Modulation of the immune system by *Listeria monocytogenes*

by

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BSc, King Abdulaziz University, 2008

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 2013

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Abstract
We had previously shown that attenuated *Listeria monocytogenes* (*Lm*) vaccines induce a strong Th1 response in neonatal mice, and prophylactically protect against histopathological signs of asthma.

To test the ability of *Lm* as a therapeutic vaccine to diminish allergic sensitization in a mouse model of ovalbumin (OVA)-induced asthma, adult mice were sensitized intraperitoneally and challenged intranasally with OVA to induce asthma. Mice were then immunized intraperitoneally with a single dose of either live attenuated *Lm* vaccine that expresses OVA (*LmOVA*), live *Lm* expressing no allergen, or NaCl. Six weeks later, anesthetized mice were challenged intranasally with OVA and evaluated for cellular infiltration and tissue remodeling. We found that vaccination with live-attenuated *Lm* strains did not reduce already established allergic inflammation in all of the parameters we measured except that it did significantly reduce the total BALF eosinophil count in the *LmOVA* group.

To test the functional impact of our prophylactic vaccine approach in vivo, we immunized mice as newborns with live *LmOVA* or *Lm*, followed 6 weeks later by allergic sensitization with OVA and evaluation by FlexiVent to determine airway hyperreactivity. We found that prophylactic vaccination reduced airway hyperreactivity, in an antigen-specific (i.e. *LmOVA*) as well as non-specific (i.e. *Lm* not expressing OVA) manner. Thus, our neonatal prophylactic vaccine approach holds promise as a powerful tool to modulate early life immune ontogeny and effectively prevent asthma.

In order to study any potentially harmful effect of neonatal immunization with *Lm*, newborn mice were immunized intraperitoneally with the live *Lm* attenuated strain or with saline. Six weeks later, anesthetized mice were challenged intranasally with *Saccharopolyspora rectivirgula* antigen (SR-Ag) on three consecutive days per week for three weeks. We were unable to detect any changes on the HP phenotype comparing vaccinate vs. unvaccinated mice.
In conclusion, prophylactic neonatal immunization with *Lm*-based vaccines provided functional protection from asthma *in vivo*, and may also provide some therapeutic benefit. Importantly, *Lm*-induced early life immune modulation did not exacerbate the development of some Th1/Th17-biased diseases later in life.
Preface

This study was based on preliminary work of Dr. Tobias Kollmann and Charis-Patricia Segeritz. Dr. Tobias Kollmann and Dr. Ashley Sherrid were involved in the design and optimization of the experiments described in this thesis. The performance and analysis of this research project was Sheka Aloyouni’s work. The outcome of this thesis benefitted greatly from collaborative work with Dr. Kelly McNagny’s laboratory, immuopathology advice and support from Dr. Jeremy Hirota 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 A10-0293 on January 1, 2011.
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List of Abbreviations

*Lm*: *Listeria monocytogenes*

HP: Hypersensitivity pneumonitis
APC: Antigen Presenting Cells
LLO: Listeriolysin
CTL: Cytotoxic T-lymphocyte
Th1: T helper 1
Th2: T helper 2
HK*Lm*: Heat-killed *Lm*
OVA: Ovalbumin
BALF: Bronchoalveolar Lavage Fluid
SR-Ag: *Saccharopolyspora rectivirgula* antigen
BHI: Brain Heart Infusion
PBS: Phosphate Buffered Saline
*i.p.*: Intraperitoneally
*i.n.*: Intranasally
CFU: Colony Forming Unit
H&E: Hematoxylin and Eosin
RBC: Red Blood Cell
ELISA: Enzyme-Linked Immunosorbent Assay
MCh: Methacholine
Abs: Antibodies
CFA: complete freund's adjuvant
HDM: house dust mite
AD: Atopic dermatitis
IgE: Immunoglobulin E
CpG-ODNs: CpG oligonucleotides
Acknowledgements

I would like to thank Dr. Tobias Kollmann and Dr. Ashley Sherrid for their guidance, support and suggestions throughout this work.

I would also like to thank Dr. Kelly McNagny’s for providing us with the SR antigen.

Special thanks to all the members in the Kollmann’s laboratory, particularly Bing Cai for her technical expertise and support.

This work was funded by grant from CIHR and the AllerGen NCE. Sheka Aloyouni was funded by a scholarship from the Saudi Arabian Cultural Bureau.
Dedication

This dissertation is dedicated in the first place to ALLAH, without whom this work would not be possible.

Next, I gratefully dedicate this thesis to my beloved parents, may Allah preserve them, for their unlimited care, support, encouragement and prayers through my studies.
Chapter 1: Introduction

Listeria monocytogenes

*Listeria monocytogenes (Lm)* is a gram-positive intracellular bacterium that mainly infects immunocompromised people and pregnant women\(^1\)-\(^3\). Listeriosis is associated with spontaneous abortion and neonatal septicemia in both humans and animals\(^4\). *Lm* causes a highly predictable infection in mice, is easy to culture, and is relatively safe to work with. This combination makes it well-suited for studying the mammalian immune response to infection\(^5\). Following infection, *Lm* is rapidly phagocytosed by antigen presenting cells (APC) such as macrophages or dendritic cells\(^6\). Bacterial proteins expressed within the phagosome are presented via MHC II molecules to CD4 T-cells. The virulence factor Listeriolysin O (LLO) allows *Lm* to escape this phagosome and replicate in the host cytoplasm where it undergoes proteosomal degradation. Listerial proteins are transported to the host’s endoplasmic reticulum and assembled into MHC-I molecules, which are recognized by cytotoxic T-lymphocyte CD8 T cells (CTL) and elicit a cellular immune response\(^5\),\(^6\). Once the CD4\(^+\) helper T cells are activated by recognition of peptide antigens within MHC II molecules, they differentiate into T helper 1 (Th1) cells which are essential for cytokine production, mainly IFN-\(\gamma\)\(^5\)-\(^7\). Activated CD8\(^+\) cytotoxic T lymphocytes mediate identification and destruction of infected cells. Both CD4\(^+\) and CD8\(^+\) *Lm*-specific T cells pass through a contraction phase which leaves a population of long lived, protective memory T cells\(^6\),\(^8\). These biological and immunological characteristics of *Lm* provide a rationale for the design of live-attenuated *Lm* vaccines that target heterologous pathogens or tumor antigens\(^6\),\(^9\),\(^10\). See figure 1.
Figure 1. The intracellular mechanism of Listeria monocytogenes.

1) Invasion of host cells by Lm vaccine strain; 2) Phagosomal escape of Lm; 3) Production and export of protein Ag by bacteria within the host cell phagosome; 4) Processing of antigens; 5) Presentation of epitopes via MHC class I and II molecules to CD8 T cells.

Hygiene hypothesis

Microbial exposure during the first years of life has the potential to program persisting immunological phenotypes that affect the subsequent risk of allergic disease. A worldwide
increase in asthma and allergic diseases has been hypothesized to be due to improved sanitation, which reduces exposure to microbes during early childhood\textsuperscript{12, 13}. According to the hygiene hypothesis, lack of early life exposure to microbes can cause an inadequate development of immunity to infectious agents, involving insufficient stimulation of Th1 cells, thus contributing to the development of asthma and allergic diseases\textsuperscript{14, 15}. IFN-γ (Th1 response) is known to be upregulated following microbial exposure, and may play a role in diminishing the risk of allergic diseases\textsuperscript{11}. It has been shown in animal models and humans that exposure to farms or endotoxin during the development of the immune system promotes robust protection against the development of asthma and allergic diseases\textsuperscript{16, 17}.

**Immune modulation in early life**

The neonatal immune system is characterized by a Th2-dominated cytokine response, which makes the newborn immune system vulnerable to infections and allergic diseases\textsuperscript{18}. Based on the hygiene hypothesis, modulation of the immune system using specific immunomodulators such as \textit{Lm}, may have potential implications in preventing asthma and allergic diseases\textsuperscript{19}. Part of the underlying mechanisms leading to asthma relate to immune mediated, Th2-biased allergen-specific responses\textsuperscript{20} and immunomodulatory approaches such as allergen-specific immunotherapy have been successfully employed\textsuperscript{21, 22}. However, vaccine-based strategies offer an additional effective approach that could lead to long-term protection from asthma\textsuperscript{22}. Thus, we believe that the development of an immunotherapeutic vaccine, modifying asthma pathology through the deviation of detrimental allergic responses toward protective immune responses, is the best option for effective and long-lasting treatment.

**Vaccination**

Vaccines have greatly diminished the prevalence of infectious diseases\textsuperscript{23}. Researchers have applied great efforts to establish safe and effective vaccines to induce beneficial immune responses in the host\textsuperscript{6}. Vaccines can not only protect from infection, but also modulate immunity to prevent or treat cancer, autoimmune disease and allergy\textsuperscript{24, 25}. Multiple studies have shown that vaccines based on whole, heat-killed bacteria (e.g. \textit{Chlamydia muridarum}, \textit{Propionibacterium acnes}, \textit{Mycobacterium bovis}, \textit{Bordetella pertussis})\textsuperscript{26-29} or bacterial-derived Toll-like receptors-ligands given as an adjuvant along with model allergens effectively prevent
allergic inflammation following allergen challenge. However, one of the most potent inducers of IFN-γ and other non-Th2 effector molecules is live Lm. Previous publications harnessing the benefits of Lm to modify hypersensitivity reactions have relied on heat-killed Lm (HKLm). In these studies, HKLm successfully suppressed allergen-specific, Th2-dominated responses by inducing allergen-specific Th1-dominated responses in adult mice. However, these studies did not address the ability of Lm-based vaccines to modulate atopic diseases originating in early life.

Asthma

The global prevalence, morbidity, mortality and economic cost associated with asthma have been on the rise, increasing nearly 50% every decade. Asthma is one of the most common allergic diseases and is most often diagnosed in childhood. Furthermore, acute asthmatic responses are one of the most common causes of emergency room visits in children. In most cases, patients can recover from the disease and keep it under control to live a normal life, but this requires repeated and long-term administration of medication. When patients breathe in allergy-causing substances such as dust, animal dander or pollen, symptoms such as wheezing, shortness of breath, chronic cough and chest tightness become apparent.

The underlying mechanisms leading to asthma are complicated due to the interaction between host genetic and environmental factors. The pathology of asthma occurs at least partially as a consequence of increased production of Th2 cytokines such as IL-4, IL-5 and IL-13 by allergen-specific CD4+ Th2 T cells. The Th2 cytokine IL-4 induces IgE secretion by B cells, inhibits macrophage activity, blocks IFN-γ activated macrophage effects, and induces mast cell growth and development. Furthermore, the Th2 cytokine IL-5 can induce eosinophil growth and differentiation. Therefore, the imbalance of Th1/Th2 responses (including lower Th1 IFN-γ production) facilitates allergic inflammatory reactions. The functional consequence of the abnormal Th1/Th2 responses leading to a Th2 bias described above are: airway hyperresponsiveness, eosinophilia, mucus hyper-secretion in the bronchial airways and tissue remodeling. The airway smooth muscle becomes thick in response to tissue remodeling that includes hypertrophy and hyperplasia, an increase in collagen deposition and angiogenesis and also an increase in the goblet cell numbers in the airway epithelium.
Asthma treatment options
Anti-inflammatory drugs and bronchodilators can be used to treat asthma\textsuperscript{45,46}. However, most of these treatment options provide only temporary relief and alleviate symptoms, while sustained relief requires long-term interventions that include medical therapy as well as lifestyle modifications. Asthma imposes a large burden on society in terms of health care cost and lost productivity, as well as on the individuals for whom many aspects of daily life are impaired. Asthma is still a monumental problem despite decades of research; therefore, better asthma therapeutics are urgently needed\textsuperscript{36,47}.

Vaccine strategy for asthma and allergic diseases

Prophylactic asthma vaccine for neonates
The processes leading to clinically symptomatic asthma have been shown to begin early in life\textsuperscript{48,49}; therefore, attempts to prevent asthma would likely be most effective if initiated perinatally. Our lab has initiated the development of a highly effective \textit{Lm}-based vaccine to prevent asthma in a mouse model. We have previously found that a single prophylactic dose of live, attenuated \textit{Lm} prevents allergen induced airway inflammation (Sheka Y. Aloyouni et. al, submitted). Our findings suggest that mice immunized as newborns with our live \textit{Lm} vaccine producing the model allergen ovalbumin (OVA) were protected from allergic OVA sensitization after only one shot given around birth (Fig. 2). In addition, the live-attenuated \textit{Lm}-based vaccine was more protective than the heat-killed \textit{Lm}-based vaccine since it exhibited an almost complete inhibition of the recruitment of inflammatory cells into lungs of immunized mice after OVA challenge. Interestingly, even mice immunized with the live attenuated \textit{Lm} strain that did not express OVA were protected from total bronchoalveolar lavage fluid (BALF) cell infiltration, specifically from infiltration with eosinophils (Fig. 2).
Figure 2. Vaccination with *Lm* and *LmOVA* significantly decreases Th2 driven allergic airway inflammation.

A, Schematic of *Lm* vaccination and experimental allergic airway inflammation protocol. B, Total BALF counts from naïve and OVA challenged mice. Naïve = saline vaccination and no subsequent OVA challenge, Ctrl = saline vaccination and OVA challenge, *Lm* = *Listeria* vaccination and OVA challenge, *LmOVA* = *Listeria-OVA* vaccination and OVA challenge, HK*LmOVA* = heat-killed *LmOVA* vaccination followed by OVA challenge. C, Differential counts of H&E stained BALF infiltrates. Values are expressed as mean ± SEM. (***p≤0.001). n=3-7 mice per group.

These promising data led us to extend our concept leading to the current study by 1) conducting a FlexiVent experiment, an *in vivo* measure of the airway resistance, 2) testing the ability of this vaccine to cure already established asthma in a mouse model, with the goal of developing an asthma therapeutic vaccine for adults. Furthermore, given that the non-specific *Lm* vaccine that does not express OVA also prevented allergic inflammation by initiating a strong Th1 response, we sought to determine whether the Th1-polarizing effect of this vaccine vector inadvertently may exacerbate development of certain Th1-driven allergic diseases like Hypersensitivity Pneumonitis (HP).
Hypersensitivity Pneumonitis

HP is a Th1/Th17-mediated alveolar and interstitial lung disease that develops following repeated exposure to various causative agents in susceptible individuals. Although there are different kinds of allergens that cause HP, the pathogeneses in response to these antigens are similar. In this project, we specifically used the Farmer’s lung model, which is the classic example of HP disease. Farmer’s lung is caused by repeated exposure to *Saccharopolyspora rectivirgula* antigen (SR-Ag). SR is a thermophilic gram-positive rod-shaped species of bacteria, commonly found in moldy bales of hay. However, not everyone exposed to SR-Ag develops HP; the underlying factors that lead to development of disease in some but not others are not well understood. Given the uncertainty about underlying pathogenesis, the impact of factors such as intensity of exposure (i.e., dose-response as well as duration and frequency of exposure) are not well understood. When susceptible persons inhale the SR, the inhaled Ag enters the alveolar space and crosses the alveolar endothelium and then binds to circulating antibodies (Abs). This process can lead to immune complex deposition in the lung and initiates an inflammatory response. Although HP has been thought to be a mostly immune complex-mediated process, subsequent studies showed that cell-mediated immunity is more important. Here, the resulting pathology relates to a Th1/Th17 response mediated by CD4+ cells and the cytokines IL-17 and IFN-γ, resulting in chronic lymphocytosis and granuloma formation in the lung. This is supported by HP in mice, a disease characterized by overproduction of IFN-γ and IL-12 by Th1 cell and also by overproduction of IL-17A by Th17 cell which can lead to mononuclear cell infiltration into the lung and eventually granuloma formation. Granuloma formation is a chronic inflammatory reaction involving macrophages, giant cells and lymphocytes. Upon SR-Ag exposure macrophages engulf and process antigen, leading to activation and giant cell formation. These activated cells then become surrounded by activated lymphocytes to prevent dissemination of infection.
Figure 3. Schematic diagram of granuloma formation.

When a susceptible individual is exposed to SR-Ag, antigen presenting cells promote the naïve T cells to differentiate into Th1 or Th17 cells. The Th1 cells then induce secretion of IFN-γ or IL-17 that lead to the activation of macrophages, monocytes and lymphocytes, as well as recruitment of neutrophils. As a result, granulomas are formed. (Graph courtesy of Pathology Department of Shantou University Medical College)
Hypotheses

We utilized two different models of allergic, immune mediated diseases in my thesis project. The first one was the asthma mouse model which we used to: 1) expand our understanding of the prophylactic asthma vaccine approach for neonates, 2) study the effect of *Lm* as a therapeutic vaccine for asthma. The second one was the Hypersensitivity pneumonitis (HP) mouse model to assess whether the neonatal dose of live, attenuated *Lm* has any detrimental effects on the development of HP later in life.

We set out to test three different hypotheses in these models:

A) **Prophylactic neonatal vaccine for asthma**: The virulence-attenuated strain *Lm*OVA will reduce airway hyperresponsiveness *in vivo* upon challenge with the allergen.

B) **Therapeutic adult vaccine for asthma**: The virulence-attenuated strain *Lm*-OVA will induce a strong Th1 immune response in adult mice, and therefore will cure already established allergic inflammation.

C) **Lm vaccine vs. HP**: The non-specific *Lm* vaccine strain will induce a strong Th1 immune response in newborn mice, leading to exacerbated allergic reactions to SR antigen in the HP mouse model.
Chapter 2: Materials and Methods

Animals
We used murine F1 pups (H-2\textsuperscript{b} x H-2\textsuperscript{d}) derived from crosses between C57BL/6 (H-2\textsuperscript{b}) and C57B10.D2 (H-2\textsuperscript{d}) purchased from the Jackson Laboratories (Bar Harbor, ME). H-2\textsuperscript{b} x H-2\textsuperscript{d} F1 mouse strains were used to match our previous work on the development of our neonatal vaccine platform based on \textit{Lm} Δ(\textit{trpS actA})/pSPO-PS\textsubscript{bb}OVA\textsuperscript{19}. F\textsubscript{1} mice were vaccinated at 6 weeks old or at 6 days old (Fig. 2) and (Fig 7). All animals were housed under pathogen-free conditions at the Child and Family Research Institute of the University of British Columbia (Vancouver, B.C.) according to animal experiment protocols approved by the Institutional Animal Care and Use Committee.

Bacterial strains and growth conditions
We have recently reported the design of the novel, live, highly-attenuated \textit{Lm}-based vaccine platform \textit{Lm} Δ(\textit{trpS actA})/pSPO that is particularly safe and well-tolerated in newborn mice. It is characterized in particular by a strong and broad immune response against ovalbumin (OVA), including CD4 and CD8 T cell responses as well as humoral responses\textsuperscript{19}. For immunization experiments (Asthma and HP), \textit{Lm} platform strains were grown to the late logarithmic phase (optical density at 600 nm (OD\textsubscript{600}), 1.0) at 37°C in brain heart infusion (BHI) medium, washed and resuspended in endotoxin-free isotonic saline solution (0.9% NaCl) and stored in 20% glycerol at -80°C prior to injection as described below.

Experimental setup

Immunization and induction of airway inflammation
A) Asthma
1) Prophylactic neonatal vaccine for asthma:
Immunization of animals. Mice were immunized intraperitoneally (\textit{i.p.}) with \textit{Lm}, which was resuspended in 0.1mL endotoxin-free 0.9% NaCl. Heat-killed \textit{Lm} was prepared by boiling 1x10\textsuperscript{7}
bacteria at 110°C for 30 min. Bacterial viability and colony forming unit (CFU) counts were
determined by plating serial dilutions on BHI agar plates.

**Induction of airway inflammation.** Asthma induction was achieved using the following
protocol: six weeks after immunization with *Lm* strains, mice were sensitized twice *i.p.* with 100
µg OVA (Worthington) absorbed onto alum hydroxide gel (Sigma) – this marked day 1 and 8 in
the experimental schedule (Fig. 1A). Non-immunized naïve mice received alum hydroxide gel
alone on the same schedule. Subsequently, anesthetized mice were challenged intranasally (*i.n.*)
on days 22, 23, 24, 26 and 28 with 200 µg OVA in 100 µL PBS prior to sacrifice on day 29.

**2) Therapeutic adult vaccine for asthma:**

**Induction of airway inflammation**

Six week old mice were intraperitoneally (*i.p.*) sensitized twice (day 1 and 8) as shown in (Fig. 4) with 100µg OVA (Worthington) dissolved in phosphate buffered saline (PBS) and adsorbed onto Alum hydroxide gel (Alum) (Sigma) 63; naïve mice received only PBS with Alum. Two weeks later, mice were intranasally challenged with 200µg OVA dissolved in 100µl PBS (days 22, 23, 24, 26 and 28) while naïve mice were challenged with PBS only.

**Immunization of animals**

Twenty-four hours after the last challenge, mice were immunized intraperitoneally (*i.p.*) with either NaCl (Naïve and Ctrl groups), or the following attenuated *Lm* vaccine strains: *Lm* Δ(*trpS actA*)/pSPO (*Lm* group), *Lm* Δ(*trpS actA*)/pSPO-PS_hly OVA (*Lm*OVA group). Bacterial viability and colony forming unit (CFU) counts were determined by plating serial dilutions on BHI agar plates to confirm vaccine dose.

Six weeks after immunization, mice were intranasally (*i.n.*) challenged with 200µg OVA dissolved in 100µl PBS (days 71, 72, 73, 75, 77). Naïve mice were challenged with PBS only.

**B) Hypersensitivity pneumonitis**

**Immunization of animals**
Mice were immunized at day 6 after birth with either NaCl (Naïve and Ctrl groups), or the attenuated \textit{Lm} vaccine strain: \textit{Lm} $\Delta$\textit{(trpS actA)}/pSPO (\textit{Lm} group). Bacterial viability and CFU counts were determined by plating serial dilutions on BHI agar plates to confirm vaccine dose.

**Induction of airway inflammation**

Six weeks after immunization, mice were intranasally primed and challenged with 40ul of 4mg/ml endotoxin-free SR antigen for three consecutive days per week for three weeks to induce acute HP. Four days after the last challenge mice were sacrificed. These studies were conducted according to the established and published mouse model of HP by our collaborator Dr. McNagny \textsuperscript{58}. This model mimics most features of Hypersensitivity pneumonitis developed by other researchers \textsuperscript{52,58,64,65}. This disease is characterized by a chronic lymphocyte cell infiltration into the lung, a Th1/Th17-biased cytokine response involving cytokines such as IFN-\(\gamma\), IL-12 and IL-17, the production of antigen specific IgG2a and loose granuloma formation \textsuperscript{58}. Based on these previous publications by other groups, our HP induction in the HP group (positive control group) was confirmed by a significant increase in the total BALF count (primarily lymphocyte), elevated anti-SR serum IgG2a levels, histology to confirm granuloma formation, and antigen-specific IFN-\(\gamma\) production in comparison to the naïve group.

**Analysis**

- **Prophylactic neonatal vaccine for asthma.**

  **FlexiVent:** We repeated the prophylactic neonatal vaccine experiment as described in figure 1A to determine the airway responsiveness to methacholine (MCh) (Sigma) challenges using a constant phase model flow employed by the FlexiVent (SCIREQ Scientific Respiratory Equipment Inc., Montreal).

  On day 29 after the last OVA challenge, mice were anesthetized with Avertin (Tribromoethanol, Sigma, Canada) (150mg/kg, \textit{i.p.}) and tracheotomized with a cannula ensuring a constant flow of air. Mice were given pancuronium bromide (Sigma) (0.8 mg/kg, \textit{i.m.}) to block spontaneous breathing. After baseline airway resistance stabilized (\(R_{\text{base}}\)), increasing doses of MCh (62.5\(\mu\)g/kg, 125\(\mu\)g/kg, 250\(\mu\)g/kg, 500\(\mu\)g/kg, 1000\(\mu\)g/kg, 2000\(\mu\)g/kg) were infused via the jugular vein. Total respiratory system resistance was determined immediately before MCh infusion (\(R_{\text{min}}\)), and then every 2 seconds following infusion until the maximum response was reached.
(R_max). MCh-induced increases in resistance were calculated as follows: % increase in airway resistance = \[(R_{max}-R_{min})/R_{base}\] \times 100. Significant differences in airway hyperresponsiveness (AHR) were determined using a one-way ANOVA with a Bonferroni’s post-hoc test and visualized by establishing the area under the curve of the AHR response.

- **Therapeutic adult vaccine for asthma and HP experiments.**

On day 78 of the therapeutic asthma experiment and on the 4th week of the HP experiment, mice were sacrificed by halothane over-exposure and the following fluids and tissues were harvested:

1) **Bronchoalveolar lavage fluid (BALF)**

BALF fluid was collected in order to determine the total number of live cells residing in the bronchial and the alveoli and quantify the different cell types (eosinophils, macrophages, neutrophils and lymphocytes) using H&E stain (Hema 3® Stain Set, Fisher Scientific). The increase in the total number of cells in BALF - specifically the eosinophils (asthma) and the lymphocytes (HP) - are the major indicator of these diseases.

Twenty-four hours after the last challenge, a micro-incision was made in the trachea and a catheter was inserted. A surgical thread was used to fix the catheter into the trachea. Three microsyringes were filled with 1mL of 1 x PBS and attached to the catheter to gently wash the lungs. All three washes were pooled into one 15ml falcon tube and stored on ice. The volume of the extracted BALF was recorded. The BALF was centrifuged at 1200rpm and 4°C for 5min. 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 viable cell count was determined by trypan blue exclusion. For HP experiments, H&E results were confirmed using flow-cytometry based quantitation of neutrophils (7.4 antigen +), eosinophils (SiglecF+/CD11c-), macrophages (SiglecF+/CD11c+), T cells (CD3+) and B cells (B220+) using antibodies purchased from BD-Pharmingen (San Diego, CA).

2) **Serum Enzyme-linked immunosorbent assay (ELISA)**
IgG1 and IgG2a OVA-specific serum ELISAs were performed in order to quantify antigen-specific antibody titers as an indicator of a Th2 or Th1 response, respectively. Twenty-four hours after the last challenge, blood was collected from the abdominal aorta in a tube for capillary blood collection (Microtainer®, BD) and stored on ice. The blood was then centrifuged at 14,000rpm for 5min and the serum supernatant was transferred into small tubes and frozen down to -20°C until subsequent antibody analysis via ELISA. To quantify IgG1 and IgG2a antibody level in serum, ELISAs were performed. Plates were coated with 100ul of coating buffer (5.3g Na₂CO₃, 4.2g NaHCO₃, 3.25mg NaN₃, pH 9.6) containing 10µg/mL OVA (asthma experiment) or 10µg/mL SR (HP experiment) then wrapped with parafilm and incubated overnight at 4°C. Twenty-four hours later, plates were washed four times in PBS-Tween (0.05% Tween-20 in PBS) then blocked with 200ul of blocking solution (PBS with 2% BSA) and incubated for 2hr at 37°C. Plates were washed again as in the previous step then 50ul of diluted serum samples were added to the plates and incubated overnight at 4°C (serum samples were run in serial 4-fold dilutions from 1:200 to 1:3276800). The next day, plates were washed again and 100ul of diluted HRP Anti-mouse IgG1 or HRP anti-mouse IgG2a (BD Pharmingen, San Diego, CA) were added and incubated for 1hr at room temperature protected from light (Abs were used at a concentration of 1:1000). Plates were washed and 100ul of TMB Substrate Reagent Set (BD OptEIA™, Biosciences San Diego, CA) substrate was added and incubated for 5-30min for color development. The reaction was stopped by adding 100ul of 1M H₂SO₄ and absorbance was measured at 450nm.

3) Spleen
Spleen cells were stimulated for 48h with OVA/R10, HKLm/R10 or R10 alone (Therapeutic vaccine experiment) or SR/R10, HKLm/R10 or R10 alone (HP experiment) to analyze the ex vivo cytokine production. Twenty-four hours after the last challenge, the spleen was extracted and stored on ice. The spleen was smashed between two ethanol-sterilized, frosted glass slides in 7mL RBC lysis buffer. To stop the reaction, 7mL RPMI medium was added. Then, the whole solution was centrifuged at 1200rpm and 4°C for 5min. The cell pellet was resuspended in 4mL R10 (RPMI 1640 supplemented with 10% FCS, streptomycin, penicillin) and run through a 70µm cell strainer. Viable cell counts were determined by trypan blue exclusion. The remaining cell suspension was centrifuged again at 1200rpm at 4°C for 5min and resuspended in R10 medium to a final
concentration of $2 \times 10^7$ cells/mL. These cells were then cultured in 200 µl of complete medium R10 in the presence or absence of either OVA (500 µg/ml) or HKLm for the asthma experiments, or either endotoxin-free SR (10 µg/ml or 100 µg/ml) or HKLm for the HP experiment, then incubated in a 37°C tissue culture incubator (5% CO2) for 48 hours. Supernatants were collected and transferred to a new plate. The new plate was sealed with an aluminum membrane (Nalgene Nunc International) and frozen at -80°C until batch analysis for cytokine production was evaluated using a 6-Milliplex Mouse Cytokine / Chemokine immunoassay according to manufacturer’s instructions (Millipore Corporation).

4) Lungs

Lungs were harvested for two purposes, A) restimulation assay; B) lung histology and morphology.

A) Restimulation assay:

Lung cells were stimulated for 48 hours with OVA/R10, HKLm/R10 or R10 alone or SR/R10, HKLm/R10 or R10 alone (HP experiment) to analyze the ex vivo cytokine production. Twenty-four hours after the last challenge, the right lung was harvested and stored on ice in 7ml RPMI. The lung tissue was then cut into small pieces with sterilized scissors and digested at 37°C for 1h under constant horizontal shaking (300rpm) 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 at 300g at 4°C for 5min. The cell pellet was resuspended in 5mL RBC lysis buffer and incubated at room temperature for 2-5min. The reaction was stopped by adding 5mL of R10, and then the solution was centrifuged again at 300g, 4°C for 5min. The cell pellet was resuspended in 5mL R10 and viable cells were counted by trypan blue exclusion. The remaining cell suspension was centrifuged again with 1200rpm at 4°C for 5min and resuspended in R10 medium to a final concentration of $2.5 \times 10^6$ cells/mL. These cells were then cultured in 200 µl of complete medium R10 (RPMI 1640 supplemented with 10% FCS, streptomycin, penicillin) in the presence or absence of either OVA (500 µg/ml) or HKLm for the asthma experiments, or either endotoxin-free SR (10 µg/ml and 100 µg/ml) or HKLm for the HP experiment, then incubated in a 37°C tissue culture incubator (5% CO2) for 48 hours. Supernatants were collected and transferred to a
new plate. The new plate was sealed with an aluminum membrane (Nalgene Nunc International) and frozen at -80°C until batch analysis for cytokine production was evaluated using a 6-Milliplex Mouse Cytokine / Chemokine immunoassay according to manufacturer’s instructions (Millipore Corporation).

B) Lung histology and morphology

1) Lung histology
Left lungs were inflated with 500ul 10% formalin and fixed with 10ml of the same fixative for 24 hours. Following fixation, the lung was bisected into superior and inferior sections. The superior half of the left lobe was subjected to a vertical cut and embedded faced down for morphology of airways distal to the primary bronchus as previously described. The inferior portion was embedded with the bisected area face down to obtain transverse cross sections of the primary bronchus for large airway morphology. Both superior and inferior left lung sections were embedded in the same paraffin wax tissue block. Five-micron thick sections were stained with H&E to determine the degree of cellular infiltration around the airways, vessels and parenchyma, and stained with Alcian Blue/PAS to assess goblet cell metaplasia (asthma experiment). Lung sections from the HP experiment were stained with H&E only to determine the degree of presence of extensive mononuclear cell infiltration around the airways, vessels and parenchyma.

2) Lung morphology
Stained tissue sections were viewed and images collected via light microscopy (Olympus BX40; Carsen Group INC., Markham Ontario) under 20x objective magnification for the parenchyma and 40x objective magnification for the airways and vessels. Image collection and analysis was performed by two blinded individuals. Lung sections were scored according to a scale of 0 to 5 according to cellular infiltration. 0 indicated that there was no cell inflammation/ goblet cell metaplasia, 5 referred to the highest amount of cell infiltration/ goblet cell metaplasia.

Statistics
The displayed results represent data from three separate neonatal asthma prophylactic vaccine experiments, a single FlexiVent experiment, a single adult asthma therapeutic vaccine
experiment and data from a single HP experiment. The results are expressed as mean ± standard error mean (SEM) deviation. Statistically significant differences in comparison to the control group were analyzed using ANOVA and marked with asterisks according to significance (*p≤0.05, **p≤0.01, ***p≤0.001). When analyzing the asthma model, we did not detect any difference between males and females for any of the parameters tested, and thus present the results of both genders combined. We did however detect a significantly higher response in female vs. male mice to SR-antigen in the HP model for all parameters tested and thus present the results of both genders separately.
Chapter 3: Results

Prophylactic immunization of newborn mice with live-attenuated *Lm* results in lower airway hyperresponsiveness and airway resistance *in vivo*.

To determine overall airway hyperresponsiveness (AHR), we used the FlexiVent small animal ventilator with live, intubated, and anesthetized mice to directly measure airway resistance in response to a non-specific stimulus methacholine, (MCh) \(^{68,69}\). We focused on mice immunized as neonates, as this approach formed the core of our study; we also omitted the HK*Lm*OVA group since we failed to observe a reduction of inflammatory infiltrates in the BALF of this group (Fig 2) and thus were unlikely to observe protection from AHR with MCh challenges. Dose-response curves of increasing MCh challenges revealed that at the highest dose of 2 mg/kg, the allergen exposed group responded with a far greater AHR than the “Naïve” group (Fig. 4A). Indeed, the allergic control group was the only cohort that exhibited statistically significant hyperresponsiveness above the naïve controls. To highlight the dose-response relationships between the experimental vaccine groups, we calculated the area under the curve of these responses (Fig 4B). This revealed that neonatally vaccinated mice from both the *Lm*OVA and the non-allergen-specific *Lm* groups exhibited reduced AHR following MCh challenge. Thus, our data suggest that neonatal immunization with *Lm* ameliorates this major hallmark of allergic inflammation.
Figure 4. Prophylactic immunization with Lm and LmOVA prevents significant increase in airway hyperresponsiveness (AHR) to methacholine challenge.

Allergic inflammation was induced in experimental groups, Naïve = saline vaccination and no subsequent OVA challenge, Allergen exposed (AE) = saline vaccination and OVA challenge, Lm = Listeria vaccination and OVA challenge, LmOVA = Listeria-OVA vaccination and OVA challenge, followed by OVA challenge as described in the Materials and Methods and in Figure 1. On the day of analysis, A) mice were administered increasing doses of MCh intravenously. Airway resistance (R % increase) was measured for each dose of MCh. B) Data from A re-evaluated as area under the curve. Values are expressed as mean ± SEM. n=8 mice per group.

Therapeutic immunization with live-attenuated LmOVA vaccine strain significantly reduces eosinophil infiltration into the lung bronchioles and alveolar spaces.

To test whether Lm-based vaccines could cure already established allergic airway disease, adult mice were sensitized twice with OVA-alum i.p. and challenged two weeks later with five intranasal instillations of OVA. Twenty-four hours after the last challenge, mice were immunized with either saline, parental Listeria monocytogenes platform strain (Lm), or the platform strain expressing ovalbumin (LmOVA) as described in the Materials and Methods. Six weeks after vaccination, mice were again challenged with five daily intranasal instillations of OVA (Fig. 5A). One day after the final instillation, BALF from the murine airways was collected and evaluated for total cell numbers (Fig. 5B), as well as differential cell counts of infiltrates (Fig.
Mice that were treated with the attenuated *Lm* strains exhibited similar total numbers of cells infiltrating the airways when compared to the allergen-exposed group (Fig. 5B).

Next, we investigated the type of inflammatory cells recruited to the lungs with particular attention to the types of cells that produce the wide array of pro-inflammatory mediators involved in asthma pathogenesis. Most strikingly, mice from the *Lm*OVA group exhibited significantly fewer eosinophils in BALF; however, mice that were treated with the *Lm* strain that does not express OVA exhibited similar numbers of eosinophils when compared to the control group (Fig. 5C). We found no significant difference in the number of macrophages, lymphocytes or neutrophils between mice from any of the vaccine groups compared to mice from the negative or allergen-exposed controls. These data suggest that therapeutic immunization with *Lm* did not alter allergic sensitization in our asthma model.

**Therapeutic immunization with live-attenuated *Lm* vaccine strains did not produce OVA-specific antibodies associated with a Th1-driven immune response.**

To further investigate alterations in the adaptive immune response resulting from *Lm* immunization, we investigated the levels of allergen-specific serum antibodies in the various test groups. Compared to naïve mice, all experimental groups subjected to OVA-based asthma induction exhibited elevated circulating anti-OVA IgG1 antibodies (Fig. 5D). However, the circulating levels of anti-OVA IgG2a antibody did not differ between the experimental and allergen-exposed (AE) groups (Fig 5E). In summary, these results suggest that therapeutic immunization with *Lm* strains did not change antibody response in our asthma model.
Figure 5. Vaccination with *Lm* and *LmOVA* did not inhibit cellular infiltration into the lung bronchioles and alveolar spaces nor produce OVA-specific antibodies associated with a Th1-driven immune response.

A, Schematic of experimental allergic airway inflammation and *Lm* vaccination protocol. B, Total BALF counts from naïve and OVA challenged mice. Naïve = saline vaccination and no subsequent OVA challenge, Allergen exposed (AE) = saline vaccination and OVA challenge, *Lm* = *Listeria* vaccination and OVA challenge, *LmOVA* = *Listeria*-OVA vaccination and OVA challenge. C, Differential counts of H&E stained BALF infiltrates. D, Relative concentration of OVA-specific IgG2a. E, Relative concentration of OVA-specific IgG1, serum was diluted 1:1000 and then serial dilutions were evaluated for OVA-specific antibody reactivity by ELISA as described in Materials and Methods. Values are expressed as mean ± SEM. (**p ≤ 0.001). n=4-8 mice per group.
Therapeutic immunization with live-attenuated *Lm* vaccine strains did not attenuate cell infiltration or goblet cell mataplasia in lung tissues.

After examining the degree of inflammatory cell infiltration into the bronchial airspaces, we set out to investigate the extent of cell infiltration into lung tissue (particularly the airway submucosa), as well as the degree of goblet cell metaplasia in airways. On the day of analysis, left lungs were inflated, fixed, segmented, sectioned and stained with H&E or Alcian Blue/PAS as described in the Materials and Methods. The H&E stained sections were then graded for the degree of cell infiltration into the lung airways, vessels and parenchyma (Fig. 6) while the Alcian Blue/PAS stained sections were graded for the amount of goblet cells in the airways (Fig. 7). A score of “0” indicated that there was no cell inflammation while a score of “5” referred to the highest amount of cell infiltration observed. We observed no significant differences in the degree of inflammatory cell infiltration into lung airway, vessels and parenchyma between the vaccinated groups and the allergen-exposed group (Fig.6). Similarly, lung sections that were stained with Alcian Blue/PAS exhibited no significant differences between the allergen-exposed and the vaccinated groups (Fig.7)
Therapeutic Lm vaccination did not inhibit OVA-induced cellular infiltration into airways, vessels, or parenchyma.

Allergic inflammation was induced in experimental groups as described in the Materials and Methods. Naïve = saline vaccination and no subsequent OVA challenge, Allergen exposed (AE) = saline vaccination and OVA challenge, Lm = Listeria vaccination and OVA challenge, LmOVA = Listeria-OVA vaccination and OVA challenge. On the day of analysis, left lobes of the lungs were formalin fixed, bisected, and sectioned and stained with H&E as described in the Materials and Methods. A score of “0” indicated that there was no cell inflammation while a score of “5” referred to the highest amount of cell infiltration observed. Airways and vessels images were captured at 40× magnification power while parenchyma images were captured at 20× magnification power. Values are expressed as mean ± SEM. n=4-8 mice per group.
Figure 7. Therapeutic *Lm* vaccination did not inhibit OVA-induced goblet cell metaplasia into the airways.

Allergic inflammation was induced in experimental groups as described in the Materials and Methods. Naïve = saline vaccination and no subsequent OVA challenge, Ctrl = saline vaccination and OVA challenge, *Lm* = *Listeria* vaccination and OVA challenge, *Lm*OVA = *Listeria*-OVA vaccination and OVA challenge. On the day of analysis, Left lobes of the lungs were formalin fixed, bisected, and sectioned and stained with Alcian Blue/PAS as described in the Materials and Methods. A score of “0” indicated that there was no metaplastic goblet cells while a score of “5” referred to the highest amount metaplastic goblet cells. Images were captured at 40× magnification power. Values are expressed as mean ± SEM. n=4-8 mice per group.

**Prophylactic immunization of newborn mice with live-attenuated *Lm* did not alter susceptibility to Th1/17 driven hypersensitivity pneumonitis.**

Given that neonatal *Lm*-vaccination non-specifically (i.e. non-OVA allergen-specific) reduced allergen-induced eosinophil numbers in BALF (Fig 2), lowered IL-5 production from lung-resident cells and dampened AHR following the MCh challenge (Sheka Y. Aloyouni *et al*, submitted), we considered the possibility that neonatal *Lm* vaccination may lead to broad immune modulation away from a Th2 response and enhance susceptibility to Th1/17 driven disease. To this end, we investigated the effect of neonatal *Lm*-immunization on susceptibility to *Saccharopolyspora rectivirgula* (SR)-induced hypersensitivity pneumonitis, a classical model for Th1/17 dependent pulmonary disease. Neonatally vaccinated mice were primed and challenged with SR antigen intranasally three times per week for three consecutive weeks (Fig. 8A). Four days after the final instillation, bronchoalveolar lavage fluid (BALF) from the murine airways was collected and evaluated for total cell numbers (Fig. 8B,C), differential cell counts of infiltrates (Fig. 8D,E). Furthermore, serum was collected to detect the IgG2a (Th1 response) and IgG1 (Th2 response) serum antibody levels (Fig. 9 and 10).

We were unable to detect a significant alteration in cellular – and in particular lymphocytic - infiltration into the lung bronchioles and alveolar airspaces following neonatal *Lm*-immunization and subsequent SR-induced HP (Fig. 8). Correspondingly, anti-SR serum IgG1 and IgG2a antibody levels were unaltered by neonatal *Lm*-immunization (Fig 9 and 10). From these experiments, we conclude that although neonatal immunization with *Lm* protects against subsequent Th2 mediated allergic disease, it does not broadly increase the sensitivity to Th1/Th17-driven lung inflammatory disease.
Figure 8. Immunization of newborn mice with live-attenuated *Lm* did not increase the total BALF count particularly lymphocytes.

A, Schematic of vaccination and SR-induced Hypersensitivity Pneumonitis (HP) induction protocol. Naïve = saline vaccination and no subsequent SR challenge, Ctrl = saline vaccination and SR challenge, *Lm* = *Listeria* vaccination and SR challenge. B,C Total BALF counts from naïve and SR-challenged mice were determined as shown in Fig. 1. D,E, Differential counts of H&E stained BALF infiltrates in response to HP induction. Values are expressed as mean ± SEM. n=6-16 mice per group.
Figure 9. Immunization of newborn mice with live-attenuated *Lm* did not increase the anti-SR-specific IgG2a levels in serum.

Anti-SR specific IgG2a antibody titers in diluted serum from the indicated groups were determined by ELISA as described in the Materials and Methods. Naïve = saline vaccination and no subsequent SR challenge, Ctrl = saline vaccination and SR challenge, *Lm* = *Listeria* vaccination and SR challenge. Values are expressed as mean ± SEM. n=6-16 mice per group.

Figure 10. Immunization of newborn mice with live-attenuated *Lm* did not alter the anti-SR-specific IgG1 levels in serum.

Anti-SR specific IgG2a antibody titers in diluted serum from the indicated groups were determined by ELISA as described in the Materials and Methods. Naïve = saline vaccination and no subsequent SR challenge, Ctrl = saline vaccination and SR challenge, *Lm* = *Listeria* vaccination and SR challenge Values are expressed as mean ± SEM. n=6-16 mice per group.
\textit{Lm} vaccination did not exacerbate HP-induced cellular infiltration into airways, vessels, or parenchyma.

HP was induced in experimental groups as described above. Left lobes of the lungs were formalin fixed, bisected, sectioned and stained with H&E. Histological sections were evaluated for the presence of mononuclear cell infiltration around the airways, vessels and parenchyma and scored according to the scheme described in the Materials and Methods. We observed no significant differences in the degree of inflammatory cell infiltration into lung airway, vessels and parenchyma between the vaccinated groups and the control (Fig. 11A, 11B, 11C respectively).

A)
### Vessels

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### Parenchyma

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Figure 11. *Lm* vaccination did not exacerbate HP-induced cellular infiltration into airways, vessels, or parenchyma.

Allergic inflammation was induced in experimental groups as described in the Materials and Methods. Naïve = saline vaccination and no subsequent SR challenge, Ctrl = saline vaccination and SR challenge, *Lm* = *Listeria* vaccination and SR challenge. On the day of analysis, left lobes of the lungs were formalin fixed, bisected, and sectioned. Lung sections were then stained with H&E then graded for the degree of mononuclear cell infiltration into the lung airways, vessels and parenchyma. A score of “0” indicated that there was no cell inflammation while a score of “5” referred to the highest amount of cell infiltration observed. Values are expressed as mean ± SEM. *n*=6-14 mice per group.
Chapter 4: Discussion

While evidence supports both genetic and environmental contributors to the development of asthma, the precise underlying etiologies of this disease are still obscure. In the absence of such mechanistic insight, attempts at empirical intervention continue to represent a relevant approach to slowing this growing epidemic. We therefore set out to investigate live, virulence-attenuated *Lm* as an immune-modulatory vehicle for the delivery of antigens to redirect the inflammatory immune trajectory of atopic asthma. Atopic hypersensitivity reactions such as asthma appear to often originate early in life and therefore suggest that intervention strategies that are suitable and effective during infancy are preferred.

Our previous findings suggested that a single perinatal immunization with attenuated *Lm* is effective at prophylactically reducing the impact of asthma sensitization in a murine model. In continuation of this work, we now determined that while neonatal *Lm*-based vaccination appears to have allergen-specific as well as non-specific components contributing to its protective effect, a single dose of this vaccine does not cure already established asthma. Importantly, we also found that a single dose of this vaccine at birth does not increase the risk for Th1/Th17 mediated hypersensitivity pneumonitis. The efficacy and safety of neonatal administration of *Lm* observed here support further exploration of early life immune modulation using *Lm* as a preventive strategy for allergic asthma and potentially other Th2-driven allergic diseases.

Our discovery that the live-attenuated *Lm* prophylactic vaccine prevents allergic inflammation in mice promised to offer additional tools to help stem the tide of this growing epidemic. However, our previous studies had not yet addressed several important aspects of asthma, most importantly the *in vivo* airway hyperreactivity following allergic sensitization. To this extent, we sought to first complete the previous study by focusing on the functional outcome in repeat experiments. To this end, we repeated the neonatal prophylactic vaccine experiment to examine the degree of airway resistance in vaccinated mice in response to methacholine challenge by FlexiVent. FlexiVent is an invasive technique used to measure the pulmonary function in mice specifically AHR. The functional FlexiVent evaluation is however considered to be the gold-standard of any modality aiming to reduce allergic inflammation and asthma in this model. We found that
neonatally vaccinated LmOVA as well as the non-allergen-specific Lm groups exhibited reductions in AHR following MCh challenge. To clarify these data, we calculated the area under the curve, which showed us that the only group that exhibited a statistically significant hyperresponsiveness above the naïve controls was the allergic control group. More specifically, vaccination with Lm or LmOVA both prevented the significant increase in the airway hyperresponsiveness when compared to the naïve group. Thus, our most functional data verified that neonatal immunization with Lm decreased the airway resistance.

Our murine asthma model replicated most pertinent features of human asthma. While inflammation is generally a valuable repair mechanism of the body in response to tissue damage, in allergic inflammatory conditions like asthma, the factors initiating and sustaining inflammation persist, thereby exacerbating inflammatory symptoms and preventing resolution. Eosinophils in the BALF were the most prominent pro-inflammatory cell in our asthma model. Heavy infiltration of this cell type into the airways in particular is strongly associated with the pathogenesis of asthma. Eosinophil-derived mediators like the major basic protein (MBP) or the eosinophilic platelet activating factor (PAF) cause damage to the mucosal epithelium, which subsequently affects their barrier function. Eosinophils and their factors have been shown to induce vasodilation, increase microvascular permeability, exert contractile effects on the smooth muscle cells, trigger enhanced mucus hypersecretion of goblet cells and encourage further eosinophil recruitment.

Encouraged by our results with the prophylactic approach, we next sought to determine whether the attenuated Lm vaccine could cure already established allergic inflammation, i.e. as a therapeutic vaccine. While mice in our positive control exhibited the severe eosinophilia and resultant phenotypes associated with allergic inflammation, mice that received live, virulence-attenuated Lm-OVA vaccination displayed a significant reduction in eosinophils (Fig. 5C). The most common type of extracted antigen-presenting cell within the BALF was the pulmonary macrophage residing on the respiratory surface of the lung alveoli. Although pulmonary macrophages have been shown to mediate airway inflammation in asthma, all our experimental groups exhibited no significant difference in macrophage BALF cell count (Fig. 5C) yet manifested different degrees of allergenic responses. Inflammation was therefore probably modulated independent from changes in the quantity of alveolar macrophages. There is
typically a strong correlation between allergen-specific serum IgG1 levels and asthma in mouse models of Th2 airway disease \(^{75}\); however, asthmatic mice that were treated with our Lm strains displayed no difference in the level of anti-OVA IgG1 antibodies when compared to the allergen-exposed group which was not surprising since the allergen exposed group and the Lm vaccinated groups were exposed to OVA allergen. Elevated levels of anti-OVA IgG2a were also not observed in response to Lm treatment (Fig 5D and 5E). Similarly, immunopathology analysis for lung tissue showed no difference in the level of cell infiltration or goblet cell metaplasia in the lung between the Lm treated groups and the allergen-exposed control (Fig 6 and 7). Furthermore, we found that adult mice that were induced to have allergic inflammation, treated with only one dose of live Lm (non-OVA) strains and then challenged with OVA exhibited no difference in the total number of cells infiltrating the bronchial airways (Fig. 5B). However, for the LmOVA vaccinated group we observed a significant reduction in BALF infiltrating eosinophils (Fig 5C), which are known to be major contributors to asthma-induced pathological changes in lung structure and function \(^{76},^{77}\). Considering that the LmOVA vaccine strain was able to significantly reduce the number of eosinophils infiltrating into the alveolar airspaces with only one dose, antigen-specificity is suggested. These unexpected findings would argue that either our single dose of Lm vaccine or the concentration of Lm in each dose were not sufficient to inhibit already established allergic inflammation. Based on these reasons, we are now testing if increasing the number of treatment doses or increasing the concentration of Lm vaccine per dose might further increase the efficacy of this therapeutic vaccine. Vaccination with a high dose is known to decrease the eosinophil infiltration, the production of Th2 cytokines, and the OVA-IgE and OVA-IgG1 levels in serum and also reduced AHR. \(^{78}\). In addition, vaccinating after acute symptoms have diminished might lead to increased therapeutic efficacy of the vaccine. We thus are exploring the option to administer therapeutic Lm-OVA vaccine to mice at different time points post challenge.

Given the promise of a prophylactic approach to prevent allergic sensitization early in life, and in particular the ability to provide effective protection from asthma without need for specific antigen involvement, i.e. non-specific protection, we realized we needed to investigate whether our Lm-based vaccine approach might have detrimental effects, such as an increase in risk to develop Th1/Th17-driven lung inflammatory disease such as Hypersensitivity pneumonitis (HP)
In this model, we found that gender strongly affected the intensity of the immune response. Specifically, females showed a stronger immune response than males, by higher total cell count in the BALF, higher lymphocyte number and also higher level of IgG2a in comparison to males. Thus, we presented results separately based on gender for all the parameter we measured. HP induction in the positive control was confirmed by a significant increase in the number of total BALF count (in particular, lymphocytic infiltration), an elevated level of anti-SR IgG2a in serum, and granuloma formation compared to the negative control. In our screen for potential adverse events of early life Lm-based immunization, we recapitulated the same approach (i.e. dose and timing of vaccination) we detected non-specific protection with in our asthma model (i.e. single dose of $10^4$ CFU of Lm at 6 days of age). This allowed us to test our hypothesis that a single dose of our Lm prophylactic vaccine would exacerbate the immune response to SR-Ag challenge. We found neither exacerbation nor protection from HP disease in the vaccinated groups using all of the parameters designed to measure the key attributes of disease.

In particular, the Lm vaccinated group exhibited no difference in the total number of cells, or specifically lymphocytes, infiltrating the bronchial airway (Fig 8B,C and 8D,E respectively) when compared to the control group. Consistently, we saw no difference in the levels of anti-SR specific IgG2a nor anti-SR specific IgG1 between control and Lm vaccinated groups (Fig 9 and 10 respectively). Furthermore, lung histopathologic analysis was performed to ensure that the neonatal prophylactic Lm vaccine had no effect on cellular infiltration into the lungs. We found that there was no difference between the Lm vaccinated group and the positive control group in the level of cell infiltration within the lung airways, vessels or parenchyma (Fig 11A, 11B, 11C). It is however possible that our experimental design for the HP model reached the maximum level of cell infiltration already, and that further enhancement following Lm immunization would thus not be detectable. We will have to repeat this experiment by challenging with a lower dose of SR-Ag to firmly establish whether Lm could increase the cell infiltration in the lung. However, the current results suggest that neither systemic (serological response to the SR antigen), nor pulmonary (BALF, histology) parameters worsened in neonatally vaccinated mice. This would negate the hypothesis and instead indicate that the non-specific effects of Lm-vaccination early in life have an immune-balancing instead of -biasing impact. Clearly, to fully rule out adverse
events following early life immunization with \textit{Lm}, we not only will have to test multiple doses, multiple time points and different models of cell-mediated disease.

It is important to note that previous human association studies examining the prevalence of asthma as a function of specific microbial exposures in early life found a reduction of symptoms in subjects exposed to microbes (and importantly that included \textit{Lm}) \textsuperscript{80, 81}. Likewise, additional studies in mice have suggested that perinatal, and in some instances prenatal, exposure to bacteria or antibiotic-induced shifts in host flora can have protective or exacerbating effects on future susceptibility to allergic airways disease, respectively \textsuperscript{82-84}.

We did not include the HK\textit{Lm} strain in our therapeutic asthma model and in the HP model because prophylactic exposure to HK\textit{Lm} vaccine did not prevent airway inflammation in our previous work, which would argue that mere exposure to listerial components is insufficient to change the trajectory of atopic diseases, and that asthma prevention requires the more robust response generated in response to live \textit{Lm}. Yet it is unclear to us why HK\textit{LmOVA} did not confer protection in our experimental model despite its positive outcomes observed by other research groups, as they observed an inhibition in the AHR, reduction in the airway inflammation, reduction in the total cell count in the BALF specifically eosinophils and an increase in the Th1 response \textsuperscript{32, 33, 85}. Different mouse strains ie, BALB/c mouse strain or different protocols used to prepare heat-killed \textit{Lm} could potentially explain these opposing findings, we prepare the HK\textit{Lm} by incubating it for 30 min at 110C° but others incubate it for one hour at 80C° \textsuperscript{32}. Also it is possible that vaccination with HK\textit{Lm} may require more concentrated dose to have a positive effect than the live \textit{Lm}.

In previous studies, the hygiene hypothesis has been postulated as a mechanistic explanation for asthma susceptibility. According to this view, allergy and asthma have become an “epidemic in the absence of infection” or perhaps more correctly, in the absence of perinatal colonization with a broad spectrum of bacteria \textsuperscript{86}. Although the mechanisms are still unknown regarding which microbial stimuli – such as \textit{Lm} – could possibly protect children from developing asthma and atopy, changes in the innate immune system likely play a role in mediating immune responses away from allergic responses \textsuperscript{29, 87-89}. Our study shows that early life exposure to a single live-
attenuated bacterial strain can reduce susceptibility to asthma without exacerbating the development of HP later in life. This sets the framework to test multiple hypotheses about the mechanism of immune skewing towards or away from the development of asthma and other atopic diseases in early life. The many available tools in the Lm-based experimental system will permit detailed dissection of molecular mechanisms underlying Lm’s ability to prevent allergic airway inflammation. These efforts will open the door to development of rationally targeted specific therapeutic interventions mimicking the effect of our live Lm vector.
Conclusion and Outlook

Our previous study shows that early life exposure to a specific, live bacterial strain can reduce susceptibility to asthma. We confirmed this protective efficacy here, using the gold standard of *in vivo* airway hyper-reactivity. We also determined that a therapeutic *Lm*-vaccine based approach might provide benefit, but likely only after repeat immunization. While our findings suggested that *Lm* decreased a Th2 response, we here discovered that neonatal prophylactic *Lm* vaccination did not exacerbate the development of the Th-1/17 driven disease, HP. Together this argues that *Lm* immunization balances rather than biases immune ontogeny. This sets the framework to test multiple hypotheses on the mechanism of immune skewing towards or away from the development of asthma and other atopic diseases.

The aims for our future experiments are:

A) Prophylactic *Lm* vaccination

1) Most vaccine studies have been conducted in mice because of the ease of analyzing immune function in this model. However, larger animals such as pigs are more similar to humans with respect to their physiology, organ development and immune responses \(^90\). Thus, we will attempt to validate the efficacy of our *Lm*-based prophylactic asthma vaccine in large animal models in collaboration with Vaccine and Infectious Disease Organization (VIDO). VIDO is the leader in large animal vaccine development of therapeutic or prophylactic vaccines aimed to improve human and animal health. The particular advantage of pigs relates to their close approximation of newborn immune and respiratory physiology \(^91,92\). Of particular importance for us, assessment of lung function in these large animals provides data that is more readily extrapolated to humans \(^91,92\). For example, pigs have already been used to study the pathophysiology of asthma, specifically eosinophilia, airway obstruction and cytokine production following airway allergen challenge \(^93\). Asthma in pigs also allows dissection of the role of eosinophils and neutrophils in the airways in allergen-sensitized pigs \(^94\). In addition, similar to our experimental set up, ovalbumin sensitized and challenged pigs have been used to measure contractile responses to acetylcholine (bronchoconstriction inducer) in bronchial segments \(^95\). Thus, the pig model offers multiple advantages and supports our efforts to translate murine findings to the clinic.
2) We also have begun to expand the target range of our prophylactic *Lm*-vaccine vector to include other allergens (peanut, cat, house dust mite); these projects are currently underway in collaboration with Dr. Mark Larche and the Karolinska Institute. Specifically,

- Peanut allergy is associated with fatal anaphylaxis\(^96, 97\) and its prevalence is increasing worldwide\(^98\). Therefore, a vaccine strategy is urgently needed to help prevent the suffering and possible death following peanut exposure. Our *Lm*-based peanut allergy vaccine is based on expressing recombinant protein that utilizes mutated forms of the highly allergenic peanut proteins Arah 1, Arah 2 and Arah 3, which can cause severe IgE-mediated reactions\(^99\). These mutated versions of the main peanut allergens themselves do not induce allergic reaction, but have the potential to prophylactically direct immune responses directed against them away from an allergic response. We have already cloned the Arah 3 (m Arah 3) into our *Lm* strain, and now in the process of cloning Arah 1 and Arah 2. Once the cloning is completed, we will start setting up experiments to test the efficacy of this vaccine on preventing peanut allergy. Similar to our *Lm*-OVA construct, we hypothesize that *Lm* will act as a bacterial adjuvant that modulates immune responses in an antigen-specific manner towards inhibiting the allergic reaction in the course of peanut allergen challenge.

- Cat allergy is also a major risk factor for asthma development\(^100\). Fel d 1 is the major cat allergen that causes respiratory diseases such as asthma and allergic rhinitis\(^101\). Our *Lm*-based cat allergy vaccine is thus based on expression of mutated (non-allergy inducing) Fel d 1 protein in our *Lm*-vaccine vectors. The cloning of this has been completed, and the vaccine is currently being tested for prophylactic efficacy in a mouse model in collaboration with Karolinska Institute in Sweden.

- We next plan to also test our *Lm*-vaccine platform in a house dust mite (HDM)-induced mouse model of allergic airway inflammation. HDM is one of the major causes of asthma in adults and children\(^102-105\). Administration of the major allergens of HDM (*Dermatophagoides farinae* or *Dermatophagoides pteronyssinus*)\(^103\) to mice leads a robust inflammatory response with increasing eosinophil cell infiltration, mucus secretion, IL-4, IL-5 and IL-13 production and airway hyper responsiveness\(^104, 106\). This points to the strong Th2 stimulatory capacity of HDM allergens\(^104\). We predict that recombinantly expressing the major allergens of HDM in Lm will redirect this immune bias away from Th2 to a more balanced Th1/2 response.
3) Asthma and atopic dermatitis (AD) share similar pathophysiology, i.e., both diseases are Th2 driven and characterized by an increase in IgE levels, total BALF count and specifically eosinophils, as well as an increase in IL-5 and IL-4 cytokine production\(^{107, 108}\). Thus, we hypothesize that prophylactic vaccination with our \(Lm\)-based vaccine will have a positive, protective effect on preventing AD as it did on asthma. The mouse models to test this hypothesis are well established\(^{109-111}\), ensuring feasibility of testing this hypothesis.

4) To further ensure the safety of this prophylactic vaccine approach, we are planning to test its effect on other Th1 diseases such as the neutrophilic asthma. As described by Pieter Bogaert et al\(^ {112}\), we will induce neutrophilic asthma by using an OVA/CFA combination (Complete Freund's Adjuvant (CFA) is known to induce a Th1 response\(^ {112}\)). To explore the possible negative impact of further enhancing Th1 responses in this model, we will immunize our mice prophylactically with \(Lm\) strains followed by induction of neutrophilic asthma using OVA/CFA. This will allow us to investigate whether \(Lm\) vaccination early in life has detrimental effects on the development of Th1 biased diseases later in life.

**B) Therapeutic \(Lm\) vaccination:**

1) Previous asthma immunotherapy-based studies have suggested that plasmid DNA encoding the allergen of interest is the most promising immunotherapeutic strategy for treating already established allergic inflammation. Administering these constructs results in the induction of Th1 immune responses, inhibits Th2 responses, and as a result decreases the eosinophil infiltration, as well as OVA-IgE and OVA-IgG1 levels in the serum; this approach can also reduce AHR\(^ {78, 113}\). However, due to the inherent low immunogenicity of the DNA vaccine approach, multiple high doses have to be administered over prolonged periods of time to achieve sustained therapeutic efficacy\(^ {78}\). While promising, we believe our \(Lm\)-based vaccine approach offers particular advantages, namely the likely efficacy after only a few applications\(^ 7\). However, while our data suggest that \(Lm\) vaccination can prevent allergic inflammation following only a single administration, a single dose of our therapeutic \(Lm\) vaccine only had limited positive effects on already established asthma. Specifically, this single-dose therapeutic vaccine significantly reduced only eosinophil BALF infiltration in our \(Lm\)OVA vaccinated group compared to the non-vaccinated control group; the other parameters we measured exhibited no reduction in established allergic inflammation in response to \(Lm\) vaccine strains compared to the allergen-
exposed group. We currently hypothesize that similar to the DNA vaccine approach, low efficacy following a single dose relates to the need for stronger immune activation to redirect established Th-2 patterns. Contrary to the DNA vaccine approach, we do not expect to have to use multiple doses over prolonged periods of time, as our current experimental set-up used a low concentration of \( Lm/dose \) in an effort to standardize vaccine dose between neonate and adult. This, in hindsight was likely a mistake, i.e. we should have used the optimal adult dose in our therapeutic vaccine model. Thus, for our future experiment, we will be increasing the \( Lm \) concentration per dose as well as adding a vaccine boost to mimic the experimental setup of the previous immunotherapy studies \(^{114}\). Lastly, we also will alter the timing of the vaccination in relation to challenge, as the therapeutic effect of this vaccine likely will increase if administered past the time of ongoing allergic inflammation.

2) Given the likely reluctance due to safety reasons for live vaccine administration (especially early in life) we will emphasize our focus on \( Lm \)-based therapeutic vaccines for chronic conditions (like asthma), but expand the range of targets to include viral infection (specifically Herpes and Hepatitis B viruses), as well as chronic bacterial infections such as \( Mycobacterium tuberculosis \). Even partial efficacy of such therapeutic vaccines would offer tremendous benefit, given the immense burden on health care systems all over the world due to these conditions. Our collaborator Advaxis, the leading industry entity for \( Listeria \)-based vaccines, has already brought into clinical trials multiple immunotherapeutic vaccines for cancer using our live, attenuated \( Lm \) that secrete specific antigens i.e. (HPV)-16E7, HER2/neu, prostate specific antigen \(^{115-117}\). Advaxis’ data suggest that our \( Lm \)-based vaccine strategy has the ability to inhibit the activity of suppressive cells, including regulatory T cells and myeloid-derived suppressor cells, which are the major cause of suppressing immune cells from killing cancer cells \(^{115}\). Establishing the paradigm of \( Lm \)-based vaccination in this (therapeutic) realm, we believe, will deliver the necessary human safety data to subsequently advance possible prophylactic vaccines.
References


