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Environmental influences during pregnancy, immune system development and offspring asthma susceptibility

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Table of Contents

Abbreviations

A. lwoffii – Acinetobacter lwoffii

Aluminum hydroxide – Alum

BAL – Bronchoalveolar lavage

CAS - Casein

DOHaD – Developmental origins of health and disease

Fc – Fragment crystallizable

FcRn – Neonatal Fc receptor

FGR – Foetal growth restriction

G – Gestation day

HSC Hematopoeitic stem cell

IP - Intraperitoneal

N – Neonatal day

OVA – Ovalbumin

PBS – Phosphate buffered saline

PN – Postnatal day

SC - Subcutaneous

SCFA – Short chain fatty acid

TLR – Toll-like receptor

1. Introduction

Asthma is the most common chronic inflammatory disease of childhood, with approximately 4 million children affected in Germany {Bergmann, 2016 #1736}. Often beginning with wheeze during early childhood, the clinical manifestation of allergic asthma is characterized by respiratory tract inflammation and airway hyperreactivity which can considerably reduce quality of life. Considering the origins of asthma, it is recognized that dysfunctional immune tolerance mechanisms trigger the immune system to react against harmless airborne allergens such as pollen. The causes of immune tolerance dysfunction, however, remain to be elucidated.

The immune system is developed in early life, and it is becoming increasingly evident that the foetal and neonatal life stages in play a substantial role in this process {Stiemsma, 2018 #1718;Drever, 2010 #1982}. Studies on environmental influences and childhood allergy lead David Strachan in 1989 to propose the Hygiene Hypothesis, which states that a lack of early childhood exposure to microbes may increase allergy susceptibility {Strachan, 1989 #14}. Similarly timed with the formation of the Hygiene Hypothesis, research by Barker and colleagues on birth weight and heart disease provided the basis for the Developmental Origins of Health and Disease (DOHaD) Hypothesis {Barker, 1986 #15}. Combining the concepts of developmental plasticity and early programming, DOHaD suggests that environmental exposures during the perinatal time period can influence foetal and/or neonatal development, thus influencing susceptibility for diseases such as allergic asthma in early life {Aris, 2018 #1983}.

During gestation, the prenatal environment plays a substantial role in maintaining the foetus, and alterations to this environment undoubtedly affect foetal and early childhood development {Peters, 2013 #12}. Demonstrating this, epidemiological studies show that certain environmental influences during pregnancy are strongly associated with childhood asthma susceptibility {Kashanian, 2017 #1698}. Prenatal exposure to traditional farming environments, in particular, farms with cows, haying procedures or consumption of raw milk are associated with protection against allergy in children {Stein, 2016 #1763;Ege, 2006 #461}. Conversely, risk factors for childhood asthma include maternal asthma exacerbation during pregnancy {Mirzakhani, 2018 #1798;Robijn, 2019 #1789} and prenatal exposure to antibiotics {Loewen, 2018 #1722;Wu, 2016 #1699}. Though it is clear that several extrinsic factors during pregnancy are influential in shaping the foetal immune system, the pathways contributing to this process are not fully understood.

In addition to the prenatal environment, particular exposures during neonatal life also influence immune system development. Indeed, current research suggests a strong link between the composition of the neonatal gut microbiome, the maturation of the immune system and susceptibility to allergic asthma in children {Takiishi, 2017 #1796;Johnson, 2016 #1624}. In first three months of life, children born with low gut microbial diversity had immune cell populations that diverged greatly from healthy children {Olin, 2018 #1965}. The absence of an early life microbiome in germ free mice also a has a profound effect on immune system development, and these animals also display increased asthma susceptibility {Herbst, 2011 #1804}. Considering the malleable nature of the microbiome in early life, an increased understanding of the intestinal colonization process is an important first step in assessing healthy immune system development. This information can be used for the eventual design of prenatal or early life interventions used to reduce the incidence of childhood allergic diseases.

Environmental influences during pregnancy undoubtedly elicit their affects on offspring asthma susceptibility through the mother. The placenta, which is a major organ formed de novo during pregnancy, can influence foetal development in two ways. If the development of the placenta is restricted in any way, the growth of the foetus is also constrained in a manner that could influence lung or immune system development {Ortqvist, 2017 #2005;Tedner, 2012 #2006}. In the case of a fully functioning placenta, nutrients, metabolites and maternal immune mediators such as short chain fatty acids, cytokines and antibodies are continuously transferred to the foetus {Dilworth, 2013 #1720}. During prenatal life, the maternal environment undoubtedly contributes to foetal development {Lai, 2019 #2013}. After birth, the early microbiome of the infant comes from the mother and the surrounding environment. During the neonatal time period, breast milk also plays a major role in immune system development. Not only does the breast milk provide protective antibodies while the neonatal immune system is still incompetent, it contains a myriad of bioactive compounds that guide the formation of the intestinal microbiota and the subsequent development of the immune system {Turfkruyer, 2015 #2015}.

To better understand how the perinatal environment influences offspring immune system development and subsequent allergic asthma susceptibility we have established three proof-of-concept mouse models that mimic different exposures observed in human populations. Our

primary focus is to use these mouse models to investigate several mechanisms, illustrated in Figure 1, that contribute to offspring asthma protection and risk.

1.1 Research questions

The research questions posed by my lab are as follows:

i) How do prenatal environmental influences such as exposure to bacteria, maternal asthma or antibiotic treatment during pregnancy affect maternal systemic immune mediators during gestation?

ii) Which maternal immune mediators (i.e. cytokines or antibodies) are transferred to the foetus and amniotic fluid, and how can these molecules influence foetal lung and immune system development?

iii) How are the maternal and neonatal intestinal microbiomes changed by different environmental exposures during pregnancy?

iv) How do alterations to the neonatal microbiome influence early life immune system development and subsequent immune tolerance?

We hypothesize that environmental influences during pregnancy alter maternal systemic immunity at specific time points, and that transfer of specific immune or metabolic mediators from mother to offspring plays a significant role in shaping early immune system and lung development. Further, we propose that in the neonatal period, the consumption of breast milk as well as the initial seeding of the neonatal gut microbiome has a strong influence on the establishment of immunity in early life, the development of tolerance mechanisms and asthma susceptibility.

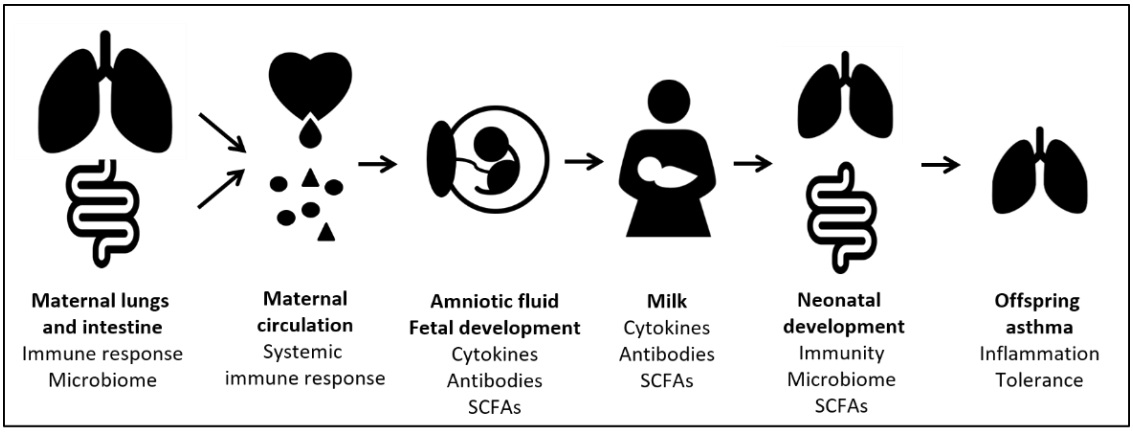

Figure 1: Graphical abstract illustrating how environmental exposures during pregnancy could influence the developing lungs or immune system and subsequent offspring asthma susceptibility. Maternal exposures during pregnancy can occur through inhalation or ingestion of bacteria from farming sites (lung and intestine), exacerbation of maternal asthma (lung) or

maternal antibiotic exposure (intestine). These exposures alter the maternal systemic immune response and mediators such as cytokines, antibodies and short chain fatty acids (SCFAs) are subsequently transferred to the foetus that can be measured in the amniotic fluid. Compounds in the amniotic fluid contribute substantially foetal development, due to their presence in the foetal intestine (via swallowing) and the lungs (through practice breathing motions). The early neonatal gut microbiome is seeded by the mother and maintained during lactation, thus alterations to the maternal microbiome and breast milk can also strongly influence the neonatal microbiome and immune system development in early life.

1.2 Project Descriptions

The Conrad lab uses three different mouse models to examine how extrinsic influences during pregnancy affect asthma severity in the offspring:

1) Prenatal exposure to non-pathogenic bacteria during pregnancy and protection against asthma in the offspring (Protection Model).
2) Maternal asthma exacerbation during pregnancy and increased asthma risk in the offspring (Risk Model).
3) Prenatal exposure to antibiotics and increased offspring asthma severity (Risk Model).

In the following paragraphs, our progress with each of these mouse models will be described.

1.2.1 Adjuvant-free mouse experimental airway inflammation models – Adjuvant is not required for murine asthma induction

Mouse models of experimental airway inflammation are well established, and mimic the clinical asthma phenotype in humans to a great extent. These models universally induce lung tissue and bronchoalveolar lavage inflammatory cell influx, goblet cell hyperplasia/metaplasia, airway hyperreactivity, and in allergy models, the production of allergen specific antibodies is also observed [24]. Though mouse asthma models produce similar phenotypes, the levels of inflammation tend to vary due to the wide variation in protocols. These include: non-allergy asthma models (inhalation of papain) [25] as well as allergic asthma, induced by a sensitization phase which stimulates antibody production, followed by and challenge phase that induces allergic airway inflammation. Within the protocols used for allergic asthma, further differences include: allergen used - ovalbumin (OVA), casein (CAS), house-dust mite, birch pollen etc; use of adjuvant – aluminum hydroxide (alum), incomplete Freund's adjuvant; route of sensitization -

intraperitoneal (IP), subcutaneous (SC) or intranasal; as well as route of challenge – intranasal or aerosol. Finally, different mouse strains also display differing degrees of asthma severity, likely due to different type 1 and type 2 immune response profiles. Due to this large variation in protocols, my early work set out to compare how different factors within the murine asthma induction protocols altered the phenotype of airway inflammation [26].

In our analysis of two widely used mouse strains, we confirmed that BALB/c mice (which exhibit a strong tendency towards type 2 immune responses, showed more severe allergic airway inflammation than C57BL/6 mice. Different parenteral routes of sensitization (IP versus SC) and different allergens (OVA versus β-galactosidase) showed comparable asthma phenotypes with only small differences. Importantly, our research clearly demonstrated that though most murine asthma induction protocols use the adjuvant alum, this adjuvant is categorically not required to induce asthma [26]. This finding is important, as deposition of this compound may have downstream effects on different treatments aimed at preventing or alleviating asthma symptoms. In order to maintain consistency in our upcoming investigations on how the environment during pregnancy contributes to offspring asthma severity, we chose to induce asthma in BALB/c mice, using an adjuvant-free model using OVA as the allergen. In this model mice are sensitized subcutaneously with 10µg OVA in 200µl of phosphate buffered saline (PBS) once weekly for three weeks. Then, after a 12-day rest period, sensitized mice are challenged 20 minutes per day for three days using a plexiglass chamber in which 1% OVA is nebulized (Figure 2). This protocol results in consistent, robust airway inflammation and is used in all subsequent transgenerational models of pregnancy and offspring asthma risk.

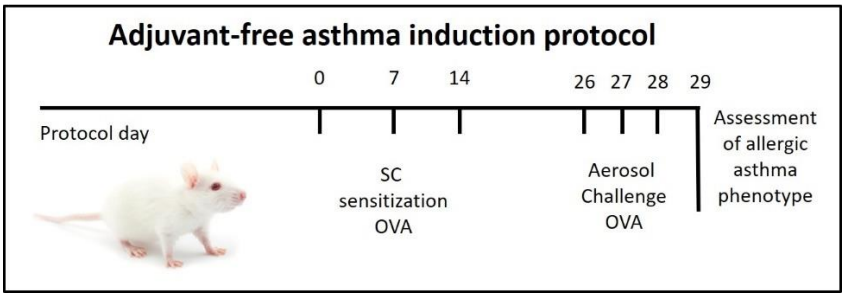

Figure 2: The adjuvant-free allergic asthma protocol used in all experiments. Mice are subcutaneously (SC) sensitized once weekly for three weeks with OVA, then allergic airway inflammation is elicited with three days of an OVA aerosol challenge, 20 minutes per day.

1.2.2 Prenatal exposure to non-pathogenic bacteria during pregnancy protects against offspring allergic airway inflammation.

Both human and animal studies show strong correlations between maternal and childhood exposure to farm-related microbes and protection against allergy. The 'allergy in farm children' studies associate prenatal exposure, via mothers working in farm sheds during pregnancy, with protection against asthma development in children [9, 27, 28]. Animal models support the allergy protective nature of a farming environment; particularly regarding the bacteria *Acinetobacter lwoffii*, which is found in high quantities at farming sites [29]. Using a model that closely mimics prenatal microbial exposures in the traditional farming situation, our previous work demonstrated that when pregnant mice were intranasally exposed to *A. lwoffii*, the elicitation of the asthma phenotype in the offspring was prevented to a large extent, model shown in Figure 3. In further investigation into the underlying mechanisms of prenatal asthma protection, our research demonstrated that treatment with *A. lwoffii* resulted in altered Toll-like receptor (TLR) expression in the maternal lung and placenta, and a local (lung) and systemic cytokine response in the mother. Furthermore, the use of knockout mice revealed that in the absence of maternal TLRs, the maternal immune response and the asthma protective effects seen in the offspring were abolished [30].

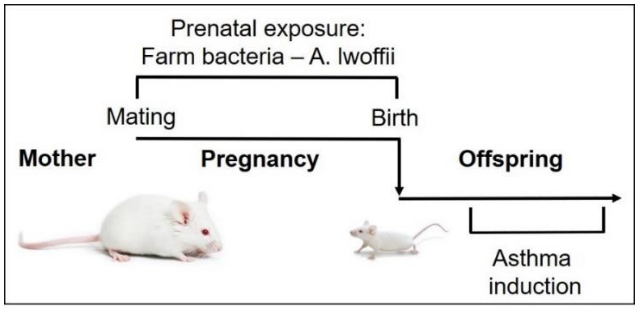

Figure 3: Mouse model of prenatal protection against asthma in the offspring. Mothers are exposed intranasally to the non-pathogenic, farm-derived bacteria *Acinetobacter lwoffii* every two days during pregnancy and asthma is induced in the offspring at weaning. Allergic offspring that were prenatally exposed to *A. lwoffii* have significantly less severe asthma than allergic offspring from control mothers.

In our follow up work to this publication, we next reasoned that in order for the maternal systemic immune response to influence the developing foetus, particular mediators must be

transferred from the maternal circulation through the placenta to the foetus. Following this line of thought we used flow cytometry to immunophenotype the cells in the maternal blood at several different gestational time points. Additionally, we measured a panel of 23 cytokines present maternal serum, using a multiplex bead array. At all time points during gestation, *A. lwoffii* exposure resulted in significantly increased neutrophils and decreased lymphocytes in the maternal circulation. This was accompanied by the upregulation of several cytokines that could also be detected in the amniotic fluid (unpublished data). We are presently testing how maternal exposure to these particular cytokines can influence foetal immune system development and offspring asthma susceptibility.

1.2.3 Maternal asthma exacerbation during pregnancy increases asthma risk in the offspring.

In addition to providing protection, prenatal exposures can also contribute to offspring allergy susceptibility. Maternal asthma is a higher risk factor than paternal asthma for allergy development in children, implicating a contribution of the intrauterine environment [11, 31]. Considering the maternal cytokine milieu, analysis of serum cytokine concentrations in allergic mothers during pregnancy identified IL-13 and IL-5 as risk factors for asthma-like symptoms in 6 and 12 month old infants [32]. Additionally, an unrelated birth cohort study reported an association between maternal asthma and a suppressed mucosal cytokine signature in neonates, suggesting that the maternal milieu during pregnancy strongly affects neonatal immunity [33]. In murine animal models, maternal serum IL-4 from asthmatic mothers was associated with higher asthma susceptibility and a stronger asthma phenotype in the pups [34].

To examine the mechanisms by which maternal asthma during pregnancy could contribute to offspring asthma risk, we designed a mouse model in which maternal asthma exacerbations during the second and third trimester of pregnancy resulted in increased offspring asthma severity (Figure 4). Mother mice were first made allergic to either OVA or CAS, then after mating, pregnant animals were challenged with allergen every second day from gestation day (G)6 to G16. To compare the effect of mother and offspring having similar or dissimilar allergies, asthma was induced in all offspring using OVA, resulting in control mother/OVA offspring (control-OVA), CAS mother/OVA offspring (CAS-OVA) or OVA mother/OVA offspring (OVA-OVA). The particularly interesting thing about this model was that although OVA-OVA offspring showed increased

asthma severity, we did not observe this phenotype in all clinical parameters measured. For example, OVA-OVA offspring showed significantly increased BAL inflammation, alterations in OVA specific IgG and IgE antibody titers and decreased regulatory T cells in the lung tissue, however, lung tissue inflammation, mucous production and airway reactivity remained similar to the control-OVA and CAS-OVA groups [35].

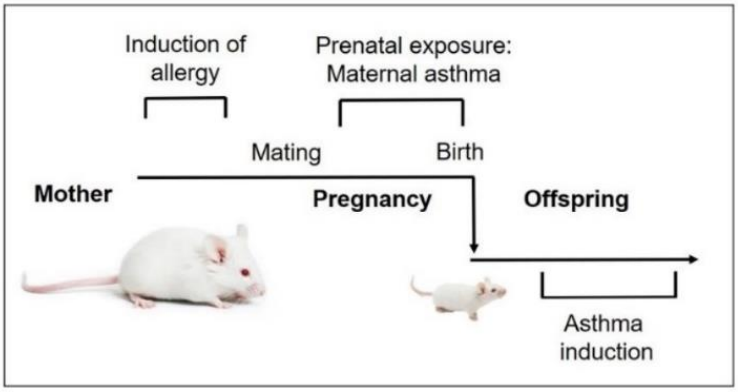

Figure 4: Maternal asthma exacerbation during pregnancy is associated with increased asthma severity in the offspring. Female mice are sensitized before mating, then during pregnancy, asthma exacerbations are induced in pregnant mothers by allergen challenge every second day from G6-G16. At weaning, asthma is induced in the offspring.

Due to the major changes found in antibody titers, we decided to additionally measure IgG glycosylation in this model. IgG antibodies can act in a pro- or anti-inflammatory manner according to the complex carbohydrate attached to the Asn-297 region of the fragment crystallizable (Fc) region. The addition of galactose or sialic acid residues to this carbohydrate chain influences antibody structure, resulting in anti-inflammatory IgG molecules that bind to inhibitory receptors [36-38]. To our surprise, we found strikingly similar IgG glycosylation patterns in the serum of both mothers and their allergic offspring. Moreover, the IgG antibodies in OVA-OVA offspring were significantly more pro-inflammatory compared to control-OVA or CAS-OVA animals, exemplified by decreased galactosylation and sialylation in the Fc region [35]. This study has awakened our interest in antibody glycosylation and we plan to examine IgG glycosylation in our other mouse pregnancy models. Our goal is to understand how antibody production in the mother could alter immune system development and contribute to offspring susceptibility for asthma in early life.

1.2.4 Antibiotic use during pregnancy increases offspring asthma severity

There is substantial evidence that antibiotic use during pregnancy contributes to increased asthma susceptibility in children, and the maternal microbiome is likely a key player in this process [13, 14, 39, 40]. The neonatal microbiome is seeded at birth from maternally derived vaginal, skin and gut bacteria, as well as during early life by contact with the mother. This colonization process is dynamic, highly susceptible to environmental influences and early bacterial presence in the intestine plays a major role in the development of immunity and central tolerance [15, 17]. Considering asthma, epidemiological studies have observed differences in the microbiome of asthmatic and non-asthmatic children [41], and reduced early life gut colonization with *Lactobacillus*, *Bifidobacterium* and *Bacteroides* genera is associated with asthma development in [42, 43]. Mouse models also show that, germ free mice have a more severe asthma phenotype than specific pathogen free mice which have a functioning gut microbiome [18]. Taken together, these studies suggest that microbial colonization in early life can influence asthma risk. Despite this growing body of evidence supporting a role for the gut microbiota in allergic disease, it is still unknown how antibiotic-induced microbiome changes affect the immune status of the host and influence disease susceptibility [44]. Additionally, 25% of expecting mothers must take antibiotics [14], and worldwide antibiotic use has risen 65% in the past 20 years [45]. If prenatal antibiotic exposure has even a small effect on childhood disease susceptibility, this could substantially influence public health.

Mouse models are ideal to study these mechanisms, due to the ability to analyze samples at various time points during pregnancy. There were previously no mouse models, however, that examined antibiotic exposure during pregnancy only; the available mouse models treated both the mothers during pregnancy and the offspring during the neonatal period [46, 47]. We have recently designed a mouse model of antibiotic treatment wherein the exposure occurs only during pregnancy (Figure 5). Using this model, we observe a concentration dependent increase in asthma severity in allergic offspring that were prenatally exposed to antibiotics versus allergic offspring from control mothers [48]. Using this model, our future studies will to assess how maternal antibiotic use alters the maternal and neonatal microbiomes as well as subsequent offspring asthma susceptibility.

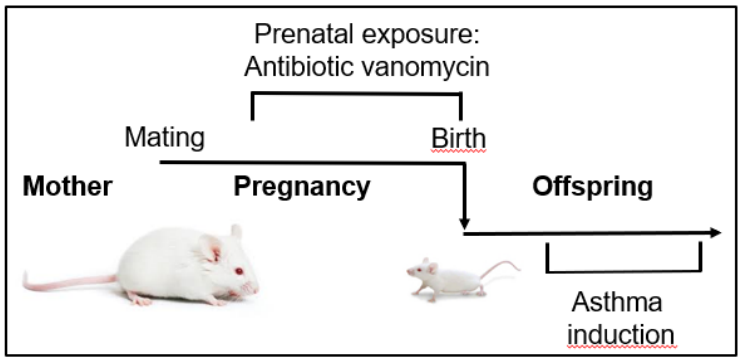

Figure 5: Antibiotic treatment during pregnancy results in increased asthma severity in the offspring. Dams are treated orally for the last 10 days of pregnancy with the antibiotic vancomycin. Allergic offspring that were prenatally exposed to antibiotic have a significantly more severe asthma phenotype than allergic offspring from control dams.

1.3 Objectives

The aim of this habilitation thesis is to examine how extrinsic influences on the mother during pregnancy and lactation can affect the development of central immune tolerance and subsequent allergic asthma susceptibility in the offspring. To investigate this question, this thesis describes the establishment of an adjuvant-free allergic asthma disease model, as well as three transgenerational models of prenatal environmental exposure that lead to either protection against, or risk for allergic asthma in the offspring. I also describe the development of a skill set for comparative placentation analysis that will be used in our future research to assess the effect of placentation on foetal development. Regarding the possible mechanisms at work in our models, our initial analyses of IgG antibody glycosylation and establishment of the gut microbiome are discussed for two of our mouse models. This work provides a strong basis for the investigation of how the perinatal environment influences offspring health and disease susceptibility.

2. Own work

2.1 Comparison of adjuvant and adjuvant-free murine experimental asthma models

Conrad ML, Yildirim AO, Sonar SS, Kilic A, Sudowe S, Lunow M, Teich R, Renz H, Garn H. Clin Exp Allergy. 2009;39(8):1246-1254 https://doi.org/10.1111/j.1365-2222.2009.03260.x

Background: Asthma is chronic lung disease affecting 300 million people worldwide. To study the mechanisms contributing this disease, mouse models replicating the clinical features of patients are widely used. Induction of asthma in a mouse model consists of a sensitization phase in which an allergen is injected to stimulate antibody production, followed by exposure to aerosolized allergen to induce allergic airway inflammation. In the sensitization phase of the protocol, the majority researchers use the adjuvant alum to stimulate antibody production, however, there is evidence that alum is not required for a functioning protocol.

Objective: The aim of this study was to compare adjuvant and adjuvant-free mouse asthma protocols to determine if adjuvant is required to induce allergic airway inflammation.

Methods: Adjuvant and adjuvant-free mouse models of asthma were compared. Intraperitoneal (IP) and subcutaneous (SC) injection were tested, as well as different allergens (ovalbumin or β-galactosidase) and different mouse strains (BALB/c or C57BL/6). Behavioural testing was also performed to investigate the effects of alum injection on mouse stress.

Results: The adjuvant alum is not required to induce allergic asthma in mice. Both adjuvant and adjuvant-free protocols showed similar degrees of airway inflammation and airway reactivity compared to the control groups. Comparison of SC and IP injection showed similar airway inflammation but lower antibody concentrations in SC sensitized mice. BALB/c mice had a more robust inflammatory phenotype than C57BL/6 mice. The adjuvant-free protocol functioned with either ovalbumin or β-galactosidase as an allergen. Finally, behavioural studies showed significant stress in mice that were treated with alum.

Conclusions: The adjuvant alum is not required to induce allergic asthma in mice.

2.2 Maternal TLR signaling is required for prenatal asthma protection by the non-pathogenic microbe *Acinetobacter lwoffii* F78

Conrad ML*, Ferstl R*, Teich R*, Brand S, Blumer N, Yildirim AO, Patrascan CC, Hanuszkiewicz A, Akira S, Wagner H, Holst O, von Mutius E, Pfefferle PI, Kirschning CJ, Garn H, Renz H. Maternal TLR signaling is required for prenatal asthma protection by the nonpathogenic microbe Acinetobacter lwoffii F78. J Exp Med. 2009;206(13):2869-2877. https://doi.org/10.1084/jem.20090845 * Equal contribution – Dr. Ruth Ferstl and Dr. Rene Teich agree that Dr. Melanie Conrad can use this publication for her habilitation

Background: Epidemiological studies demonstrate that prenatal exposure to traditional farming environments, particularly those with cows, haying procedures and consumption of raw milk, is protective effect against childhood asthma susceptibility. Studies show that cow-sheds have high concentrations of the bacteria *Acinetobacter lwoffii* F78. Though these studies provide convincing evidence as to the protective nature of the farming environment, the mechanisms involved in this process thus far are unknown.

Objective: The aim of this study was to create a proof-of-concept mouse model, demonstrating the protective effect of farm exposure during pregnancy on asthma protection in the offspring.

Methods: BALB/c mice were mated and exposed intranasally every second day during pregnancy to a lyophilized preparation of the cow-shed derived bacteria *A. lwoffii*. Control pregnant mice received a sham treatment with phosphate buffered saline. At weaning, offspring were subjected to an experimental asthma protocol and the clinical phenotype in these animals was assessed.

Results: Allergic offspring that were prenatally exposed to *A. lwoffii* had a significantly less severe asthma phenotype than allergic offspring from sham treated mothers. A time course assessment of the maternal immune response to *A. lwoffii* treatment revealed a transient immune response with an increase in lung Toll-like receptor (TLR) expression. To test maternal TLR involvement in our model, we mated wild type males with 5-fold TLR knockout (TLR2/3/4/7/9) females. *A. lwoffii* treated TLR knockout mother mice treated did not show an anti-bacterial immune response and the offspring from these animals were no longer protected from asthma.

Conclusions: We generated a mouse model demonstrating that exposure to the cow-shed derived bacteria *A. lwoffii* during pregnancy has a protective effect against asthma development in the offspring. The mechanism in this model involved TLR engagement and the maternal immune response to *A. lwoffii* exposure.

2.3 Differential spatiotemporal patterns of galectin expression are a hallmark of endotheliochorial placentation

Conrad ML*, Freitag N*, Diessler ME, Hernandez R, Barrientos G, Rose M, Casas LA, Barbeito CG, Blois SM. Differential Spatiotemporal Patterns of Galectin Expression are a Hallmark of Endotheliochorial Placentation. Am J Reprod Immunol. 2016;75(3):317-325. https://doi.org/10.1111/aji.12452 * Equal contribution – Dr. Nancy Freitag agrees that Dr. Melanie Conrad can use this publication for her habilitation

Background: Placentation is an important process in pregnancy that regulates the delivery of nutrients to the foetus and the excretion of waste. During placental development, angiogenesis is a key process that is directed by cross-talk between the mother and foetus, and galectins play a key role in these processes. There are three placental types, according to the number of membranes present between mother and foetus: epitheliochorial, endotheliochorial and hemochorial, in order from most membranes to least. Very little is known about galectin expression in the endotheliochorial placenta, information which is very important for animal models and comparative studies of placentation.

Objective: The purpose of this study was to investigate galectin (gal)-1, gal-3 and gal-9 expression in endotheliochorial placentas of dogs and cats, to gain insight into the role galectins may play in this specific type of placentation.

Methods: Immunohistochemical analysis of paraffin embedded dog and cat placentas was conducted using antibodies directed against gal-1, gal-3 and gal-9. Observations were made for both early and late gestation placentas.

Results: With respect to early canine gestation, we observed that though no galectins were expressed on the placental syncytiotrophoblast, both gal-1 and gal-9 were strongly expressed on the cytotrophoblast cells. Late gestation canine placentas did not change with respect to gal-9 expression, however gal-1 and gal-3 expression increased in both cyto- and syncytiotrophoblast cells. Feline early gestational placentas strongly expressed gal-1, gal-3 and gal-9 on the maternal vessels and the trophoblast; all measured galectins decreased in late gestation.

Conclusion: Analysis of galectins in early and late gestational endotheliochorial placentas of dogs are cats reveals a spatiotemporal regulation. This indicates that different members of the galectin family likely play a role in the regulation of several processes during placentation.

2.4 Maternal asthma is associated with persistent changes in allergic offspring antibody glycosylation

Sodemann EB, Dahling S, Klopfleisch R, Boiarina E, Cataldo D, Alhasan MM, Yildirim AO, Witzenrath M, Tabeling C, **Conrad ML**. Maternal asthma is associated with persistent changes in allergic offspring antibody glycosylation. Clin Exp Allergy. 2020. https://doi.org/10.1111/cea.13559

Background: Clinical studies indicate that maternal asthma is a risk factor for childhood asthma development, due to foetal exposure to the maternal immune response during pregnancy. For instance, maternal IgG antibodies passed via the placenta to the developing foetus can be pro- or anti-inflammatory depending on glycans attached to the Fc region of the molecule.

Objective: The objective of this research is to create a mouse model to understand 1) The effect of maternal asthma exacerbation during pregnancy on asthma development in the offspring 2) The influence on IgG antibody glycosylation in this process.

Methods: Female mice were allergically sensitized to either ovalbumin (OVA) or casein (CAS) during pregnancy or sham sensitized with (PBS). After mating, pregnant mice underwent asthma exacerbation every two days from gestation day 6-16 with OVA, CAS or PBS respectively. At weaning, offspring were subjected to allergic asthma protocol using OVA as an allergen. We tested three different groups: control mothers with allergic offspring (control-OVA), mothers and offspring allergic to different substances (CAS-OVA) and mothers and offspring allergic to the same substances (OVA-OVA). The offspring asthma phenotype was assessed, as well as maternal and offspring serum IgG Fc glycosylation.

Results: OVA-OVA offspring had more severe allergic airway inflammation than CAS-OVA offspring, shown by increased inflammatory cells in the bronchoalveolar lavage, altered antibody concentrations and decreased percentages of regulatory T cells in the lungs. The clinical differences between OVA-OVA and CAS-OVA allergic offspring were supported by our IgG glycosylation analysis. In OVA mothers and their OVA allergic offspring, the IgG Fc regions had significantly lower galactosylation and sialylation percentages, indicating a stronger pro-inflammatory phenotype.

Conclusions: Maternal asthma exacerbation during pregnancy increases offspring asthma severity when mother and offspring are exposed to the same allergen. One suspected mechanism for this is through the transfer of pro-inflammatory IgG antibodies that have less galactose and sialic acid residues on the Fc Asn-297 glycan.

2.5 Antibiotic use during pregnancy increases offspring asthma severity in a dose dependent manner

Alhasan MM, Cait AM, Heimesaat MM, Blaut M, Klopfleisch R, Wedel A, Conlon TM, Yildirim AO, Sodemann EB, Mohn WW, Bereswill S, **Conrad ML**. Antibiotic use during pregnancy increases offspring asthma severity in a dose dependent manner. Allergy. 2020. https://doi.org/10.1111/all.14234

Background: Antibiotics account for over 80% of the prescriptions given during pregnancy and epidemiological studies associate prenatal exposure to antibiotics with increased asthma susceptibility in children.

Objective: To show in mice that maternal antibiotic use during pregnancy contributes to increased asthma severity in the offspring.

Methods: Mice were treated daily during pregnancy, from gestation day (G)8 – G17, with an oral dose of vancomycin. Three different concentrations were tested and control mothers were sham treated with water. Asthma severity was tested in offspring using an experimental asthma protocol with OVA as an allergen. Feces was collected from both mothers and offspring to examine microbiome composition, and short chain fatty acid (SCFA) concentrations were assessed in the cecum of adult allergic offspring.

Results: When taken during pregnancy, the antibiotic vancomycin was associated with maternal and offspring gut microbial dysbiosis. Of note, the genus *Clostridia*, which digests fiber to produce SCFAs was significantly reduced in the maternal and offspring gut microbiota. This dysbiosis was associated with reduced concentrations of cecal SCFAs in allergic offspring. Finally, antibiotic use during pregnancy increased asthma severity in the offspring in a dose dependent manner.

Conclusions: We have designed the first mouse model showing that antibiotic use during pregnancy is a risk factor for offspring asthma. Our next studies will investigate how microbial dysbiosis affects offspring immune system development.

3. Discussion

3.1 The mechanisms contributing to offspring asthma susceptibility

Due to the transgenerational nature of our mouse models and the relatively extended period of time in which lung or immune system development could be influenced, we are quite certain that several different mechanisms contribute to offspring asthma susceptibility. The next steps in the evolution of the project and the major future focus of the lab involves understanding the mechanisms activated by particular maternal environmental exposures and whether they function predominately during the prenatal or postnatal time points. Our established mouse models are ideal to assess this information, as discovery of a possible mechanism in one model can be checked using a second model, i.e. a strong prenatal influence in the Protection Model, that also shows a significant and opposite change in a Risk Model is one way of validating candidates for future functional studies. Using this method of model comparison, it may also be possible to identify mechanisms that are unique to a particular environmental stimulus.

The future research in my laboratory is broken down into several analysis themes that will allow us to predict and assess the pathways at work, including: 1) Characterization of maternal mediators that could affect either placentation and foetal development, or breast milk composition and neonatal development, 2) Analysis of placentation and foetal growth, 3) Examination of foetal and neonatal development. 4) Hypothesis testing using fostering experiments, knockout models or supplementation procedures. The eventual goal of our research is to translate our pre-clinical findings to humans, using pregnancy cohorts.

3.2 Which maternally generated mediators have the capacity to alter offspring lung or immune system development and subsequent asthma susceptibility?

Answering this question is a first step in elucidating the mechanisms that influence maternal transference of protection and risk for offspring asthma susceptibility. During pregnancy, maternal mediators must travel via the blood stream through the placenta in order to effect changes in the foetus. Since soluble immune substances such antibodies and cytokines can cross the placenta [49] and are strongly immunomodulatory [50, 51], the potential exists for these compounds to influence foetal lung or immune system development. Particularly interesting is

the amniotic fluid, as changes to its composition could play a major role during the establishment of the lung and immune system due to its presence in the foetal lung and intestine. To begin assessing this, we have used our *A. lwoffii* protection model to investigate how maternal exposure to lyophilized bacteria changes circulating immune cells and soluble immune components at several different time points during gestation. In our analysis of 23 cytokines, we found that G-CSF was increased at all stages of gestation measured (G9, G13 and G17), and in all tissues measured including the maternal bronchoalveolar lavage, circulation and amniotic fluid (unpublished data). We plan to analyze the maternal serum and amniotic fluid cytokine profiles in our other models at these gestational time points to check if alterations in cytokine patterns can be also be observed. Additionally, we will to perform cytokine intervention experiments in the future to test if treatment with G-CSF during pregnancy could protect against asthma in a mouse model.

Although the amniotic fluid likely plays a major role in foetal development, there are relatively few studies regarding amniotic fluid composition [52-54], and those that exist predominately concentrate on pathologic conditions such as maternal infection [55, 56]. The paucity of studies regarding steady state amniotic fluid composition is likely due to the unjustifiable risk that accompanies obtaining samples from healthy human pregnancies and the difficulty and in obtaining amniotic fluid from pregnant mice. In this regard, we have developed a method to collect up to 200µl of amniotic fluid per mother from G13 and G17 mice. With the existence of multiplex bead arrays, this volume is enough to perform all the measurements required to assess amniotic fluid composition in our models.

In addition to amniotic fluid, the breast milk provides a second close connection between mother and neonate. Though the mothers in our mouse models are only treated during pregnancy, residual immune or metabolic compounds could also be present in the breast milk [57]. To examine breast milk composition in our mouse models, we have recently collaborated with Charité Universitätsmedizin Centrum Wissenschaftliche Werkstätten to construct a specially designed mouse breast milk pump based on a publication by Gomez-Gallego et al. (2013) [58]. The milk collection apparatus, used with a human breast milk pump, can obtain up to 1 ml of milk from a lactating mouse depending on litter size (shown in Figure 6). Using these samples, we

intend to measure soluble compounds such as cytokines, antibodies and SCFAs in the milk at different lactation time points.

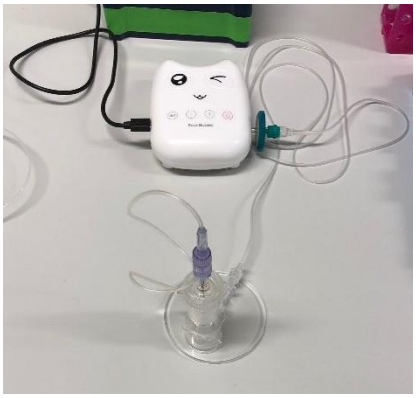

Figure 6: Newly designed mouse breast milk pump. An air tight milk collection apparatus with specially designed suction cups was constructed at Charité Universitätsmedizin - Berlin, modelled after [58]. The milk collection apparatus, combined with a human breast milk pump can gather up to 1 ml of breast milk from an anaesthetised lactating mouse.

3.3 Are placentation and foetal growth affected by maternal environmental exposures during pregnancy?

After implantation, the placenta forms through a carefully orchestrated cascade of developmental events. Once fully formed in mid-pregnancy, the placenta facilitates the delivery of nutrients and the elimination of waste from the foetus. In late pregnancy the vascularization of placenta increases substantially to accommodate foetal growth [59]. Since appropriate placental development is crucial to normal foetal development, we will use our mouse Protection and Risk Models to examine the establishment of the placenta at G13 and its vascularization at G17, using morphometric placenta analysis adapted from [60]. Additionally, we will assess the galectin signature in early and late pregnancy mouse placentas to determine the developmental progression of the placenta in each model. We hypothesize that in our protection model, we will likely not see differences in placentation. In our risk models, however, placental morphometry or galectin expression may be compromised, resulting in reduced delivery of nutrients to the foetus and foetal growth restriction (FGR). FGR is associated with delayed lung development in children and is a predisposing factor for diseases such as allergic asthma [19, 20].

In addition to changes in placental morphology, the Risk Models may also have alterations in placental receptors such as the neonatal Fc receptor (FcRn). This receptor, which transfers IgG antibodies to the foetus, could result in a reduction of protective antibodies transferred from mother to offspring, resulting in compromised immunity and predisposition to asthma. Nakata et al. (2010) found using FcRn knock out mice that maternal transfer of IgG antibodies plays a major

role in the development of immune tolerance and protection against asthma in early life [61]. Knowledge from our models regarding placental development will contribute to a growing body of literature recognizing the placenta as an essential organ with the potential alter foetal development and subsequent disease susceptibility.

The developmental stage of an embryo can be classified according to certain morphological characteristics which, in the mouse are called Theiler stages. Though all foetuses from one mother mouse are relatively similar according to their gestational age, individual differences in Theiler stage can occur between foetuses, and in adverse conditions involving FGR or dysfunctional placentation, the entire group of foetuses can be at a younger developmental stage than their gestational age [62]. To assess if the environmental exposures in our models are associated with changes in foetal growth, G13 and G17 foetuses can be preserved in Bouin solution and assessed for their Theiler developmental stage as well as their weight [63]. We have already performed this analysis in our *A. lwoffii* Protection Model and did not find any difference between foetuses from bacterially exposed or control mothers. We plan also to perform this analysis on the foetuses from our Maternal Asthma and Maternal Antibiotic Risk models, with the expectation that a significant number of foetuses may be growth, and/or developmentally restricted. We hypothesize that foetuses from antibiotic treated mothers in particular will be at an earlier Theiler stage than their actual gestational age due to the changes in maternal nutrient acquisition induced by gut microbial dysbiosis. Slower development in this manner would indicate that complex organ structures such as the immune system and lung could also be premature, thus predisposing the offspring to diseases such as asthma in early life.

3.4 How does the maternal environment during pregnancy influence foetal and neonatal development?

3.4.1 Development of the immune system

The murine immune system begins developing early in gestation with a complex migration of cells through several different anatomical sites. Through this process, hematopoietic stem cells (HSCs) are generated that can differentiate into all immune cell lineages; indeed it is proposed that sufficient numbers of HSCs are generated in the foetal liver for the lifetime of an individual [64]. HSCs therefore, provide a self-renewing cornerstone for the innate and adaptive immune

responses throughout postnatal life. After residence in the aorta-gonad-mesonephros, HSCs migrate to the foetal liver at G11, where the population expands over 40-fold between G12 and G14. From G16 to G19 HSCs egress from the foetal liver to the bone marrow where they will remain for the lifetime of the individual [65]. Since changes to early HCS population numbers in the foetal liver could influence neonatal immunity and asthma [66], we will use flow cytometry to examine the frequencies of multipotent long- and short-term HSCs as well as multipotent progenitors and common lymphoid and myeloid progenitors. These experiments will clarify if there is a timely egression of HSC from the liver to the bone marrow in foetuses whose mothers were exposed to specific stimuli such as bacterial components, antibiotics or an asthma exacerbation during pregnancy. In addition to assessing foetal liver HSCs, we will also examine these cells in the neonatal bone marrow. Bone marrow HSCs will be of special interest in our Antibiotic Risk model, since there is evidence that antibiotic induced disruption of the gut microbiome results in reduced bone marrow cellularity and suppression of the cell cycle in progenitor cells [67]. It is not yet known how maternal and/or neonatal microbial dysbiosis can affect HSCs in the foetus or neonate.

Colonization of mucosal surfaces is thought to educate the developing mucosal immune system, and the gut microbiome plays a major role in this process. The microbiota are in constant communication with immune cells in the gut, and emerging evidence highlights a gut-lung axis [50] in which migration of immune cells from the intestine to the lungs may influence homeostasis and inflammatory status [68-70]. To assess neonatal immune system development, future work using our mouse models will immunophenotype cells in the neonatal lung and intestine. In addition to communicating with our intestinal immune cells, the microbiota also produces soluble metabolites such as SCFAs from the digestion of dietary fiber. These metabolites are immunomodulatory, and mouse models of diet during pregnancy show that a high fiber diet altered the maternal microbiome, increased SCFA production and protected against asthma in the offspring. This was also observed with maternal supplementation with acetate during pregnancy [71]. In addition to this, it has been shown that supplementation with acetate, propionate and butyrate in neonatal life rescued the severe asthma phenotype in animals with an antibiotic-induced microbial dysbiosis [72]. In our Antibiotic Risk model, neonates born to antibiotic treated mothers had reduced microbial diversity in their gut microbiome and adults

had increased asthma severity. Correlated with this, animals prenatally exposed to antibiotic also had decreased SCFA concentrations in neonatal feces and adult offspring cecum [48]. Through these observations we hypothesize that SCFA play a major role in the development of neonatal immunity and analysis of these metabolites will be one of the main topics of future investigation in my laboratory.

3.4.2 Development of the lung

The lung forms in five major stages during gestation and early life, and developmental disturbance at any of these time points could predispose an individual to asthma. In mice, the Embryonic stage of lung development begins at G10 with lung bud differentiation, then proceeds through the Pseudoglandular and Canalicular stages until birth. Both humans and mice are born with immature lungs, which must proceed through the Saccular and Alveolar developmental stages, which last until after weaning [73]. Babies born prematurely have increased risk for viral infections and asthma [19, 20] and mouse models show that underdeveloped pups also have lung problems [22, 74]. To understand how the pre- and postnatal environments influence lung development, our future work will evaluate foetal and neonatal lung morphology at several different time points using our Risk Models. We have already analyzed G17 foetal lung morphology in our *A. lwoffii* protection model, and have observed that G17 foetuses from *A. lwoffii* treated mothers have increased numbers of CD3 T cells in their lungs (unpublished data). Future work will examine how these T cells could contribute to asthma protection in the offspring.

3.5 Functional studies and expansion to other disease models

The purpose of the previously described observational studies is to discover the pathways by which the maternal environment during pregnancy contributes to offspring development and subsequent asthma risk. In case that we find: 1) A pathway that is consistently and significantly altered during the time course of a particular model or, 2) Mediators that are significantly and oppositely changed in Protection versus Risk Models, we will next run functional experiments to test our newly built hypotheses. For example, significant changes in the expression of a particular mediator (cytokine, antibody, metabolite, cell type) in several different tissues and time points of a single model is a strong indication of a pathway that may contribute to the model phenotype. In this case, functional experiments would involve supplementing pregnant mother mice with the

chosen mediator to test if this alters asthma susceptibility in the offspring. In addition to functional experiments, fostering experiments can also be performed in our models to assess the influence of the pre- versus postnatal time periods. There is little doubt that both of these time points play a strong role in offspring development and disease susceptibility later in life. Future analyses will include fostering experiments to determine the contribution of the prenatal and postnatal time points in our models to offspring development and asthma susceptibility.

Finally, though our main concentration has been on asthma, we do not think that extrinsic influences during pregnancy alter offspring asthma susceptibility only. Rather, we presume that developmental disturbances alter immune tolerance mechanisms and either 1) Predispose the offspring to increased inflammatory responses to a wide variety of stimuli (in the Risk Models) or 2) Increase immune tolerance mechanisms in the offspring (in the Protection Model), resulting in decreased overall inflammatory responses. In order to test this hypothesis, our future studies will test how different maternal exposures during pregnancy influence inflammatory diseases such as colitis, arthritis or experimental autoimmune encephalomyelitis in the offspring.

4. Outlook

This habilitation thesis has summarized a body of work performed over the past decade. The development of several mouse models was highlighted, including an adjuvant-free experimental asthma model and a protection model involving maternal exposure to non-pathogenic bacteria during pregnancy and protection against asthma in the offspring. Providing contrast to the protection model, two different risk models were established, demonstrating that maternal asthma exacerbation as well as maternal antibiotic use during pregnancy are risk factors for offspring asthma development. Finally, we have also established expertise in placental analysis as well as assessment of foetal and neonatal growth and development. In future studies, we will use these transgenerational mouse models to examine the pre- and postnatal mechanisms involved in the development of offspring immune tolerance and how this influences susceptibility to diseases later in life.

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Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
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