The role of maternal immunity for prevention of allergy in offspring

Betydningen av mors immunstatus for forebygging av allergi i avkom

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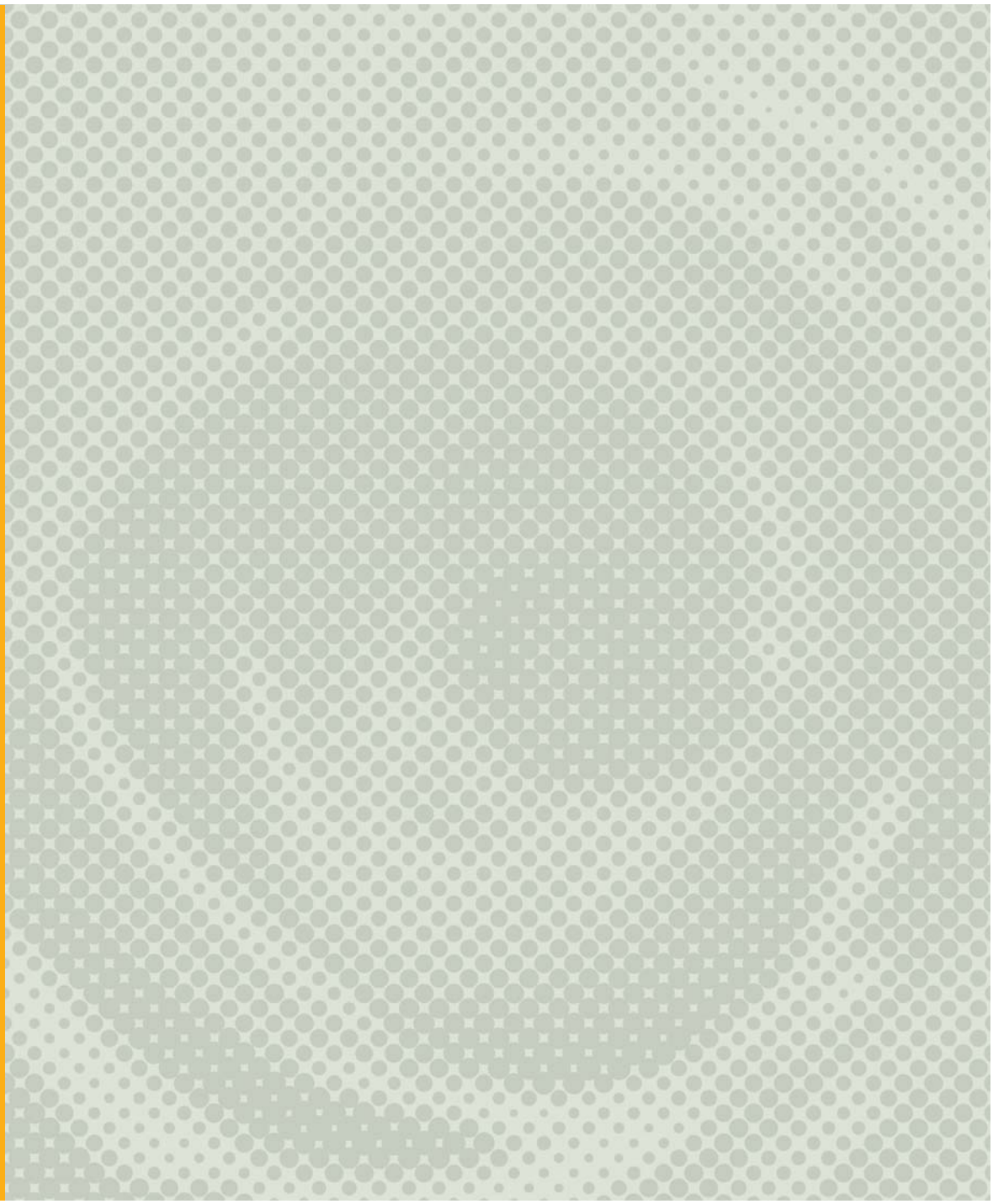

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CONTENTS

ABSTRACT

There has been an increase in the research concerned with the causes and mechanisms of allergy concurrently with allergy being recognized as an emerging public health issue. The key immunological events which lead to the development of allergic diseases are thought to occur *in utero* and during the first years of life. Experimental animal studies suggest a role for maternal transfer of allergen specific IgG1 to reduce the risk of allergy in the offspring. When dams are allergen-immunised during pregnancy, the offspring have lower IgE levels when immunised with the same allergen in young adulthood. Lowering the IgE levels may be associated with lower risk of developing allergy. In models with allergic dams there have been contradictive findings. Both the transfer of allergy protection and allergy risk has been demonstrated. Therefore it is speculated that different pathways for transfer of allergy protection and allergy risk may coexist.

The study aimed to explain how allergy risk and allergy protection is transferred from dams to offspring using a mouse model. First, the role for maternal allergen-specific IgG1 in suppressing allergy responses in offspring independent of the maternal immune response, i.e. whether the mother is immunised or allergic, was investigated. Secondly, the study aimed to clarify if allergic dams transferred an increased risk of allergy to the offspring.

Offspring of control, immunised and allergic dams were immunised with ovalbumin (OVA) as juveniles (1 - 5 week old) and adolescents (6 - 10 week old) and allergy outcomes in terms of OVA-specific antibody production (IgE and IgG1) and OVA-induced cell proliferation were compared.

Suppressed IgE-levels were observed in both offspring of immunised and allergic dams. The protection was evident in both juvenile and adolescent offspring; however it declines in the adolescent offspring. The study was not able to demonstrate a transfer of allergy risk from allergic dams.

Animal models provide an opportunity to explore mechanisms of allergy development that for ethical reasons are not possible to investigate in humans. This study offer good indications that maternal mediators facilitate beneficial conditions for allergy suppression, and suggest that this potential should be further investigated for future allergy prevention in children.

SAMMENDRAG

Det har vært en økning i forskning som fokuserer på årsaker og mekanismer til allergi, parallelt med at allergier aksepteres som et akselererende folkehelseproblem. Det antas at viktige immunologiske hendelser for utvikling av allergier inntreffer allerede *in utero* og i de første leveårene. Eksperimentelle dyreforsøk antyder videre at maternalt overført allergenspesifikk IgG1 har betydning for undertykkelse av allergi hos avkommet. Dersom mus immuniseres under svangerskapet får avkommene lavere IgE-nivåer når disse igjen immuniseres som unge voksne. Undertrykte IgE-nivåer assosieres med redusert risiko for å utvikle allergi. I modeller med allergiske mus har funnene vært motstridende. Det er vist både overføring av allergibeskyttelse og allergirisiko. Av den grunn kan det tenkes at det finnes ulike, sameksisterende, reguleringsveier for overføring av allergibeskyttelse og allergirisiko.

Denne studien ønsket ved hjelp av en musemodell å bidra til å forklare hvordan allergirisiko og allergibeskyttelse overføres fra mor til avkom. Betydningen av maternalt allergenspesifikk IgG1 for undertrykkelse av allergiresponser i avkommet ble først undersøkt. Deretter ønsket studien å klargjøre hvorvidt allergiske mødre overførte økt allergirisiko til sine avkom.

Avkom fra kontroll, immuniserte og allergiske mødre ble immunisert med ovalbumin (OVA) som unge (1 - 5 uker gamle) og som ungdom (6 - 10 uker gamle) og allergiutfall, målt i antistoffproduksjon (IgE og IgG1) og OVA-indusert celleproliferasjon, ble sammenlignet.

Undertrykte IgE-nivåer ble observert i både avkom fra immuniserte og allergiske mødre. Allergibeskyttelse var tilstedet i begge aldersgrupper, men beskyttelsen var avtagende hos de eldste. Studien demonstrerte ikke en overføring av allergirisiko fra de allergiske mødrene.

Dyremodeller muliggjør undersøkelse av mekanismer som kan forklare utvikling av allergi, som av etiske årsaker ikke er mulig å gjennomføre i mennesker. Denne studien gir en god indikasjon på at maternale faktorer skaper fordelaktige omstendigheter for allergibeskyttelse og det oppfordres til at dette potensialet undersøkes nærmere for fremtidig forebygging av allergi hos barn.

1 INTRODUCTION

1.1 Allergy

The term allergy is derived from Greek and means "altered reactivity". The immune system is designed to protect the human body and react upon infectious agents that intrude it. Under some circumstances, however, the immune system overreacts and harmless agents, termed allergens, stimulate the adaptive immune response. On subsequent exposures to the same allergen, there will be a hypersensitivity response, defined as allergy (Parham, 2009). Numerous of the allergens responsible for this reaction are common agents present in our everyday environment, including airborne plant pollen, feces of dust mites, food and drugs. Most people do not react to these allergens and may be defined as tolerant. Why some develop the allergic reaction and others do not is yet to understand. Several hypotheses have been projected and associate the "Westernized" lifestyle with the increase in allergy prevalence. For example, the hygiene hypothesis suggests that enhanced hygiene and the overuse of antibiotics have caused insufficient stimulation of regulatory mechanisms in the adaptive immune response (Yazdanbakhsh et al., 2002). Increased allergy prevalence has also been ascribed to factors in the outdoor and indoor environment, such as diesel exhaust particles and tobacco smoke (Nielsen et al., 2005; Nikasinovic et al., 2004). Further, studies have shown that children born in atopic families are more likely to develop allergic diseases compared to those with no family history of atopy. The risk is higher if both parents are allergic, and when the mother (rather than the father) has allergies (Lim et al., 2010). There seems to be no association between the types of allergy in parents and the allergic disease in their children, but more likely it is the tendency to develop allergy that is inherited, not the allergic disease itself (Lim et al., 2007). There is agreement that both heredity and environmental factors play important roles, probably in symphony (Lea, 2008).

Common allergic diseases include allergic rhinitis, asthma, rhino conjunctivitis, gastrointestinal symptoms, urticaria and eczema (Lea, 2008).

1.2 Allergy prevalence and health consequences

The prevalence of childhood allergies and asthma is escalating worldwide, particularly in industrialized countries, and are emerging as a major public health burden (Asher et al., 2006; Beasley et al., 2000; Eder et al., 2006). During one month in 2003, 29% of the working population in Norway reported complaints related to allergies (Ihlebaek et al., 2007). A Norwegian birth cohort study indicates that in Oslo every fifth 10 year old has asthma (Lodrup Carlsen et al., 2006). Respiratory allergic diseases affected 24.4 % of the European population aged 16 - 60 in 2004, with the highest prevalence among the young people (Dahl et al., 2004). In 2009, common allergic diseases affected 35 % of the adult population and 33 % of all children in Europe (Bousquet et al., 2009). The same study estimated that in six years 50 % of all Europeans will suffer from allergy. Furthermore, the prevalence of some allergic diseases, such as asthma is also increasing in developing countries as they become more urbanized (Beasley et al., 2000).

Several studies report an influence of gender on development of allergic diseases. There is substantial evidence that boys are more susceptible to allergic sensitisation than girls, although this gender difference attenuates in adulthood (Govaere et al., 2007). Also boys have a higher prevalence of asthma than girls before puberty and adulthood asthma occurs more frequently and severe among women (Postma, 2007). The gender related switch in asthma prevalence occurs around the time of puberty, and sex hormones are suggested to play an important role (Chen et al., 2008).

Allergic diseases are characterized by significant negative effects on personal life quality and may be associated with severe morbidity (Bousquet et al., 2009). Additional health problems such as neck and shoulder pain, muscular pain, headaches, and abdominal pain are more often reported in adolescents with allergic symptoms than those without, drawing the attention to a possible link between several subjective health complaints and allergy (Tollefsen et al., 2007). Sensitisation has been suggested to be a possible causal mechanism for such comorbidity (Eriksen and Ursin, 2004).

As the prevalence of allergies raise, the disease burden posed by allergic conditions is also increasing. In UK the cost of general practitioner services and hospital admissions related to allergy is estimated to more than one billion pounds per annum (Gupta et al., 2004). This calculation does not include any indirect cost related to reduced quality of life or work days lost. One study, however, demonstrated that workers with allergies show a 10 % decrease in productivity compared to workers without allergies (Burton et al., 2001). Also a study proposed that the sickness absence related to allergic diseases in the Norwegian working population have increased from 1996 to 2003 (Ihlebaek et al., 2007).

These findings indicate that allergic diseases already have a major health impact worldwide and should be allowed more attention. Special consideration should be given the primary prevention strategies in order to stop the escalating prevalence of allergies. This implies that understanding the mechanisms of allergy development is both essential and necessary.

1.3 Pathogenesis of allergy

Hypersensitivity reactions are categorized according to the underlying effector mechanism causing the reaction. Type I, II and III hypersensitivity are antibody-mediated, while type IV hypersensitivity is induced by a cellular response (Lea, 2008). All type I hypersensitivity reactions are caused by allergen-specific Immunoglobulin E (IgE) antibodies formed following exposure to antigens such as proteins from plant pollens or food inhaled or ingested, respectively (Parham, 2009). They are referred to as immediate hypersensitivity reactions due to the rapid onset of clinical symptoms occurring within 30 minutes after exposure (Lea, 2008). Only the type I hypersensitivity reaction will be assessed in the presented thesis.

The majority of allergens are small soluble proteins derived from plants and animals that are caught in the mucosa in airways or in the digestive system (Parham, 2009). The proteins are processed by professional antigen-presenting cell in the mucosa, such as dendritic cells, and presented to CD4 expressing (CD4+) T helper-cells. The cytokines present in the immediate environment of the

CD4+ T cell will influence the differentiation pathway into T helper (Th) 1 or Th2 cells. Cytokines IL-12 and TNFγ stimulate the development of Th1 cells that subsequently produce and release IL-2 and IFNγ. CD4+ T-cells differentiate into Th2 cells under the influence of cytokine IL-4. Once Th2 cells start secreting IL-4 and IL-5, they will facilitate further differentiation of CD4+ T-cells into Th2 cells. When an antigen-specific Th2 cell encounters an antigen-activated B-cell, it may provide the B-cell with signals through co-stimulatory molecules and cytokines. This will activate the B cell and stimulate antibody secretion. IL-4 secreted from the T cell will induce isotype switching to IgE (Parham, 2009). Circulating IgE binds to the high-affinity receptor FcεRI of mast cells and basophils, thus providing these cells with antigen receptors. These events constitute the process of being sensitized (Figure 1).

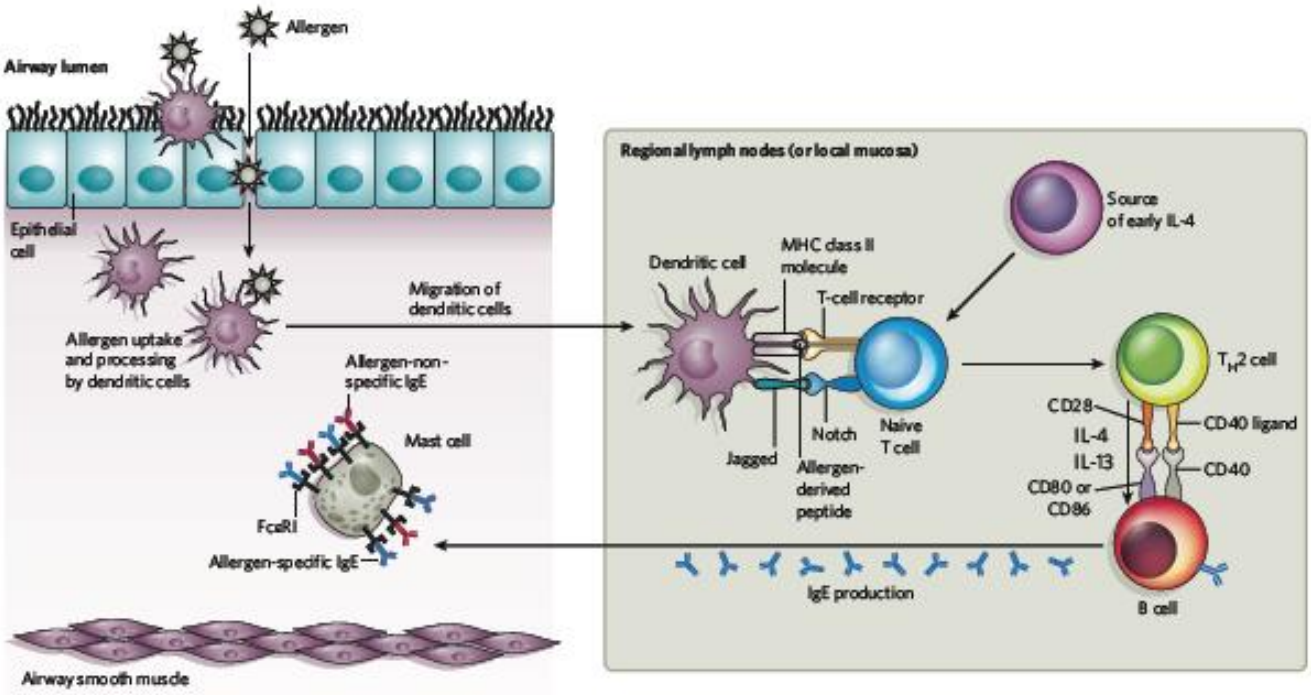

Figure 1: Sensitisation to an inhaled allergen (from Galli et al., 2008).
Allergens are taken up by dendritic cells in the mucosa of the airways. These activate naïve T cells to become Th2 effector cells. Th2 cells bind to the B cell and provide the B cell with signals through co-stimulatory molecules and cytokines, causing the B cell to secrete IgE.

On subsequent allergen encounter, the allergen will cross-link the cell-bound IgE molecules bound on mast cells, causing the cell to degranulate and release its content of chemical mediators, such as

histamine. The released mediators recruit and activate inflammatory cells such as eosinophils, and causes vasodilatation and oedema, producing the symptoms of an allergic reaction. These are sneezing and itching in hay fever and bronchoconstriction in asthma.

1.4 Allergy management

Currently, allergy management mostly embraces secondary and tertiary prevention strategies, with focus on the avoidance of allergens and treatment of the allergic symptoms in individuals that are already symptomatic (Sicherer and Sampson, 2009). Antihistamines, one of the most prescribes drugs, blocks the effect of histamine released by mast cells and basophils, and thus alleviates the allergic symptoms (Arshad, 2002). Asthma is commonly treated with bronchodilators that induce relaxations of bronchial smooth muscles (Arshad, 2002; FitzGerald and Shahidi, 2010). Several immunotherapeutic approaches are successful and have shown promising results in reducing the symptoms of allergic reactions (Passalacqua et al., 2009; Pfaar et al., 2010; Sicherer and Sampson, 2009). Immunotherapy aims to stimulate a Th1 immune response or generate regulatory T cells that will down-regulate the Th2 response. An increasing dose of allergen to which the patient is sensitized, is injected or administered sublingually (Arshad, 2002). The therapy is time-consuming and in some cases it can provoke serious adverse effects (Sicherer and Sampson, 2009).

1.5 Allergy prevention

Several strategies aimed at preventing allergy development have focused on the fetal and postnatal period. Exclusively breastfeeding for at least 4 - 6 months is demonstrated to be the most effective regimen for avoidance of food allergy and atopic eczema (Muraro et al., 2004a; Muraro et al., 2004b). Both the introduction of cow's milk protein and solid foods before 4 months of age has been associated with higher risk of atopic eczema (Greer et al., 2008). Restriction diets after 6 months of age have shown no preventive effect (Muraro et al., 2004b). A number of allergen-avoidance strategies are either proven ineffective of unverified in either mothers or children (Eder et al., 2006; Greer et al., 2008).

Epidemiological findings suggest a causal link between exposure to tobacco and asthma. Maternal smoking constitute one of the most well-described risk factors for developing allergy and asthma in children, and avoidance of exposure to smoke during pregnancy and childhood is consequently an important way of preventing allergy in children (Eder et al., 2006).

Supplements of omega 3 and 6 oils have been suggested to have anti-inflammatory effects and play important roles in the immunological mechanisms of allergy. However, systematic reviews and meta-analyses suggest that it is unlikely that omega 3 and 6 oils have any preventive effects on allergic diseases (Anandan et al., 2009).

The role of probiotics in primary prevention has also been studied. Probiotics in early life is suggested to have beneficial effect on the developing immune system. So far no studies have shown any clear preventive effect on sensitization, nor on any allergic disease, other than eczema (Prescott and Bjorksten, 2007).

1.6 Animal experiments in allergy research

There has been an increase in the research concerned with the causes and mechanisms of allergy concurrently with allergy being recognized as an emerging public health problem. Epidemiological studies are necessary to describe the extent of the problems and may give indications and correlations of the causal factors for allergy, but it may rarely demonstrate clear cause-relationships and mechanisms (Rotman, 2002). Experimental research in particular in live animals has, thus, been used to answer key questions and understand the pathogenesis and mechanisms of allergy development and pathophysiology (Berin and Mayer, 2009; Hausding et al., 2008; Kips et al., 2003; McCusker, 2004).

2 BACKGROUND FOR STUDY

2.1 Mouse models of transfer of maternal immunity

The key immunological events which lead to the development of allergic diseases are thought to occur *in utero* and during the first years of life (Holt et al., 2004). Therefore, preventive strategies should be started in early life or perhaps even *in utero*. Such strategies have been investigated experimentally in mouse models. It has been shown that allergen immunisation (vaccination) of mice during pregnancy lower the allergen-specific IgE levels in the offspring when they are sensitised in young adulthood to the same allergen (Melkild et al., 2002). Lowering the IgE levels may also lower the risk of developing allergy. In contrast, offspring from unimmunized dams develop high allergen-specific IgE levels when sensitized (Fusaro et al., 2002; Melkild et al., 2002; Victor. et al., 2003). Previous studies suggest a role for maternal allergen-specific IgG1 to mediate the IgE-suppressing effects in the offspring (Fusaro et al., 2002). IgG1 in mice is transferred in particular via breast milk from dam to offspring, although also via placenta (Fusaro et al., 2007). Breastfeeding by mothers exposed to an allergen shortly before pregnancy restrains the development of an allergic response to the same allergen in offspring, suggesting that the maternal transfer of allergy suppression is via mother's milk (Bednar-Tantscher et al., 2001). The maternal IgG1 is gradually decreasing in offspring after weaning, but is detectable at least up to 9 weeks (Victor et al., 2003). A few studies have demonstrated that maternal vaccination does not have effect in older mice (Fusaro et al., 2002; Victor et al., 2003) – again suggesting a role for maternal IgG1 on IgE suppression in offspring.

Other mouse models have investigated the effect of transfer of maternal allergy and tolerance on allergy outcomes such as allergen-specific IgE and airway inflammation in offspring. Interestingly, when comparing with the vaccination models, similar observations have been found. Thus, it has been shown that high dose allergen-tolerant dams transfer allergy protection to their offspring (Polte et al., 2008; Polte and Hansen, 2008; Verhasselt et al., 2008). The studies by Polte et al. (2008) suggested that the allergy suppression was mediated by maternal IgG1 similar to what is observed in vaccination models. However, another study found no effect of maternal IgG1 - rather the allergy

suppression in offspring was mediated by regulatory T cells and presence of TGFβ in breast milk (Verhasselt et al., 2008).

Experimental studies have been developed to investigate the observation how allergy is inherited from parents to children. Thus, it has been shown that allergic dams transfer an increased risk of allergy to their offspring in terms of higher IgE production and more severe airway inflammation and hyperreactivity (Hamada et al., 2003; Lim et al., 2007). In these allergy models there have, however, been contradictory observations. Some studies demonstrate an allergen-dependent reduction in IgE in offspring from allergic dams (Matson et al., 2009; Uthoff et al., 2003). The protective effects were also mediated by maternal allergen-specific IgG1. In contrast, other studies observe a transfer of allergy risk from allergic dams to offspring and that the transferred allergy risk is allergy-independent (Hamada et al., 2003; Lim et al., 2007).

Different models have, thus, been implemented to study the mechanisms of maternal transfer of immunity from immunised and allergic mothers. In both models, maternal allergen-specific IgG1 may be responsible for the observed suppression of allergen-specific IgE in the offspring. However, in some models of maternal transfer of allergy risk, this suppressive effect of IgG has not been observed (Hamada et al., 2003). The reasons for these findings may be due to different sensitization procedures in the offspring, the age when sensitization is induced and the use of different mouse strains. Hence, there are reasons to believe that allergy risk is not related to the transfer of allergen-specific antibodies, but rather related to the transfer of cellular responses or mediators with "allergy-signalling" potential. This raises the question whether pathways for transfer of allergy protection and allergy risk may coexist and is the focus of the present study.

3 OBJECTIVES

The project "Preventive vaccination against allergy in offspring" is a post doctor project financed by the Norwegian Research Council for three years. The main objective is to investigate if maternal immunisation may be used as a preventive strategy to transfer allergy protection from mother to child during pregnancy and early life. Knowledge of the effects of maternal immunity on allergy responses in offspring is of great importance for the implementation of allergy-preventing strategies prenatally. The study presented in this thesis was conducted as a defined and independent study within the above project to investigate, in a mouse model, the role of maternal immunity for allergy prevention. Offspring of control, immunised, and allergic dams were immunised as juveniles and adolescents, and allergy outcomes compared. Antibody production (IgE and IgG1) and cell proliferation were investigated as outcomes of allergy in dams and offspring.

First, the role for maternal IgG1 in suppressing allergy responses in offspring independent of the maternal immune response, i.e. whether the mother is immunised or allergic, was investigated:

Hypotheses:

1. If IgE suppression in immunised offspring is mediated by maternal IgG1, then this suppression will be observed in offspring of both immunised and allergic dams.

2. If IgE suppression in immunised offspring is mediated by maternal IgG1, then older mice will have less IgE-suppression than younger mice due to the decrease in maternal IgG1 after weaning.

3. If IgE suppression in immunised offspring is mediated by maternal transfer of cells or cytokines, then cellular responses in offspring may demonstrate this. Following allergen-stimulation of mediastinal lymph node cells, offspring of immunised and allergic dams wills have an increased proliferation compared to offspring of control dams.

In contrast to immunised dams, the allergic dams have an airway inflammation and previous independent studies have shown that both allergy protection and an increased risk of allergy can be demonstrated in offspring from allergic dams. Therefore the study secondly aimed to show if different pathways for transfer of protection and allergy risk may coexist.

Hypotheses:

4. If allergic dams transfer an allergy risk to offspring, then exacerbated allergy responses will be seen in offspring of allergic compared to offspring from immunised and control dams.

5. If IgE suppression is caused by maternal IgG1, then increased allergy risk in offspring (independent of, or in co-existence with, the effects of maternal IgG1) will be seen first when maternal IgG1 declines.

6. If allergy risk and protection is transferred by cells or cytokines from dams to offspring and, therefore, is independent of or in co-existence with the effects of maternal IgG1, then cellular responses in offspring may demonstrate this. Following allergen-stimulation of mediastinal lymph node cells, offspring of allergic dams wills have an increased proliferation compared to offspring of control dams.

4 MATERIALS AND METHODS

4.1 Mice

Specific pathogen free mice (*Mus musculus*) from the NIH/OlaHsd strain (Harland Ltd. UK) were used because they produce large litters (approximately 7 pups per litter) and are good antibody producers (Nygaard et al., 2005). The latter is important for the described allergy experiments. Seven-week-old females (n = 54) and 8-week-old males (n = 20), weight 25-30 grams, were supplied by plane and van in filter boxes and arrived at the animal facilities at the institute on the 26th October 2009. The mice were acclimatized for 9 days. Synchronisation of oestrus and mating were performed according to Whitten (1957). The females were kept in male-free rooms to induce anoestrus. Three days before mating, bedding from male cages was transferred into female cages to synchronize oestrus. Two to three females were moved into the cage of one male and the females were checked for a pregnancy indicator, a vaginal plug, for the next three days. After mating, all females were housed individually until weaning of pups. Males were euthanized. After weaning, offspring were housed 3-4 individuals in each cage. Older males were housed solitary or only 2-3 brothers together to avoid aggressive behaviour. All cages were provided with plastic igloos and wooden gnaw sticks for environmental enrichment.

Room temperature and humidity were regulated (22 ± 2°C, 35-75%). There was a 12/12 hours cycle of light/darkness and the animals were handled during the light phase. The light source was 60 lux at two meters above the floor. NESTPACKS produced by Datesand Ltd. (UK) was used as bedding. The mice were fed *ad libitum* with diet 2019 (Harlan Teklad). The diet was strictly free from animal protein to avoid contamination of ovalbumin. Tap water was given *ad libitum*. The experiment was conducted from 26th October, 2009, to February 19th, 2010.

4.2 Allergen, adjuvant and anaesthetics

Chicken egg albumin, ovalbumin (OVA, grade VII, Sigma, St. Louis, MO, USA), was used as model allergen. It is widely applied in mouse models of allergy (Kumar et al., 2008). $Al(OH)_3$, (Alhydrogel 2%, Brenntag Biosector, Denmark) was used as adjuvant to stimulate Th2 immune responses (Lindblad 1995). For anaesthesia, 3.5 % isoflurane gas was administered in surgical O_2. The ZRF anaesthesia contains 18.7 mg Zolazepam, 18.7 mg Tiletamine, 0.45 mg Xylazine and 2.6 ug fentanyl per ml and mice were administered 0.1 ml/10 g intraperitoneally (i.p.) prior to heart puncture.

4.3 Maternal treatment

Pregnant dams were randomized into three groups; immunisation, airway allergy and controls. The immunisation protocol has been established previously (Melkild et al., 2002). Dams (n = 7) were immunised subcutaneously on day 3 after observation of vaginal plug with 10 µg OVA in 2 mg $Al(OH)_3$ (in 0.2 ml saline) and booster immunised on day 10 with 1 µg OVA. Allergic dams (n = 8) were immunised in the same way and further received intranasal exposures of 10 µg OVA in 35 µL under isoflurane anaesthesia on day 17-19 to induce airway inflammation. Control dams (n = 9) were sham-immunised by subcutaneous injection of 0.2 ml saline on day 3 and 10. In order to follow the development of the immune response, blood samples were taken from the *vena saphena lateralis* before mating and 10 days after delivery. Blood was collected by heart puncture following administration of the ZRF anaesthesia one week after weaning of the offspring. The experimental design for both dams and offspring is outlined in Figure 2.

4.4 Offspring immunisation and tissue collection

Offspring were immunised in an established intranasal immunisation protocol (Nygaard et al., 2009; Hansen, unpublished data). The offspring were allocated to immunisations at different ages (Figure 3). Half of the offspring (n = 61) were immunised at 1 week of age, the other half (n = 67) at 6 weeks of age. The offspring were anesthetised by isoflurane gas and immunised by intranasal application of 10 µg OVA in 120 µg $Al(OH)_3$ for 3 consecutive days. Three weeks later (at weeks 4 and 9), a blood sample was collected from the *vena saphena lateralis* (100 µL) and subsequently, they

received two booster immunisations by intranasal application of 10 µg OVA under isoflurane anaesthesia. Further, a blood sample was taken from the *vena saphena lateralis* in unimmunised 6-week-old offspring to be analysed for the presence of maternal antibodies. Three days after the booster immunisations, the offspring were anaesthetised with the ZRF cocktail and blood was collected by hearth puncture. Mediastinal lymph nodes were collected according to Van den Broeck et al. (2006).

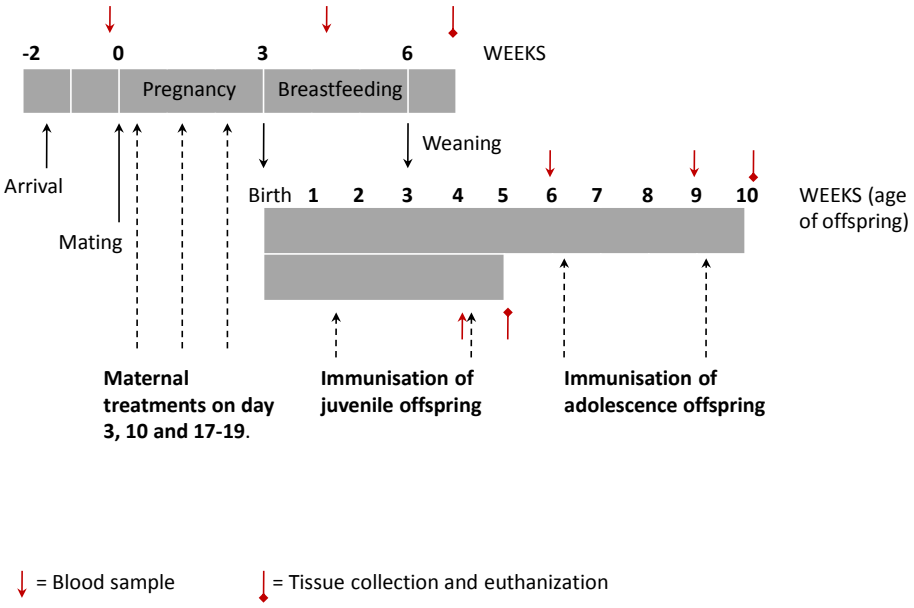

Figure 2: Overview of the experimental design

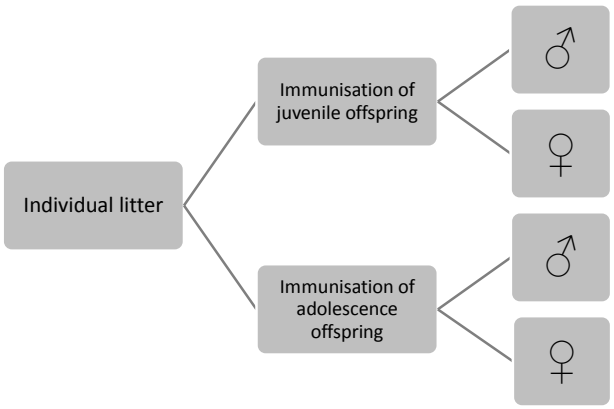

Figure 3: Allocation of offspring (males and females) from individual litters

4.5 Detection of OVA-specific IgE and IgG1 in sera

OVA-specific IgE and IgG1 levels were analysed using enzyme linked immunosorbent assay (ELISA) described in details in Appendix I and II. For detection of IgE in sera ELISA plates (MaxiSorp) are coated with monoclonal rat anti-mice IgE in 0.05 M carbonate-bicarbonate buffer (pH 9.6) in one hour at 21˚ C, then overnight at 4˚ C and washed with Tris/Tw 8. Optimally diluted sera (1:10 or 1:50) were added and incubated for 1 hour at 21˚ C. Biotin labelled OVA was added to each well after washing with Tris/Tw 8 and the plates were reincubated at 21 ˚ C . For colour development, poly horseradish peroxidase (HRP) streptavidin diluted in Tris/Tw 8 was added to each well after washing with Tris/Tw8. After incubation for 1 hour t 21˚ C the plates were washed with Tris/Tw 8 and TMB Stabilized Cromogen solution was added to each well. After 15 minutes the colour development process was stopped by adding 2N H_2SO_4 and absorbance (OD) was measured at 450 nm on a MRX Microplate reader.

For detection of IgG1 in sera, ELISA plates (MaxiSorp) were coated with monoclonal rat anti-mice IgG1 in 0,05 M carbonate-bicarbonate buffer (pH 9.6) in one hour at 21˚ C , then overnight at 4˚ C and washed with 0.05% Tween 20 in Tris/HCL 50 mM pH 8.0 (Tris/Tw 8). Optimally diluted sera (1:200 or 1:200.000) were added and incubated for 2 hours at 21 ˚ C. For colour development horseradish peroxidase (HRP) conjugated OVA in Tris/Tw 8 was added to each well after washing with Tris/Tw 8. After reincubation for 2 hours at 21˚ C the plates were washed with Tris/Tw 8 and TMB Stabilized Cromogen solution was added to each well. After 15 minutes, the colour development process was stopped by adding 2N H_2SO_4 and absorbance (OD) was measured at 450 nm on a MRX Microplate reader.

For the IgE standard curve, mouse anti-OVA IgE (MCA2259) was purchased with a known concentration and the IgE levels were given in ng/ml. The standard curve for the IgG1 assays was generated from a serum pool from OVA and $Al(OH)_3$ immunized Balb/c mice and values were given as arbitrary units (AU).

4.6 Ex vivo quantification of cell proliferation

Single-cell preparations from mediastinal lymph nodes (MLNs) were cultured *ex vivo* as described in Appendix III and VI. Lymph node cells were cultured with OVA (100 μg/ml) or a T cell mitogen, Concanavalin A (ConA) to induce cell proliferation, and analysed with a bromodeoxyuridin (BrdU) incorporation assay to give indications of suppressed or enhanced immune responses due to the maternal treatments. After 120 hours of incubation at 37° C, proliferating cells were labelled with BrdU that replaces thymidine during DNA synthesis. Anti-BrdU-POD was added and the complex of BrdU-anti-BrdU-Pod was detected using a colorimetric substrate reaction. Absorbance (OD) was measured at 450 nm on a MRX Microplate reader.

A stimulation index (SI) was calculated, in that OD values for OVA and ConA stimulated cells respectively were divided by OD values for unstimulated cells cultured in medium.

4.7 Statistical analyses

All parametric analysis was performed with Minitab (version 15). SigmStat Statistical Analysis system for Windows (version 3.5) was used for non-parametric analyses.

The aim of the statistical analysis was to compare a number of outcomes between the three different maternal gestational treatments and also to examine sex differences upon the same outcomes. An initial analysis compared whether there were any overall differences in the outcome. If there were a significant overall difference, additional post-hoc tests were used to compare between each pair of treatments. Antibody levels in dams were compared with the General Linear Model with treatment as fixed factor. Antibody levels in blood samples from offspring were also compared using the General Linear Model procedure with both maternal treatment and sex as fixed factors. Both main and interaction effects were investigated. Overall significant differences in outcomes for both dams and offspring were then tested with a Bonferroni adjusted post-hoc test (as described below). The General Linear Model assumes equal variance and normal distributed residuals, and data sets that did not accomplish these assumptions were given a logarithmic transformation. If data transformation did not introduce equal variance and normally distributed residuals the non-parametric Kruskal-

Wallis test with the Mann-Whitneys post hoc test was used to evaluate the outcome of maternal treatment in offspring.

A feature of the data collected from the euthanized 5- and 10-week-old offspring (antibody levels and proliferation indexes) was that there were several offspring from the same mothers. A consequence of this was that it was likely that results from offspring with the same mother would be more similar than mice from different dams. Therefore, the assumption made by most standard statistical methods that the results from each mouse would be independent of each other might not be valid in this case. To account for the structure of the data, multilevel statistical methods were performed by a statistical consultant. Two-level models were used, with offspring nested within dams. Again, data were transformed to obtain normal distribution of residuals and equal variance if necessary.

Due to the continuous nature of the outcomes, multilevel linear regression was used in the multilevel analyses. Initially the interaction between the sex and treatment was included in the regression model. If this interaction was not significant, it was removed from the analysis, and only the main effects were considered. An initial analysis compared whether there was any overall difference in outcomes between the three treatments. If there was no overall difference, no additional comparisons were performed. However, if there was a significant overall difference, additional post-hoc tests were used to compare between each pair of treatments. There are multiple pairwise comparisons, and thus a greater likelihood of finding a significant result due to chance alone. Therefore, to counter this, the p-values from the post-hoc tests were given a Bonferroni adjustment. This involves multiplying the p-values by the number of comparisons made (three in this case).

Differences between outcomes were considered significant when p-values were < 0.05.

4.8 Ethical considerations

To present mothers with airway allergies and to investigate the subsequent allergy responses in children would not be an option for obvious ethical reasons. *In vitro* experiments alone would not provide the sought information regarding the interaction between mother and child. Mice have been chosen as the model organism for several reasons. Inbreed mice are genetically similar and have a short lifespan. The chosen strain is an easy breeder and excellent antibody producer. The vast amount of previous mouse studies constitutes a solid ground for comparisons (Baumans, 2004).

The three R's in animal experiments, proposed by Russel and Burch in 1959, and recently republished by Balls et al. (2009) have been assessed in the present study; Replacement of animal experiment with alternative experiments, reduction of the number of animals used and refinement of the animal experiment. As already argued, no other experimental method could have replaced the animal experiments in order to investigate the relationship in question between dams and offspring. The numbers of animals were reduced to an absolute minimum by implementation of a factorial design, and the number calculated by resource equation. Multilevel statistical analyses enabled the use of multiple offspring from each dam. With respect to refinement, a well-established model was used so that animals would not be scarified without a scientific contribution. Further, animals were attended to 30 minutes after all immunisations for observation of general well being. Anaphylactic shock is regarded as unlikely to occur, and if severe it would lead to immediate euthanization.

The experiments were performed in conformity with laws and regulations for experiments with live animals in Norway, and they were approved by the Experimental Animal Board under the Ministry of Agriculture in Norway.

5 RESULTS

5.1 OVA-specific IgE and IgG1 responses in dams

Blood samples were taken from dams before mating, 10 days after delivery and one week after weaning of offspring, and OVA-specific IgE and IgG1 levels were compared with respect to treatment (allergic, immunised and control). As expected, OVA-specific antibodies were not detected in serum sampled before mating (data not shown). In serum collected from dams 10 days after delivery, there was a significantly higher level of IgE in both immunised and allergic dams compared to control dams, with allergic dams having significantly more OVA-specific IgE than immunised dams (Figure 4a). The OVA-specific IgG1 level was also higher in both immunised and allergic dams compared to control dams (Figure 4b). The same trend of antibody responses was observed in dams after weaning of offspring. There was a significantly higher level of OVA-specific IgE in immunised and allergic dams than in control dams. Also, allergic dams had significant more OVA-specific IgE than immunised dams (Figure 4c). With respect to OVA-specific IgG1 there were significantly higher levels in immunised and allergic dams than in control dams. Allergic dams also had significantly more OVA-specific IgG1 than immunised dams after weaning of offspring (Figure 4d).

5.2 OVA-specific IgE and IgG1 responses in juvenile offspring

Blood was collected three weeks after the primary immunisation and OVA-specific IgE and IgG1 levels in juvenile offspring from allergic, immunised and control dams were compared. There was a significantly higher level of OVA-specific IgE in 4-week-old offspring from control dams compared to offspring from both immunised and allergic dams (Figure 5a). OVA-specific IgG1 was significantly higher in 4-week-old offspring from allergic and control dams compared to those from immunised dams. Further, offspring born to allergic dams had significantly more OVA-specific IgG1 than offspring from control dams (Figure 5b).

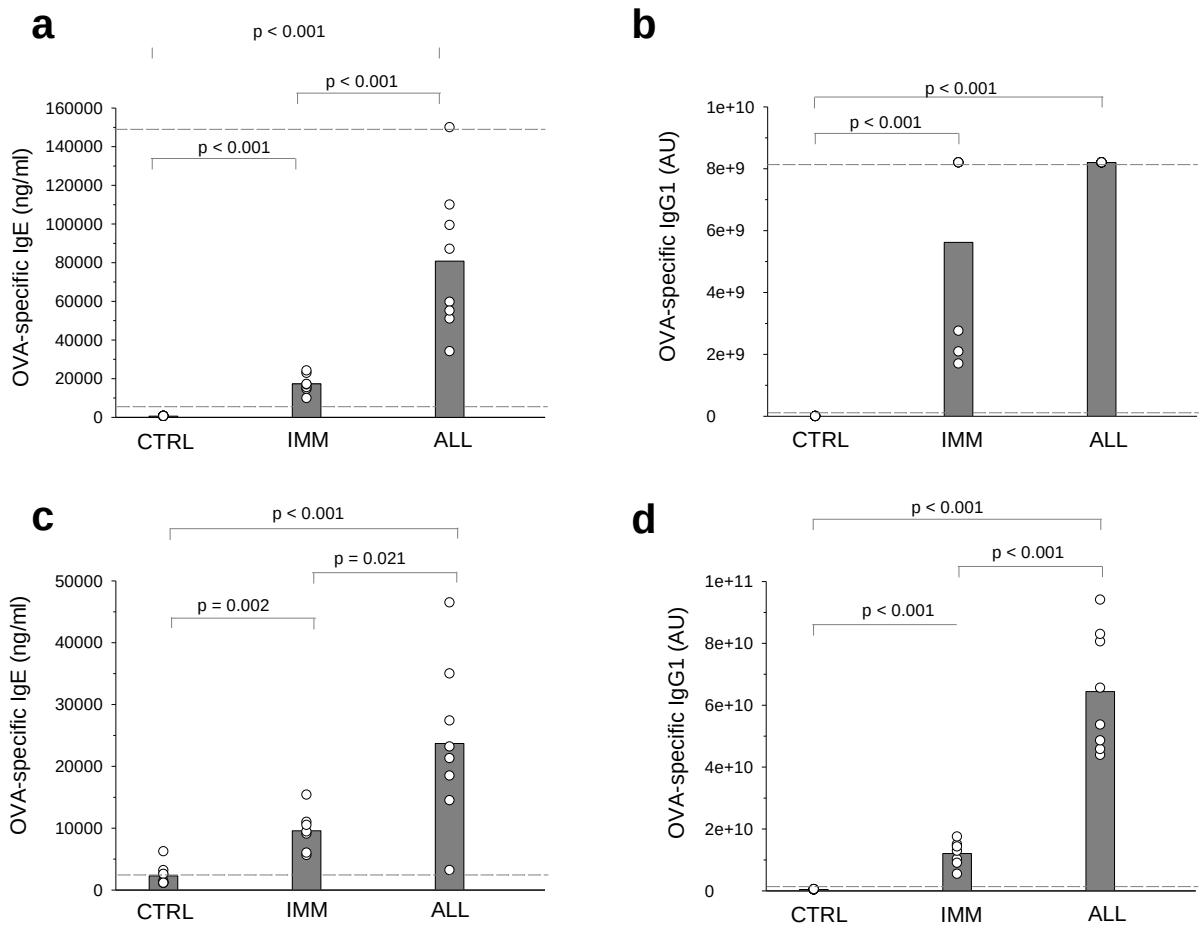

Figure 4: OVA-specific IgE in dams 10 days after delivery (a) and one week after weaning (c) and IgG1 responses in dams 10 days after delivery (b) and one week after weaning (d)
Pregnant dams received different treatments with the allergen ovalbumin (OVA); immunisation (IMM), airway allergy (ALL) and controls (CTRL). OVA-specific IgE and IgG1 were analysed in serum collected 10 days after delivery of offspring and one week after weaning of offspring. An effect of treatment on both OVA-specific IgE and IgG1 was found at both time points (Kruskall-Wallis, $p < 0.001$). The result of the post hoc test is shown on the figure.

Three days after the booster immunisations, blood was collected and OVA-specific IgE and IgG1 levels in juvenile offspring from allergic, immunised and control dams were compared. In 5-week-old offspring, the analyses of OVA-specific IgE revealed that there was a significant interaction between sex and maternal treatment suggesting that the effect of treatment varied between males and females. As a consequence, effects of maternal treatments were analysed separately for males and females.

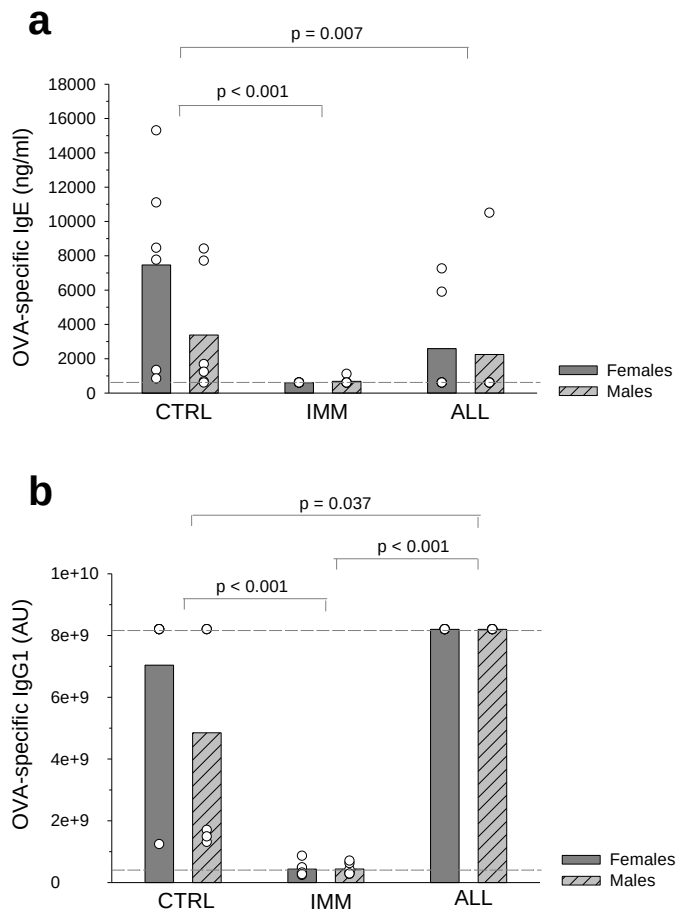

Figure 5: OVA-specific IgE (a) and IgG1 (b) responses in 4-week-old offspring
Juvenile offspring from control (CTRL), immunised (IMM) and allergic (ALL) dams were immunised with the allergen ovalbumin (OVA) emulsified in $Al(OH)_3$ at age 1 week and levels of OVA-specific IgE (a) and IgG1 (b) were compared at 4 weeks of age. An effect of maternal treatment on both OVA-specific IgE and IgG1 was found (Kruskall-Wallis, $p < 0.001$). The result of the post hoc test is shown on the figure.

There was a significant overall effect of maternal treatment for both male and female offspring with the largest effect for females. Both male and female offspring from immunised and allergic dams had IgE below or around the detection limit of the assay, which was significantly lower than IgE from offspring of control dams (Figure 6a). For OVA-specific IgG1 in 5-week-old offspring the analyses suggested that the effect of treatment was equivalent for both males and females, and that there was a significant overall difference between the three maternal treatment groups. Offspring from control and allergic dams had significantly elevated OVA-specific IgG1 levels compared to offspring born to immunised dams. Males were found to have significantly lower OVA-specific IgG1 values than females at this time point (Figure 6b).

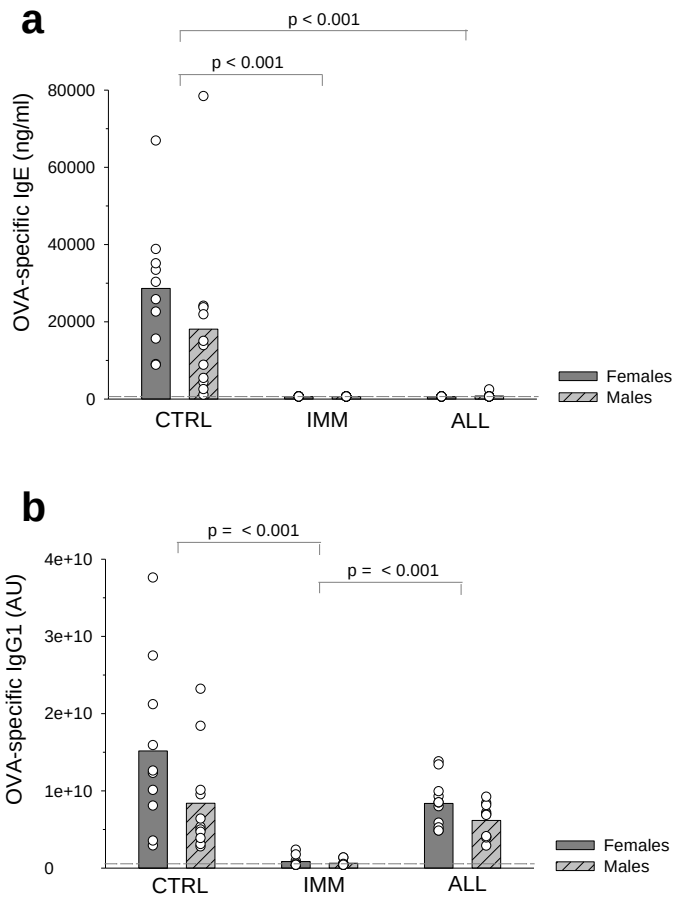

Figure 6: OVA-specific IgE (a) and IgG1 (b) responses in 5-week-old offspring
Juvenile offspring from control (CTRL), immunised (IMM) and allergic (ALL) dams were immunised with the allergen ovalbumin (OVA) emulsified in $Al(OH)_3$ at 1 week and booster immunised with OVA at 4 weeks of age. Levels of OVA-specific IgE and IgG1 were compared at age 5 weeks. An interaction between sex and maternal treatment was found for OVA-specific IgE (interaction effect, $p = 0.02$). Both in females and males, the effect of maternal treatment was significant (main effect, $p < 0.001$) and post hoc tests were performed to reveal which groups differed. An effect of sex was only seen in control offspring, where females from control dams responded with significantly higher levels than males (main effect, $p = 0.001$). An effect of maternal treatment (main effect, $p = < 0.001$) and sex (main effect, $p = 0.02$) on OVA-specific IgG1 was found. The results of the post hoc tests for maternal treatment are shown on the figure.

5.3 OVA-specific IgE and IgG1 responses in adolescent offspring

Blood were collected from adolescent offspring (6-10 weeks old) at different time points; in unimmunised 6-weeks-old offspring, three weeks after the primary immunisation and three days after the booster immunisations. Serum levels of OVA-specific IgG1 and IgE in offspring born to allergic,

immunised and control dams were compared. Serum analyses from 6-week-old offspring revealed that there was no detectable OVA-specific IgE previous to the primary immunisation regardless of maternal treatment (data not shown). There was, however, a significantly higher level of OVA-specific IgG1 in offspring born to allergic and immunised mothers compared to offspring from control dams. Further, offspring from allergic dams had significant higher levels that offspring from immunised dams (Figure 7).

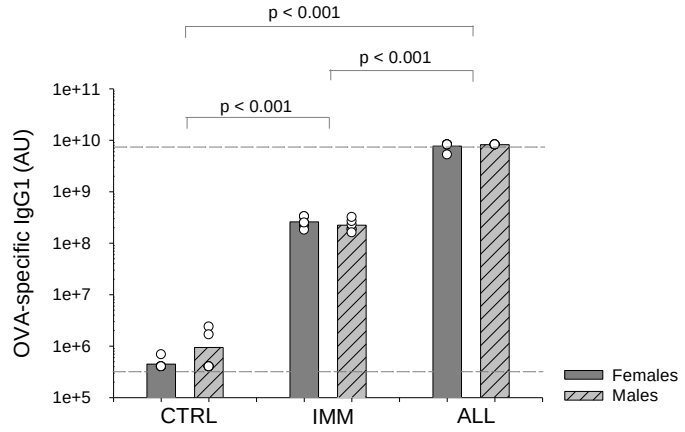

Figure 7: OVA-specific IgG1 responses in non-immunised 6-week-old offspring
Levels of OVA-specific IgG1 in adolescent offspring from control (CTRL), immunised (IMM) and allergic (ALL) dams were compared before immunisation at 6 weeks. An effect of maternal treatment was found (main effect, $p < 0.001$). The result of the post hoc test is shown on the figure.

After the primary immunisation, the OVA-specific IgE-level in 9-week-old offspring was not significantly different between the maternal treatment groups (Figure 8a). Offspring from control mothers had significantly more OVA-specific IgG1 in serum than offspring born to allergic mothers (Figure 8b).

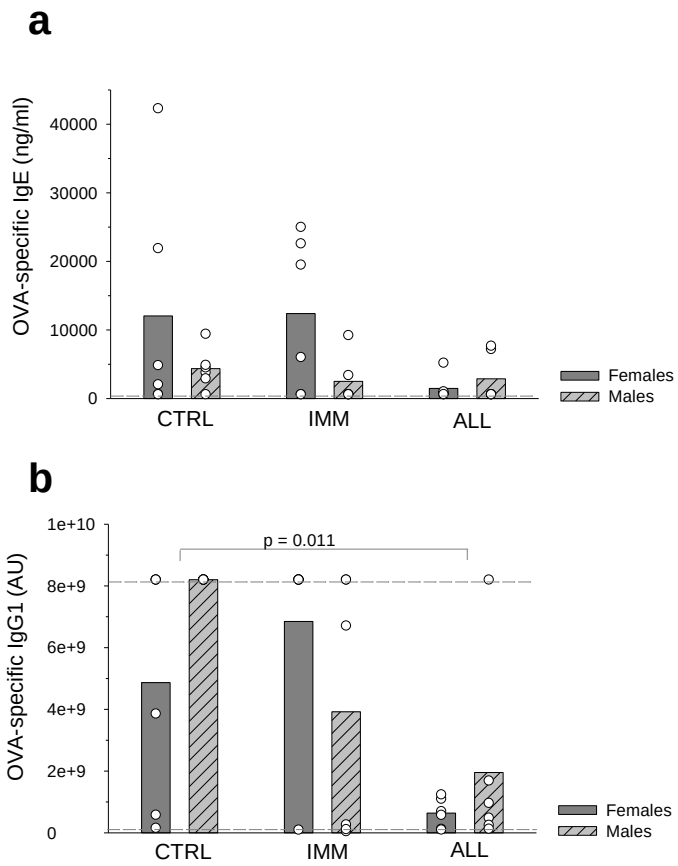

Figure 8: OVA-specific IgE (a) and IgG1 (b) responses in 9-week-old offspring
Adolescent offspring from control (CTRL), immunised (IMM) and allergic (ALL) dams were immunised with the allergen ovalbumin (OVA) emulsified in $Al(OH)_3$ at age 6 weeks and levels of OVA-specific IgE and IgG1 were compared at 9 weeks of age. An effect of maternal treatment on OVA-specific IgG1 was found (main effect, $p = 0.014$). The result of the post hoc test is shown on the figure.

Analysis of OVA-specific IgE in sera collected three days after booster immunisation of adolescent offspring suggested that there was a significant overall difference between the three maternal treatment groups and that the effect of maternal treatment were equivalent for both males and females. Offspring from allergic dams had significant lower OVA-specific IgE values than offspring from control dams. Also, females responded with significantly higher values than males at this time point (Figure 9a).

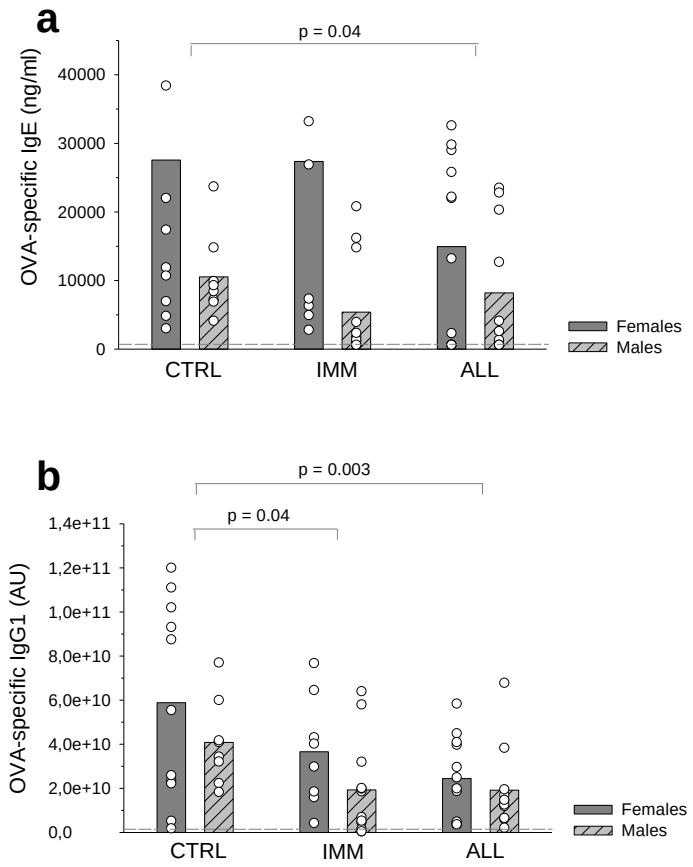

Figure 9: OVA-specific IgE (a) and IgG1 (b) responses in 10-week-old offspring
Adolescent offspring from control (CTRL), immunised (IMM) and allergic (ALL) dams were immunised with the allergen ovalbumin (OVA) emulsified in $Al(OH)_3$ at age 6 weeks and booster immunised with OVA at 9 weeks. Levels of OVA-specific IgE and IgG1 were compared at 10 weeks of age. An effect of maternal treatment on both OVA-specific IgE and IgG1 was found (main effect, $p < 0.05$) and also an effect of sex on OVA-specific IgE (main effect, $p = 0.004$) with females significantly more affected by maternal treatment than males. The result of the post hoc test for maternal treatment is shown on the figure.

With respect to OVA-specific IgG1 in 10 week old offspring, the effects of maternal treatment were comparable for males and females. Offspring from immunised and allergic had significant lower OVA-specific IgG1 values compared to offspring from control dams. Further the results suggested that males had lower OVA-specific IgG1values compared to females, although the result was of borderline statistical significance (main effect, $p = 0.05$) (Figure 9b).

5.4 Ex-vivo quantification of cell proliferation

Cell proliferation (given as Stimulation Index, SI) was calculated for lymph nodes cells cultured *ex vivo* in OVA- and ConA-enriched media, and SI was compared for both juvenile (5-week-old) and adolescent (10-week-old) offspring born to allergic, immunised and control dams. The results of all analyses indicated that there were no significant interactions between sex and maternal treatment for either outcome at either of the time periods. Maternal treatment and sex did not affect OVA-stimulated cell proliferation in juvenile offspring (Figure 10a). In adolescents, however, offspring from immunised dams showed a significantly higher cell proliferation following OVA-stimulation compared to offspring from allergic dams. No significant effects of maternal treatment or sex were observed in cells stimulated with ConA (Figure 10b).

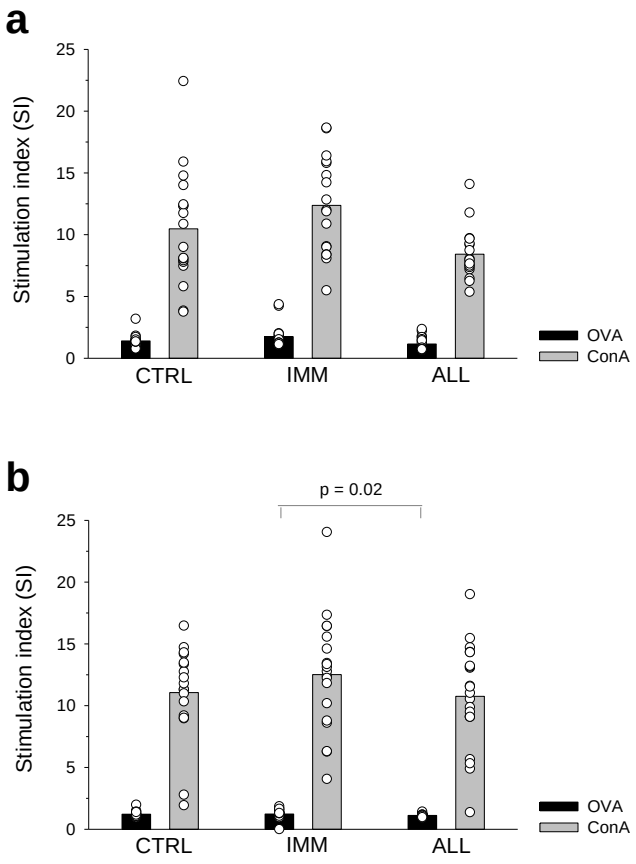

Figure 10: Cell proliferation in 5- (a) and 10-week-old offspring (b)
Ovalbumin (OVA) and Concanavalin A (ConA) stimulated lymph node cells were harvested and cell proliferation (SI) was compared for 5- and 10-week-old offspring born to control (CTRL), immunised (IMM) and allergic dams (ALL). There was a significant effect of maternal treatment in 10-week-old offspring (main effect, $p = 0.03$). The result of the post hoc test for maternal treatment is shown on the figure.

6 DISCUSSION

The presented study suggested a role for maternal IgG1 in suppressing allergy responses in offspring independent of the maternal immune response. The study was, however, not able to show that different pathways for transfer of allergy protection and risk coexist.

6.1 Immunised and allergic dams transfer protection of allergy

It was hypothesized that if maternal OVA-specific IgG1 caused the suppressive effect on OVA-specific IgE responses, then IgE would be suppressed in offspring from both immunised and allergic dams. Several animal experimental studies have suggested that maternal allergen-specific IgG1 transferred via placenta and breast milk is mediating the protective effect in offspring immunised with the same allergen as the dam (Fusaro et al., 2002; Fusaro et al., 2007; Melkild et al., 2002), although the mechanisms are not fully understood. Uthoff et al. (2003) demonstrated that allergen-specific IgG1 transferred via the placenta indeed was responsible for the suppressed allergen-specific IgE responses observed in sensitised young offspring. The protective effect of maternal IgG1 was also investigated by Fusaro et al. (2002) showing both placental transfer of allergen-specific IgG1 to the foetus, and maternally transferred IgG1 in milk collected from the stomachs of 5-day-old offspring from immunised dams. Injection into dams of allergen-specific IgG1 without any further immunisation also suppressed IgE responses in offspring after allergen-immunisation (Seeger et al., 1998; Uthoff et al., 2003). The suppression of OVA-specific IgE in offspring from both immunised and allergic dams supports the first hypothesis that maternal allergen-specific IgG1 may cause this suppression.

Siegrist (2003) have evaluated the mechanisms by which maternal antibodies influence the infant vaccine responses in humans. It is proposed that epitope masking by maternal antibodies prevents the binding of antigen to the infant B cells and thus hinder the production of allergen-specific antibodies. It is relevant to note that the ratio of maternal antibodies and antigen present will be

decisive, in that an excess of maternal antigen will prevent B cell responses, while an excess of antigen will maintain B cell responses in the offspring due to freely available antigen/epitopes. Thus, it may be speculated that the inverse relationship between maternal OVA-specific IgG1 and offspring OVA-specific IgE observed in the present study may be explained by this mechanism. With age, maternally derived IgG1 will be metabolised and eliminated from the circulation, and equivalent to the mechanism suggested above, we demonstrated a corresponding decline in IgE suppression. This observation supports the second hypothesis of the thesis that the IgE suppression in the offspring wanes with decreasing levels of maternal IgG1.

Interestingly, allergic dams transferred allergy protection to an even greater extent than did immunised mothers. Allergic dams responded to the treatment with higher IgG1 levels than immunised mothers both during lactation and after weaning. In line with this, higher levels of OVA-specific IgG1 were detected in unimmunised offspring from allergic compared to immunised dams. It is plausible that offspring from allergic dams received more maternal IgG1 than those from immunised dams, which facilitated the protective effect seen in adolescent offspring from allergic, but not immunised dams. This confirms the role of maternal IgG1 to cause allergy protection, and further suggests an inverse relationship between the quantity of maternally derived IgG1 and the produced IgE in the offspring.

In mouse models investigating mechanisms behind transfer of allergy protection from dam to offspring, a role for other mediators than maternal antibodies have been shown. INFγ (Polte and Hansen, 2008) and TGFb (Verhasselt et al., 2008) were shown to influence offspring T cell responses and to be crucial for mediating allergy protection. In the present study, the maternal treatments had no consistent effects on *ex vivo* cell proliferation in immunised offspring as suggested in the third hypothesis. Only 10-week-old offspring from immunised mothers had increased cell proliferation compared to those from allergic mothers. This suggests that maternal mediators transferred to offspring did not affect allergen-specific T cell proliferation. Recently, it was shown that the inhibitory FcγRIIb receptor was up regulated on splenic B cells in neonates from immunised dams (Victor et al., 2010). Thus, other mechanisms involving offspring B cell responses should be taken into consideration.

Taken together, the present study suggests that maternal allergen-specific IgG1 has a profound influence on the observed IgE-suppression in offspring immunised with the same allergen.

6.2 Co-existing pathways of allergy risk and allergy protection

We induced airway inflammation and a strong antibody response in dams to investigate the influence of maternal airway allergy on allergy responses in the offspring. Comparing allergy responses in terms of allergen-specific IgE and cell proliferation in offspring from allergic and immunised dams (identical immunisation protocol but without the airway challenge), we aimed to demonstrate that an increased allergy risk in offspring from allergic dams was explained by their strong antibody response and/or inflammation. However, with the used mouse model, exacerbated allergy responses could not be demonstrated in offspring of allergic dams as proposed by the fourth hypothesis.

In line with human data showing that children born to allergic parents are predisposed to develop allergies (Kurukulaaratchy et al., 2005; Lim et al., 2010), previous studies in mouse models have demonstrated that allergic dams transfer an allergy risk to their offspring in terms of increased levels of IgE and more severe airway inflammation and hyperreactivity than offspring born to naive dams (Hamada et al., 2003; Lim et al., 2007). In contrast, studies by Uthoff et al. (2003) and Matson et al. (2009) demonstrated attenuated allergic responses in offspring from allergic dams. These discrepancies may be explained by different treatments of the dams and of the offspring as well as by the offspring being immunised at different ages. The present study was designed to facilitate a demonstration of both conditions in the offspring. In the fifth hypothesis, it was suggested that a co-existing transfer of allergy risk will be seen in offspring first when maternal IgG1 disappears. Therefore, the offspring were immunised at two different time points. There is already some evidence that maternal IgG1 is responsible for the observed allergy protection, and as argued earlier, the inhibition of B-cell responses caused by maternal antibodies (Siegrist, 2003) may explain the prolonged protection in adolescent offspring from allergic dams (with an excess of maternal IgG1) compared to offspring from immunised dams. However, comparing IgE levels in offspring from control dams and allergic dams, it becomes evident that the IgE is less suppressed in adolescent than

in juvenile offspring. This indicates that the protection transferred to the offspring diminishes with age, but it was impossible to show an increased IgE response at any of the investigated time points. Therefore, to be able to show an increased risk of allergy, it is possible that the offspring should have been immunised when maternal antibodies were no longer detectable.

As discussed by Siegrist (2003), T cell responses in children immunised under the "umbrella" of maternal antibodies may be independent of the children's antibody responses. This may be due to an increased presentation of antigen in complex with maternal antibodies by antigen-presenting cells such as dendritic cells. If this was the case or if maternal mediators had exerted any "educational" effects on T cells in offspring as demonstrated by Polte et al. (2008) and Verhasselt et al. (2008), an increased cell proliferation could have been observed in offspring of allergic dams. However, in reference to the sixth hypothesis, the maternal treatments had no consistent effects on *ex vivo* cell proliferation in offspring from allergic dams.

This study was not able to demonstrate an increased allergy risk neither on antibody nor on proliferative responses in offspring of allergic dams, and thus, a co-existing transfer of both allergy protection and risk. There was evidence that the protective effect from allergic dams attenuated with age.

6.3 Sex differences

Since the prevalence of allergic diseases in humans differs between boys and girls and in women and men (Govaere et al., 2007; Postma, 2007), it is relevant to evaluate the influence of sex in the presented mouse model. Overall, the analyses indicated that males responded with less IgE than females. Because of the higher antibody production in females, the maternal treatment had superior impact on juvenile females and imposed an even greater suppression of IgE compared to juvenile males. With age, the effect of maternal treatment was less pronounced and this may explain why different IgE responses due to maternal treatments were absent in adolescent males and females. The fact that adolescent females responded with more IgE than males is in consistence with other

studies. One study showed that female adolescent mice (aged 8-10 weeks) developed a more pronounced allergy than male mice after OVA challenge (Melgert et al., 2005). Further, Ma et al. (2008) demonstrated a sex specific modulation in 12-week-old offspring, where females responded with more robust delayed-type hypersensitivity responses than males.

6.4 Methodological considerations

In line with several previous studies, the presented work provided indirect evidence of the role for maternal antibodies for allergy protection. However, it should be noted that other methods might have offered further evidence. If maternal IgG1 is the key mediator for the allergen-specific IgE suppression, then control dams could be injected with OVA-specific monoclonal IgG1. Their offspring should then show signs of OVA-specific IgE suppression after immunization with OVA. This has been demonstrated previously by Uthoff et al. (2003). Also the role of maternal IgG1 could have been investigated in FcRn-deficient mice which cannot transfer IgG1 to their offspring via placenta or where the offspring cannot take up maternal IgG via the gut. Within the limitations of this thesis, it was not possible to perform any further mechanistic studies.

Older offspring had less IgE suppression than younger offspring in accordance with the second hypothesis. However, in older offspring from allergic dams, the level of IgG1 is still high enough to maintain the protective effect. Maternal allergen-specific IgG1 has been detected in offspring up to 9 weeks old in a study by Victor et al. (2003), but not in 13 to 20-week-old unimmunised offspring in the mouse model used here (Hansen, unpublished data). It may be speculated that if other mediators causes allergy protection in offspring, it will not be apparent until the maternally derived IgG1 is eliminated.

For several reasons, we cannot be certain that our method is optimal for identifying a co-existing pathway of increased allergy risk. As discussed, it is possible that an increased allergy risk cannot be demonstrated as long as maternal antibodies are present. Further, one could argue that the maternal airway challenge was administrated too late for having an optimal effect on the offspring. The airway

challenge was administrated just before delivery in comparison to several other studies (Matson et al., 2009; Uthoff et al., 2003) where dams were immunised prior to conception and airway challenged during gestation. Also, the offspring immunisation induces a robust antibody response, which may be difficult to increase any further in the 10-week-old offspring from allergic dams.

In general, it is possible that T cell proliferation is not an optimal measurement of T cell responses. An optimal protocol for measuring proliferation in spleen cells from intraperitoneally immunised mice was already established in the lab and the four days of *ex vivo* stimulation was based on this. In the present study, draining lymph nodes from intranasally immunised mice were used. These differences may cause different kinetics following *ex vivo* stimulation and it is possible that proliferation should have been measured at another time point to give optimal responses. Also, release of Th1, Th2 or regulatory cytokines following *ex vivo* stimulation is another functional response of the T cells that may give more differentiated data.

The immunisation of the offspring was performed solely by airway exposure. This is in contrast to common murine airway allergy models, where mice are immunised by intraperitoneal injection and booster-immunised via the airways (Kumar et al., 2008). This provides a more relevant model for human airway allergy, when effects of the maternal immune system on allergy development are investigated.

6.5 Further studies

IgG2a in mice have been associated with reduced levels of IgE in previous studies (Melkild et al., 2002) and for publication purposes of this study OVA-specific IgG2a will be analysed from the same blood samples. Also, cytokine release from OVA-stimulated mediastinal lymph node cells will be investigated as well as influx of inflammatory cells in bronchoalveolar lavage fluid to evaluate if maternal IgG1 modulate protection also relevant for clinical responses. The protective effect observed was declining with age and additional experiments will be conducted to evaluate the

potential of long-lasting allergy protection in adult offspring (> 20 weeks) when allergen-specific maternal antibodies are no longer present.

6.6 Public health perspectives

Although is tempting to speculate that allergies can be prevented prenatally, there are several aspects that must be taken into considerations. First, these findings cannot be extrapolated directly to humans. The immunoglobulin G has evolved differently in mice and humans and may have different functions (Callard and Turner, 1990; Mestas and Hughes, 2004). IgG is mainly transferred via the placenta in humans and to a greater extend via breast milk in mice. Further, IgG1 in mice has been demonstrated to possess anaphylactic properties (Finkelman et al., 2005). Inbreed mice are more genetically similar and will respond more homogenous than would be expected in a human population. These factors needs to be further evaluated in the view of the findings of the presented study.

Still, animal studies may have valuable contributions. It has been estimated that 50 % of the European population will suffer from allergic diseases within few years, and there are several unanswered questions with regard to why some people develops allergies and others do not. Optimal mouse models for specific disease traits are considered useful for understanding mechanisms behind and optimal time points for therapeutic interventions (Kips et al., 2003). Immunological events *in utero* and early life is thought to be of particular importance, as the mechanisms behind allergy transfer still are poorly understood. Animal models provide an opportunity to explore these mechanisms that for ethical reasons are not possible to investigate in humans. From this study, there are good indications that maternal mediators facilitate beneficial conditions for allergy suppression, and this potential should be explored further for future allergy prevention strategies in children.

7 CONCLUSIONS

The present study demonstrates a potential to prevent allergy in offspring with allergen immunisation of the mother. It was not possible to demonstrate an increased risk of allergy in terms of increased IgE or proliferative responses in offspring from allergic dams.

The study demonstrates that immunised and allergic dams transfer allergy protection to their offspring in terms of reduced allergen-specific IgE production, when the offspring are immunised with the same allergen as their mothers. However, the protective effect diminishes with age. This coincides with declining levels of maternal IgG1, which is transferred from the mother to the offspring via the placenta and breast milk and gradually declines after weaning. Further, maternal IgG1 is transferred both to offspring of immunised and allergic dams, but is found at much higher levels in offspring from allergic dams, where the protective effect remains also in adolescent. Overall, this suggests that maternal IgG1 has a direct role on IgE suppression and, thus, allergy protection in offspring. We were not able to detect an increased or decreased cell proliferation that could have explained protection mediated by activity of T-cells, and the limited extent of this thesis did not allow further investigation of cells and cytokines.

In conformity with several other studies, maternal allergen specific IgG1 is suggested as the most likely mediator of the observed allergy protection in the offspring. However, it cannot be excluded that the allergy protection could be mediated by other maternal factors not measured in this study. The work presented indicates that maternal mediators may facilitate a beneficial condition for allergy prevention, and suggests that more attention should be given to prenatal and early age prevention strategies when the immune system is under the influence of maternal mediators. It is important to note that the results must be carefully interpreted and cannot be directly generalised from mice to humans.

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9 APPENDIX

I. ELISA - Specific mouse IgE against ovalbumin

AVD. FOR MILJØIMMUNOLOGI

Prosedyre nr.: 1 Rev. nr.: 6 Rev. dato: 30.10.08 Ant sider: 4	**Spesifikt mus IgE mot ovalbumin ELISA**	Utarbeidet av: PIG Dato: 24.08.94 Endret dato:27.01.10
Ansvarlig ingeniør: Else-Carin Groeng Ansvarlig forsker: Berit Granum		

INNLEDNING

Assay for kvantitativ bestemmelse av spesifikt IgE mot ovalbumin i musesera.

Capture ELISA med monoklonalt rotte anti-mus IgE som primært antistoff (coat) og et biotin-merket ovalbumin som detektor. Fremkalling med Poly-HRP-Streptavidin.

Hele oppsettet som beskrevet nedenfor går over 3 dager, men kan utføres på to dager.

METODE

1. Alle brønner på platen tilsettes 100 µl monoklonalt rotte anti-mus IgE med en konsentrasjon på 2 µg/ml (*intern nr. 298 H*, 1 mg/ml) i 0,05 M karbonat/bikarbonatbuffer pH 9,6.

   Plate, lotnr.: ……..

   Buffer, lotnr.: …….

   Hver plate forsegles med tape. La stå i 1 time på benk ved 21°C (flowrommet), deretter over natten ved $+4^0$C.
2. Vask platene x5 med Tris/Tw-8. Bruk vaskeprogram A+5*W (se forøvrig bruksanvisning på vaskeren). Deretter dunkes platene "tørre" mot cellestoff.
3. Blokkering:

4. 3% skummet melk i PBS/ 0,05 % $Tw_{20.}$ 300 µl/brønn. La stå 1 time på benk ved 21°C (flowrommet)
5. *Standard:*
   Brønnene A2-3 til H2-3 tilsettes i duplikat 100 µl fortynnet mus anti-ovalbumin renset IgE fra Serotec, batch nr 0707, MCA 2259, 1mg/ml (*internnr.455*) fortynnet 2-fold fra 3000 ng/ml til 23 ng/ml i 4% BSA/PBS (jmf.templat). NB: Start konsentrasjon er avhengig av batch nr. og må testetes ut for hver batch.
6. *Ukjente sera:*
   Fortynnes i 4% BSA/PBS, f.eks 1:10, men eget titreringsforsøk med utvalgte sera kan settes opp for bedre å avgjøre egnet fortynning. Prøvene settes opp i duplikat. Tilsettes 100 µl pr. brønn.
7. Brønnene A1-D1 tilsettes alle reagenser utenom serum. Vi tilsetter fortynningsbufferen (4% BSA/PBS) istedenfor serum.
8. *Positiv kontroll:*
   Brønnene E1 og F1 tilsettes 100 µl positiv kontroll (imm OA 3 fra 22.12-03) fortynnet 1:1600 i 4 % BSA/PBS. Denne fortynningen skal være den samme i alle oppsett.

   Negativ kontroll:
   Brønnene G1 og H1 tilsettes 100 µl negativ kontroll (velges etter musestamme i forsøket) fortynnet med samme fortynning som de ukjente prøvene i 4 % BSA/PBS
9. Hver plate forsegles med tape. La platene stå i 1 time på ristemaskin ved 21°C (flowrommet), evt. deretter over natten ved +4 °C.
10. Vask platene x5 med Tris/Tw-8. Bruk vaskeprogram A+5*W (se forøvrig bruksanvisning på vaskeren). Deretter dunkes platene "tørre" mot cellestoff.
11. Hver brønn tilsettes 100 µl biotin-merket ovalbumin (*internnr. B-OA 22.05.08)* bruksfortynning 3 µg/ml i Tris/ Tw-8. NB. (B-OA 30.10.08) bruksfortynning 2 µg/ml.
12. Hver plate forsegles med tape. La platene stå i 1 time på ristemaskin ved 21°C (flowrommet).
13. Vask platene x5 med Tris/Tw-8 Deretter dunkes platene "tørre" mot cellestoff.
14. Poly-HRP-streptavidin:
    Fortynnes 1: 40 000 i 4% BSA/PBS. 100µl i hver brønn .
    Hver plate forsegles med tape. La platene stå i 1 time på ristemaskin ved 21°C (flowrommet).
15. Vask platene x5 med Tris/Tw-8. Bruk vaskeprogram A+5*W (se forøvrig bruksanvisning på vaskeren). Deretter dunkes platene "tørre" mot cellestoff.
16. Hver brønn tilsettes 100 µl TBM løsning (ferdig til bruk).
    Platene inkuberes ved romtemperatur[1] i 15 min.

[1] Ved spesielt lave eller høye temperaturer på lab'en, bruk flowrommet også her.

17. Stoppløsning- 2N H_2SO_4 Tilsett 50µl i hver brønn
18. Platene avleses i Dynatech plateavleser ved 450nm. Program:
    f:/Revelation/Assay/OVA/spesIgE mot OVA HRP.asy
    Datafilene lagres automatisk på F, men skal overføres til den enkeltes hjemmeområde på nettet.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std.1 3000ng/ml		T1		T9		T17		T25		T33
B		Std.2 1500		T2		T10		T18		T26		
C		Std.3 750		T3		T11		T19		T27		T34
D		Std.4 375		T4		T12		T20		T28		
E	Pos kontr	Std.5 187		T5		T13		T21		T29		T35
F		Std.6 94		T6		T14		T22		T30		
G	Neg.ktr.	Std.7 47		T7		T15		T23		T31		T36
H		Std.8 23		T8		T16		T24		T32		

UTSTYR/FORBRUKSVARER/REAGENSER

- Skan Washer 400 Platevasker, Skatron
- Dynatech Laboratories MRX Plateleser, revelation(Programvare), Pedersen & Sønn
- Pipetter: 0,5-10 µl, 100 µl, 200-1000 µl, multipipette

FORBRUKSVARER

- Maxisorp, Nunc-Immuno Plate (Kat. nr. 439454, VWR)

KJEMIKALIER

- 0,05 M karbonat/bikarbonat-buffer pH 9,6, BABG
- Blokkeringsløsning: Skim Milk Powder (FLUKA)
- 50 mM Tris/HCL-buffer pH 8.0 (ved 21^0C) med 0,05% Tween 20 (=Tris/Tw-8)
- PBS med 4% BSA (Sigma kat.nr.A7030-100G)
- Poly-HRP-streptavidin. (Thermo Scientific Kat. nr. N200) Diagen AS
- TBM (Biosource kat.nr. SB02) Invitrogen.
- Stoppløsning: 2N H_2SO_4

ANTISTOFFER

- Monoklonalt rotte anti-mus IgE (klone LO-ME-3), Experimental Immunology Unit, University of Louvain, Belgia, fax nr +32 27 64 95 20
- Mus anti-ovalbumin renset IgE, MCA 2259 1mg/ml (batch 0707 fra Serotec)
- Positiv kontroll: Serum med høyt titer av IgE anti-ovalbumin fra den musestammen som analyseres. Vi bruker Serumpool fra BALB/c mus immunisert med ovalbumin, Imm OA-3 fort.1:1600
- Negativ kontroll: Normal serum fra den musestammen som analyseres

ANDRE REAGENSER

- Biotinylert ovalbumin 500 μg/ml (Se egen protokoll)

JOURNALFØRING OG BEARBEIDELSE AV DATA

- Oppsett og data noteres i lab-journal.
- Utskriftene fra ELISA-avleseren arkiveres.
- Filer fra ELISA-avleseren lagres automatisk på f, men skal overføres til den enkeltes hjemmeområde på nettet for å sikre back-up av data.

II. ELISA- Specific mouse IgG1 against Ovalbumin

AVD. FOR MILJØIMMUNOLOGI

Prosedyre nr.: Rev. nr.: 7 Rev. dato:16.02.2010 Ant sider: 3	**Spesifikt mus IgG1 mot ovalbumin ELISA**	Utarbeidet av: Linda/Unni/Berit Dato: Endret dato:
Ansvarlig ingeniør: Else-Carin Groeng Ansvarlig forsker: Unni C. Nygaard		

INNLEDNING

Assay for kvantitativ bestemmelse av spesifikt IgG1 mot ovalbumin i musesera.

Capture ELISA med monoklonalt rotte anti-mus IgG1 som primært antistoff (coat) og et HRP-merket ovalbumin som detektor. Fremkalling med Substratløsning, Stabilized Chromogen , TMB fra Biosource kat.nr SB02-1.

Hele oppsettet som beskrevet nedenfor går over 3 dager, men oppsettet kan utføres på to dager ved å inkubere med sera og sekundært antistoff i 1-2 timer ved 21°C.

METODE

NB! Alle buffere skal ha romtemperatur ved tilsetting.

1. Plater **(Maxisorb)** coates med rotte anti-mus IgG1 (internnr.380, 1 mg/ml) fortynnet til 2 µg/ml i 0,05 M karbonat/bikarbonatbuffer pH 9,6. Tilsett 100 µl til alle brønnene. Hver plate forsegles med tape. La stå 1 time på benk ved 21°C (flowrommet), deretter over natten ved 4°C.

2. Vask platene x5 med vaskebuffer (Tris/Tw8). Bruk vaskeprogram A+5W. Dunk platene tørre mot cellestoff.

3. Se templat nedenfor
   - *Ukjente sera:* Tilsettes i duplikat, 100 µl pr. brønn, fortynnet i fortynningsbuffer (1% BSA/PBS). Eget titreringsforsøk med utvalgte sera bør settes opp for å avgjøre egnet fortynning.
   - *Standard*: Tilsettes i duplikat 100 µl fortynnet ImmOA3, **8** punkter, fortynnet **4**-fold i fortynningsbuffer (1% BSA/PBS) fra 1: 10 000 til 1: 163,84 x 10^6
   - *Blank:* Brønnene A1-D1 tilsettes alle reagenser utenom serum. Fortynningsbufferen (1% BSA/PBS) tilsettes istedenfor serum.

- *Negativ kontroll:* Brønnene G1 og H1 tilsettes 100 µl negativ kontroll (velges etter musestamme i forsøket) fortynnet med samme fortynning som de ukjente prøvene i fortynningsbuffer (1% BSA/PBS).
- *Positiv kontroll:* Brønnene E1 og F1 tilsettes 100 µl positiv kontroll (099, internnr.453) fortynnet til 25 ng/ml i fortynningsbuffer (1% BSA/PBS). Denne fortynningen skal være den samme i alle oppsett.
- Hver plate forsegles med tape. Inkuber i 2 timer på ristemaskin ved 21°C (flowrommet)

4. Vask platene x5 med vaskebuffer (Tris/Tw8). Bruk vaskeprogram A+5W. Dunk platene tørre mot cellestoff.

5. HRP-konjugert OVA (internnr.469) fortynnes 1:300 (**batch 309 NB: Må prøves ut ved ny batch)** i Tris/Tw (”vaskebuffer”). Tilsett 100 µl til hver brønn.
   Hver plate forsegles med tape. Inkuber i 2 timer på ristemaskin ved 21°C.

6. Vask platene x5 med vaskebuiffer (Tris/Tw8). Bruk vaskeprogram A+5W. Dunk platene tørre mot cellestoff.

7. Hver brønn tilsettes 100 µl substratløsning (**Stabilized Chromogen ,TMB fra Biosource kat. nr. SB02-1)**. Ferdig til bruk. Hver plate forsegles med tape. La platene stå mørkt i 15 minutter ved romtemperatur.

8. STOPP-LØSNING
   Hver brønn tilsettes 50 µl stoppløsning: 2 N H_2SO_4 ”Bland ved å banke forsiktig på platen”.

   NB: Må leses av innen 10 minutter.

9. Platene avleses i Dynatech plateavleser, ved bølgelende **450nm.** Program: c:/Revelation/assay/ spesIgG1 mot OA_HRP.asy
   Datafilene lagres automatisk på C, men skal overføres til den enkeltes hjemmeområde på nettet.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B l a n k	Std.1 1:10 000										
B		Std.2 1:40 000										
C		Std.3 1:160 000										
D		Std.4 1:640 000										
E	Pos. ktr. 25 ng/ml	Std.5 1:2560 000										
F		Std.6 1:10 240 000										
G	Neg. Ktr	Std.7 1:40 960 000										
H		Std.8 1:163840000										

UTSTYR/FORBRUKSVARER/REAGENSER

UTSTYR

- Skan Washer
- Dynateck Laboratories MRX Plateleser
- Pipetter; 0,5-10 µl, 100 µl, 200-1000 µl, 1-5 ml

FORBRUKSVARER

- Maxisorb mikrotiterplater (kat.nr. 439454)

KJEMIKALIER

NB! HRP påvirkes av azid, derfor brukes BSA i pulverform og vaskebuffer uten azid!

- 0,05 M karbonat/bikarbonat-buffer pH 9,6 (ved 21 °C), BABG

- PBS
- Vaskebuffer: 0.05% Tween 20 i Tris/HCL, 50 mM, pH 8.0 = Tris/Tw 8 i beskrevet metode.
- Albumin, bovine, Sigma A-7030 PULVER TIL OPPVEIING
- Fortynningsbuffer: 1% BSA i PBS, pH 7.2-7.4, 0.2 µm filtrert
- Substratløsning (Stabilized Chromogen, TMB), Biosource, kat.nr. SB02-1. Ferdig til bruk
- Stopp-løsning 4 N H2SO4 (nødvendig for å få gul farge, og anbefalt av leverandør)

ANTISTOFFER

- Monoklonalt rotte anti-mus IgE (LO-MG1-13 (*internnr. 380*, 1 mg/ml)), Experimental Immunology Unit, University of Louvain, Belgia, fax nr +32 27 64 95 20
- Standard: ImmOA3 (serumpool fra Balb/c mus immunisert med ovalbumin og Al(OH)3)
- Positiv kontroll: Mus anti-ovalbumin (chicken, nativt, subkl IgG1) (HYB 099-01),1 mg/ml (*internnr. 453*)
- Negativ kontroll: Normal serum fra den musestammen som analyseres

ANDRE REAGENSER

- HRP-konjugert OVA (AbDSerotec, kat.no. BUF048), internnr.469 . NB må prøves ut ved ny batch.

JOURNALFØRING OG BEARBEIDELSE AV DATA

- Oppsett og data noteres i lab-journal.
- Utskriftene fra ELISA-avleseren arkiveres.
- Filer fra ELISA-avleseren lagres automatisk på c, men skal overføres til den enkeltes hjemmeområde på nettet for å sikre back-up av data.

III. Lymph nodes in mice: Collection, preparation and *ex vivo* stimulation in culture

AVD. FOR MILJØIMMUNOLOGI

Prosedyre nr.: 44 Rev. nr.: 1 Rev. dato: Ant sider: 6	**Uttak, preparering og *ex vivo*-stimulering i kultur av lymfeknuter - mus**	Utarbeidet av: JIHA, UCNY Dato: 09.11.09
Ansvarlig ingeniør: Ansvarlig forsker: UCNY		

INNLEDNING

Uttak og stimulering *ex vivo* av celler fra lymfeknuter og milter fra mus brukes ofte i allergimodeller, hvor en allergen-spesifikk reaksjon ønskes underbygget mhp. cytokinproduksjonen i drenerende lymfeknuter eller i milt. Celler fra lymfoide organer prepareres til enkel-cellesuspensjon. Cellene kan stimuleres i kultur med det relevante allergen, mitogener eller CD3/CD28-coatede kuler. Ofte vil cytokiner som IL-4, IL-5, IL-13, IFN-γ og IL-10 analyseres i cellekultursupernatanten som et uttrykk for om immunresponsen er TH2, TH1 eller muligvis regulatorisk. Celleproliferasjon kan måles vha. BrdU-inkorporering (se egen SOP) og ulike celletyper fra organene kan også bestemmes vha. flowcytometrisk analyse av ekspresjonen av overflatemarkører (se egen SOP).

Denne SOP'en beskriver uttak av lymfeknutene superficial cervical (SLN), deep cervical (DLN), mediastinale (MLN), tracheobronchiale (TBLN) og popliteale (PLN). Uttak av milt er beskrevet i egen SOP. Deretter beskrives preparering av vevet til enkeltcellesuspensjon ved hjelp av enzymatisk eller mekanisk disaggregering. Som et eksempel beskrives *ex vivo*-stimulering av cellene med ovalbumin (OVA) som modellallergen. Andre konsentrasjoner og stimuleringer kan være aktuelle, dette må avgjøres for hvert enkelt forsøk

Fordelen ved å preparere enkeltcellesuspensjon fra lymfeknuter (LNs) vha den enzymatiske i forhold til den mekaniske metoden er et bedre utbytte av celler, inkludert dendrittiske celler (DC) og makrofager i tillegg til lymfocytter. Hvis en spesifikt ønsker å studere DC'er brukes EDTA for å hindre DC'er og T-celler i å aggregere igjen (Vremec and Shortman 1997, de Heer *et. al.* 2004) – ikke inkludert her. EDTA hemmer dessuten også DNase- og collagenase-aktiviteten.

For en oversikt over plasseringen av de ulike lymfeknuter henvises til følgende gode oversikt:

van Den Broeck, W., Derore, A., and Simoens, P. (2006). Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice. *J Immunol Methods* **312,** 12-19.

METODE

- Forsikre deg om at musen er ordentlig bedøvet eller avlivet ved å klype den i tærne med en klopinsett og se at den ikke reagerer.
- Skinnet på halsen trekkes forsiktig til side, så de to spyttkjertlene kommer til syne. SLNs er plassert som perler oven på spyttkjertlene og på rad ut mot ryggen. Nappes ut ved hjelp av spiss pinsett med bøy.
- DLNs finnes ved å dra til side spyttkjertlene. På hver side av trachea krysser to halsmuskler. DLNs finnes rett i krysset mellom musklene og trachea. Nappes ut ved hjelp av spiss pinsett med bøy.
- MLNs finnes på (din) venstre side (musens højre) av thymus. Lymfeknutene kan være vanskelig å skjelne fra thymus-vevet, men beholder kule-formen når de tas ut. Thymusvev er mere som fettvev. Vi finner vanligvis to LNs på den siden av thymus og en LN på den motsatte siden. Nappes ut ved hjelp av spiss pinsett med bøy.
- TBLN finnes ved å legge hjertet over på (din) venstre side. LN vil da ligge i "gropa" ("krysset") mellom lunge-lobene, en knapp centimeter lenger ned enn thymus.
- PLN finnes i knehasen til dyret. Med en liten skarp saks klippes et snitt i huden "ved akilles", og huden trekkes oppover benet med en klopinsett. Ved å "knekke" kneet fremover vil PLN "stikke seg frem". Taes ut ved hjelp av liten saks eller pinsett med bøy. (Ved uttak av PLN kan tomtapping gjøres etter normal prosedyre uten å åpne dyret).
- Lymfeknutene plasseres i 5 mL meineckerrør med 1 mL hentebuffer på is.
- Hold rørene på is inntil enkeltcellesuspensjon er oppnådd.

Enzymatisk preparering av enkelcellesuspensjon (LNs):

- Alt må gjøres i sterilbenk, hvis LN-cellene skal brukes til kultur.
- LNs overføres til små petriskåler/brønner i 24-brønns-brett og deles med skalpellblad eller kanyler.
- Tilsett 1 mL digestion medium etter preparering av hver enkelt LN for å unngå uttørking.
- Sett i CO_2-inkubator ved 37 °C i 30 min.
- Etter 15 minutter kan tilsettes ytterligere 0,5 mL digestion medium og løsningen kan pipetteres for å oppløse klumper. Inkuberes da ytterligere 15 min. eller til vevet er oppløst.
- Etter inkubasjon pipetteres celleløsningen opp og ned i en 1 mL-sprøyte for å ødelegge klumper. Filtrer cellene gjennom en 70 μm-sil ned i et 50 mL sentrifugerør på is. Brønnen skylles med 1 ml hentebuffer, som også overføres til silen. Skyll med ca. 10 mL iskald hentebuffer. Hold på is resten av tiden.

Mekanisk preparering av enkelcelle-suspensjon (LNs)

- Alt må gjøres i sterilbenk, hvis cellene brukes til kultur

- LNs fra hvert rør helles over i 70 μm sil (cell strainer) i 50 mL sentrifugerør, hvoretter mediet bortkastes. Lymfeknutene presses gjennom silen vha stemplet fra en 2 mL-sprøyte og det skylles med ca. 10 mL iskald hentebuffer for å oppnå enkeltcellesuspensjon. Hold på is resten av tiden.

Telling av celler

- Rørene sentrifugeres ved 1400 rpm, 10-15°C, i 5 min. Supernatanten helles av og cellene resuspenderes i 0,5 mL dyrkningsmedium.
- 20 μL celleløsning tilsettes 10 mL isoton (løsning til telling) i telleglass. Antall celler per ml i utgangsløsningen, med diameter 6-14 μm, bestemmes ved hjelp av Coulter partikkelteller, profil B. Total celletall (konsentrasjonen ganget opp med resuspensjonsvolumet) brukes også til bestemmelse av antallet celler per LN i annen databehandling.

Eksempel på utsåing til *ex vivo*-kultur med OVA stimulering

- Etter telling kan cellene evt. pooles for å oppnå et passende antall celler til utsåing.
- Etter pooling etterfylles rørene evt. med dyrkningsmedium til samme antall mL for å balansere sentrifugen.
- Sentrifuger ved 1400 rpm, 10-15°C, i 5 min.
- Supernatanten helles bort og cellene resuspenderes i dyrkningsmedium, tilsvarende en konsentrasjon på $3 * 10^6$ celler/mL.
- Cellene sås ut med sluttkonsentrasjonen $2,7 * 10^6$celler/mL og det bør brukes 4 brønner (2 ustimulerte brønner og 2 OVA-stimulerte brønner) til ett oppsett. Avhengig av hvor mye supernatant man trenger til videre analyser (se under), kan man sette opp i 24 eller 96-brønners brett.

Utsåing i 24-brønns-brett

- Cellene sås ut i 24-brønns-brett (2 medium og 2 OVA-stimuleringer pr. oppsett), 1 ml pr brønn. En trenger derfor ca. $10,8 * 10^6$ celler pr. oppsett. Hvis det er for lite celler kan det sås ut med halve volumet dvs. 4 x 500 μl per oppsett, eller redusere til en ustimulert og en stimulert brønn per oppsett.
- Tilsett 900 μL cellesuspensjon (NB 450 μL ved 500 μL oppsett!) til brønnene.
- Tilsett 100 μL OVA-løsningsmiddel eller OVA-løsning til brønnene. Her brukes en OVA-løsning på 10.000 μg/mL, hvilket svarer til at cellene stimuleres med 1000 μg/mL. (NB 50 μL ved 500 μL-oppsett!)

Utsåing i 96-brønns brett

- Cellene sås ut i 96-brønns-brett (2 medium og 2 OVA-stimuleringer pr. opsett). En trenger derfor ca. 2,16 * 10^6 celler pr. oppsett
- Tilsett 180 µL cellesuspension til brønnene.
- Tilsett 20 µL OVA-løsningsmiddel eller OVA-løsning til brønnene. Her brukes en OVA-løsning på 10.000 µg/mL, hvilket svarer til at cellene stimuleres med 1000 µg/mL.

Inkubasjon

- Cellene inkuberes i CO_2-inkubator ved 37 ºC.

Høsting av supernatant

- Supernatanten høstes etter 2-5 dager (5 dager vanligst for allergen-stimulering, mens 2-3 dager vanligst for uspesifikke stimuleringer)
- For å unngå kontaminering av supernatanten fra cytokiner i spytt: unngå å snakke, evt. bruk munnbind, ved høstingen. Kan gjøres på benk.
- Spinn ned platene ved 1200 rpm i 5 min ved romtemperatur (18 °C). Supernatanten suges av med pipette eller med multikanalpipette slik at en unngår å ta med celler. Deretter overføres supernatanten til eppendorfrør eller cellekulturplater. Platene dekkes med sealingtape for å unngå fordampning til nabobrønner før lokket settes på.
- Supernatantene fryses ved -80 °C (om cytokiner skal måles)

UTSTYR/FORBRUKSVARER/REAGENSER

UTSTYR

- Celleteller (Coulter Counter Z1) + telleglass
- Sentrifuge (Hettich Rotanta 460R-sentrifuge med 5624-rotor)
- Begerglass til å ha sprit til sterilisering av instrumenter
- Stump saks, spiss saks, pinsetter (anatomisk og kirurgisk), peang, fine bøyde pinsetter
- 37 ºC CO_2-inkubator

FORBRUKSVARER

- Bedøvelse http://labanimals.no/ADFDSop/09SOP-024.pdf
- Is
- Merkede sterile 5 mL meineckerrør
- Evt. 25G kanyler og 1 mL sprøyter til tomtapping fra hjertet
- 50 mL plast sentrifugerør (kat.no. 227261, Greiner bio-one)
- 70 µm sil (nylon cell strainer, kat.no. 352350, BD Falcon)
- Stempler fra 2 mL plastsprøyter
- Pipetter + pipettespisser
- Isoton-løsning for celletellinger
- 24 brønner cellekulturplate (kat.no. 3524, Corning Incorporated)
- 96 brønner cellekulturplate (kat.no. 167008, Nunc, Denmark)
- Sealingtape (kat.no. 236366, Nunc, Denmark)

KJEMIKALIER

- Hentebuffer: HBSS med 2% FCS og 1% pencillin/streptomycin (P/S kan utelates hvis det ikke trenger å være steril preparering)
- Digestion medium (lages samme dag): RPMI 1640 med 5% FCS og 1% pencillin/streptomycin, 1 mg/mL collagenase type 2 og 0,02 mg/mL DNase I
- Collagenase type 2, Katalognr.: LS004174 Worthington Biochemical Corp./Laborel
- DNase I, grade II from bovine pancreas, Katalog nr.:11284932001, Roche Applied Science. Kan rekonstitueres i sterilt dobbeldestillert vann til 10mg/mL og fryses.
- Dyrkningsmedium: RPMI 1640 med 1% pencillin/streptomycin og 10% FCS
- Ovalbuminløsning, 1mg/mL i HBSS, LPS-renset, i -20 ºC fryser

EGNE NOTATER

Vi har vist at collagenasen ikke har trypsin-liknende aktivitet på overflatemarkører. En tre gange høyere collagenase-konsentrasjon hadde ingen effekt på CD4- og CD8-ekspresjonen på thymocytter.

Den enzymatiske disaggregering kan også brukes på lungevev etter tilpassning av metode fra: Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med* 2001; 193:51-60.

Vi har vist at FCS i hentebufferen medfører mindre celledød enn HBSS alene eller HBSS tilsatt BSA.

Ved Cytometric Bead Array (CBA)-analyser av cytokinnivåer i cellekultursupernatant gir 96-brønnene en rikelig mengde supernatant til analysene. Hvis ELISA skal brukes (evt. fordi cytokinet ikke finns i CBA) må 24-brønnsbrett benyttes for å få tilstrekkelig med prøvemateriale

IV. ELISA - Cell proliferation BrdU

AVD. FOR MILJØIMMUNOLOGI

Prosedyre nr.: 39 Rev. nr.: Rev. dato: Ant sider: 4	**Cell Proliferasjon ELISA, BrdU (colorimetric)**	Utarbeidet av: ANPL Dato: 10.05.07 Endret dato: 02.06.09
Ansvarlig ingeniør: Else-Carin Groeng Ansvarlig forsker: Solvor Berntsen		

INNLEDNING

Denne analysen er en kolorimetrisk metode for kvantifisering av celleproliferasjon, basert på måling av pyrimidin analogen BrdU. BrdU inkorporeres i DNA ved at thymidin erstattes av BrdU under DNA-syntese i prolifererende celler. Anti-BrdU-POD vil deretter binde seg til BrdU i det nysyntetiserte DNAet. Dette immunkomplekset detekteres så ved hjelp av en substratreaksjon (fargereaksjon). Reaksjonsproduktet kvantifiseres ved å måle absorbansen ved hjelp av en ELISA-leser. Den målte absorbansen vil korrelere med mengde nysyntetisert DNA. Dette vil gi et relativt mål på mengde prolifererende celler og/eller proliferasjonshastighet.

Selve analysen utføres i løpet av en dag. Før analysen kan gjennomføres må cellene dyrkes.

Antall celler som bør brukes er avhengig av celletype og inkuberingstid. Anbefalt antall celler er 0,1 – 1,0 * 10^4 celler/brønn ved bruk av cellelinjer, eller 1 – 40 * 10^4 celler/brønn ved bruk av primære lymfocytter.

METODE

Blankprøve:
Det skal inkluderes blank i hvert oppsett. Blank gir et mål på uspesifikk binding av BrdU og anti-BrdU-POD til mikroplaten. Absorbansen til blankprøven trekkes fra absorbansen til prøvene. Absorbansen til blankprøven skal ikke overstige 0,1. Blankprøven skal inneholde det samme som cellesuspensjonene, men uten celler/kulturmedium.

Bakgrunnskontroll
Bakgrunnskontroll trenger bare utføres en gang med det respektive cellesystemet. Bakgrunnen gir informasjon om uspesifikk binding av anti-BrdU-POD til cellene uten BrdU presentert i prøven. Absorbanseverdien skal ikke overskride 0,1. Absorbansen kan stige ved økt cellekonsentrasjon. Bakgrunnskontrollen skal inneholde celler og anti-BrdU-POD, og behandles videre som de andre prøvene.

Celleproliferasjonskontroll

Oppsettet bør inneholde en spontan celleproliferasjons kontroll. Denne består av ustimulerte celler og kulturmedium.

- Brønnene i en flatbunnet 96 brønners mikroplate tilsettes 100 µl cellesuspensjon som inneholder ulike løsninger av testsubstanser (eks. mitogener, vekstfaktorer, cytokiner, cytostatic drugs) som inkuberes i 37 ºC i CO_2-atmosfære. For de fleste eksperimentelle oppsett er en inkuberingstid fra 24 til 120 timer tilstrekkelig.

- BrdU labeling solution lages ved at BrdU labeling reagent fortynnes 1:100 med sterilt kulturmedium (slik at sluttkonsentrasjonen blir 100 µM BrdU). Denne løsningen er stabil i flere uker ved 2-8 ºC, for langtidslagring anbefales -15 til -25 ºC. Det anbefales å fortynne alt samtidig og fryse ned i små volumer. For en 96-brønners mikroplate med 100 µl/brønn trengs det 1 ml BrdU labeling solution.

- Dersom cellene dyrkes i 100 µl/brønn, tilsettes 10 µl BrdU labeling solution til alle brønnene unntatt bakgrunnskontrollen (sluttkonsentrasjon: 10 µM). Dersom det benyttes 200 µl/brønn cellesuspensjon må det brukes 20 µl BrdU labeling solution. Miksing gjøres ved å dunke lett på platen. Deretter reinkuberes cellene i 2-24 timer ved 37 ºC i CO_2-atmosfære med lokk. Inkubasjonstiden må tilpasses det aktuelle oppsettet. Normalt er 2 timer tilstrekkelig. Økt inkubasjonstid vil øke mengden inkorporert BrdU i det cellulære DNAet som vil resultere i økt absorbansverdier og økt sensitivitet.

- For å unngå stor variasjon av absorbansverdier, dunk lett på platen etter inkubering.

- Fjerning av labeling solution:
  For ikke-adherente celler sentrifugeres mikroplaten ved 300 g i 10 min. Labeling solution fjernes ved å snu platen raskt på hodet. Deretter dunkes platene "tørre" mot cellestoff.

  Adherente celler trenger ikke å sentrifugeres, platen skal bare snus raskt på hodet.

- Deretter tørkes cellene ved bruk av hårføner i 15 min eller varmeskap på 60 ºC i en time.

- Etter merkingen kan analysen stoppes ved at mikroplatene oppbevares opp til en uke ved 2-8 ºC. Dersom man ønsker å fortsette, gå videre til på punkt 9.

- Deretter tilsettes 200 µl/brønn FixDenat til cellene. Platen inkuberes i 30 min ved 15-25ºC (RT).

- Cellene er nå festet til brønnene. FixDenat løsning fjernes ved å snu platen raskt på hodet og tørk platen ved å dunke den mot cellestoff.

- Anti-BrdU-POD (antistoff konjugat) stock solution lages ved å løse opp Anti-BrdU-POD i 1,1 ml dobbelt destillert H_2O i 10 min ved grundig miksing. Stocken bør fordeles i mindre volum og kan oppbevares over lang tid ved -15 til -25 ºC.

  Anti-BrdU-POD working solution lages ved å fortynne stock 1:100 med antibody dilution solution.

  For 1 plate blandes 110 µl anti-BrdU-POD stock solution (fryser) i 11 ml antibody dilution solution. Hver brønn tilsettes 100 µl med anti-BrdU-POD working solution. Platen inkuberes deretter ved 15-25 ºC (RT) mellom 30 og 120 min.

- Anti-BrdU-POD fjernes ved å snu platen raskt på hodet og tørk platen ved å dunke den mot cellestoff. Platen vaskes deretter tre ganger med 200 µl/brønn Washing solution (10 ml buffer + 90 ml dest.vann). Fjern Washing solution ved å snu platen raskt på hodet og tørk platen ved å dunke den mot cellestoff mellom hver gang. Kan alternativt bruke 300 µl Washing solution.
- Det tilsettes deretter 100 µl/brønn Substrat solution. Inkuberes mørkt ved 15-25 ºC (RT) til tilstrekkelig fargeutvikling i brønnene (5 til 30 min). (Vår erfaring tilsier 30 min.)

Avlesning av absorbansverdier kan gjøres både med og uten stoppløsning.

Uten stoppløsning: Absorbansverdiene måles ved hjelp av en ELISA-plateleser ved 370 nm (referanse bølgelengde: ca 490 nm).

Med stoppløsning: 25 µl/brønn 1M H_2SO_4 tilsettes (med samme hastighet som Substrat solution) og inkuberes i 1 min mens man dunker lett på platen. (Evt. setter platen på en shaker). Absorbansen av prøvene måles ved bruk av ELISA-leser ved 450 nm (referanse bølgelengde: ca 690 nm). Absorbansen må leses av innen 5 min etter tilsetting av stopp-løsning.

Platene leses av i Dynatech plateavleser (Revelation software) i assayet: "BrdU_450nm.asy" under mappen "Proliferasjon".

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UTSTYR/FORBRUKSVARER/REAGENSER

UTSTYR

- 37 ºC CO_2 inkubator
- 60 ºC varmeskap
- Sentrifuge
- Pipetter: 0,5-10 µl, 100 µl, 200-1000 µl, multipipette
- Dynatech Laboratories MRX Plateleser, Revelation (Programvare), Pedersen & Sønn

FORBRUKSVARER

- Flatbunnet 96-brønners mikroplate, Nunclon Surface, kat.no: 167008, Nunc. Lagret på fryserrom.

KJEMIKALIER

- Roche- Cell Proliferation ELISA, BrdU (colorimetric); kat.No 11647229001
    - BrdU labelling reagent
    - FixDenat
    - Anti-BrdU-POD working solution
    - Washing buffer
    - Substrate solution
- 1M H_2SO_4 (laget på laboratoriet)