulates enhanced eotaxin expression in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med*. 2006;174:379-385.

110 Gounni AS, Wellemans V, Yang J, et al. Human airway smooth muscle cells express the high affinity receptor for IgE (fc epsilon RI): A critical role of fc epsilon RI in human airway smooth muscle cell function. *J Immunol*. 2005;175:2613-2621.

111 Gounni AS. The high-affinity IgE receptor (FcepsilonRI): A critical regulator of airway smooth muscle cells? *Am J Physiol Lung Cell Mol Physiol*. 2006;291:L312-21.

112 Victor JR, Muniz BP, Fusaro AE, et al. Maternal immunization with ovalbumin prevents neonatal allergy development and up-regulates inhibitory receptor fc gamma RIIB expression on B cells. *BMC Immunol*. 2010;11:11.

113 Coffman RL, Seymour BW, Lebman DA, et al. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol Rev*. 1988;102:5-28.

114 Tang ML, Kemp AS, Thorburn J, Hill DJ. Reduced interferon-gamma secretion in neonates and subsequent atopy. *Lancet*. 1994;344:983-985.

115 Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. *Science*. 1997;275:77-79.

116 Tang L, Benjaponpitak S, DeKruyff RH, Umetsu DT. Reduced prevalence of allergic disease in patients with multiple sclerosis is associated with enhanced IL-12 production. *J Allergy Clin Immunol*. 1998;102:428-435.

117 Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*. 1989;170:2081-2095.

118 Lipozencic J. A hundred years of "allergy" clemens von pirquet's essay "allergie", published on july 24, 1906 in munchener medizinische wochenschrift. *Acta Dermatovenerol Croat*. 2006;14:206.

119 Oshiba A, Hamelmann E, Takeda K, et al. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (ig) E and IgG1 in mice. *J Clin Invest*. 1996;97:1398-1408.

120 Saini SS, Klion AD, Holland SM, Hamilton RG, Bochner BS, Macglashan DW,Jr. The relationship between serum IgE and surface levels of FcepsilonR on human leukocytes in various diseases: Correlation of expression with FcepsilonRI on basophils but not on monocytes or eosinophils. *J Allergy Clin Immunol*. 2000;106:514-520.

121 Saxon A, Zhu D, Zhang K, Allen LC, Kepley CL. Genetically engineered negative signaling molecules in the immunomodulation of allergic diseases. *Curr Opin Allergy Clin Immunol*. 2004;4:563-568.

122 Hammad H, Plantinga M, Deswarte K, et al. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med*. 2010;207:2097-2111.

123 Malbec O, Daeron M. The mast cell IgG receptors and their roles in tissue inflammation. *Immunol Rev*. 2007;217:206-221.

124 Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol*. 2008;8:205-217.

125 Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol*. 1999;17:255-281.

126 Lehar SM, Bevan MJ. Immunology: Polarizing a T-cell response. *Nature*. 2004;430:150-151.

127 Neurath MF, Finotto S, Glimcher LH. The role of Th1/Th2 polarization in mucosal immunity. *Nat Med*. 2002;8:567-573.

128 van Oosterhout AJ, Motta AC. Th1/Th2 paradigm: Not seeing the forest for the trees? *Eur Respir J*. 2005;25:591-593.

129 Salvi SS, Babu KS, Holgate ST. Is asthma really due to a polarized T cell response toward a helper T cell type 2 phenotype? *Am J Respir Crit Care Med*. 2001;164:1343-1346.

130 Cuddapah S, Barski A, Zhao K. Epigenomics of T cell activation, differentiation, and memory. *Curr Opin Immunol*. 2010;22:341-347.

131 Umetsu DT, DeKruyff RH. Interleukin-10: The in asthma regulation? *Am J Respir Cell Mol Biol*. 1999;21:562-563.

132 Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production by T helper 2 (Th2) cells: A critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med*. 1997;186:1737-1747.

133 Hansen G, McIntire JJ, Yeung VP, et al. CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *J Clin Invest*. 2000;105:61-70.

134 Randolph DA, Carruthers CJ, Szabo SJ, Murphy KM, Chaplin DD. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J Immunol*. 1999;162:2375-2383.

135 Garn H, Renz H. Epidemiological and immunological evidence for the hygiene hypothesis. *Immunobiology*. 2007;212:441-452.

136 Kemp A, Bjorksten B. Immune deviation and the hygiene hypothesis: A review of the epidemiological evidence. *Pediatr Allergy Immunol*. 2003;14:74-80.

137 Thornton CA, Macfarlane TV, Holt PG. The hygiene hypothesis revisited: Role of materno-fetal interactions. *Curr Allergy Asthma Rep*. 2010;10:444-452.

138 Tantisira KG, Weiss ST. Childhood infections and asthma: At the crossroads of the hygiene and barker hypotheses. *Respir Res*. 2001;2:324-327.

139 von Mutius E, Radon K. Living on a farm: Impact on asthma induction and clinical course. *Immunol Allergy Clin North Am*. 2008;28:631-47, ix-x.

140 Ege MJ, Mayer M, Normand AC, et al. Exposure to environmental microorganisms and childhood asthma. *N Engl J Med*. 2011;364:701-709.

141 Riedler J, Braun-Fahrlander C, Eder W, et al. Exposure to farming in early life and development of asthma and allergy: A cross-sectional survey. *Lancet*. 2001;358:1129-1133.

142 Braun-Fahrlander C, Riedler J, Herz U, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med*. 2002;347:869-877.

143 Kollmann TR, Crabtree J, Rein-Weston A, et al. Neonatal innate TLR-mediated responses are distinct from those of adults. *J Immunol*. 2009;183:7150-7160.

144 Aumeunier A, Grela F, Ramadan A, et al. Systemic toll-like receptor stimulation suppresses experimental allergic asthma and autoimmune diabetes in NOD mice. *PLoS One*. 2010;5:e11484.

145 Bruhn KW, Craft N, Miller JF. Listeria as a vaccine vector. *Microbes Infect*. 2007;9:1226-1235.

146 Ege MJ, Bieli C, Frei R, et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol*. 2006;117:817-823.

147 Schaub B, Liu J, Hoppler S, et al. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. *J Allergy Clin Immunol*. 2009;123:774-82.e5.

148 Hilty M, Burke C, Pedro H, et al. Disordered microbial communities in asthmatic airways. *PLoS One*. 2010;5:e8578.

149 Bisgaard H, Hermansen MN, Buchvald F, et al. Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med*. 2007;357:1487-1495.

150 Gencay M, Rudiger JJ, Tamm M, Soler M, Perruchoud AP, Roth M. Increased frequency of chlamydia pneumoniae antibodies in patients with asthma. *Am J Respir Crit Care Med*. 2001;163:1097-1100.

151 Furmonaviciene R, Ghaemmaghami AM, Boyd SE, et al. The protease allergen der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: Experimental analysis of in silico substrate identification and implications in allergic responses. *Clin Exp Allergy*. 2007;37:231-242.

152 Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol*. 2009;183:1427-1434.

153 Huang YJ, Nelson CE, Brodie EL, et al. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J Allergy Clin Immunol*. 2011;127:372-381.e1-3.

154 Lefrancois L, Puddington L. Intestinal and pulmonary mucosal T cells: Local heroes fight to maintain the status quo. *Annu Rev Immunol*. 2006;24:681-704.

155 Kalliomaki M, Salminen S, Isolauri E. Positive interactions with the microbiota: Probiotics. *Adv Exp Med Biol*. 2008;635:57-66.

156 Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. Bacterial community variation in human body habitats across space and time. *Science*. 2009;326:1694-1697.

157 Adams RJ, Wilson DH, Taylor AW, et al. Coexistent chronic conditions and asthma quality of life: A population-based study. *Chest*. 2006;129:285-291.

158 Calder CJ, Lacy D, Raafat F, Weller PH, Booth IW. Crohn's disease with pulmonary involvement in a 3 year old boy. *Gut*. 1993;34:1636-1638.

159 Marra F, Marra CA, Richardson K, et al. Antibiotic use in children is associated with increased risk of asthma. *Pediatrics*. 2009;123:1003-1010.

160 Van der Waaij D. The ecology of the human intestine and its consequences for overgrowth by pathogens such as *Clostridium difficile*. *Annu Rev Microbiol*. 1989;43:69–87.

161 Ying S, Meng Q, Corrigan CJ, Lee TH. Lack of filaggrin expression in the human bronchial mucosa. *J Allergy Clin Immunol*. 2006;118:1386-1388.

162 Renz H, Herz U. The bidirectional capacity of bacterial antigens to modulate allergy and asthma. *Eur Respir J*. 2002;19:158-171.