Respiratory Syncytial Virus (RSV) and Asthma

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Respiratoir syncytieel virus (RSV) en astma

Een studie naar de impact van RSV infectie op allergische ontsteking van de luchtwegen in een muis model

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Cover illustration: Lung of mouse sensitised (days 0-12 7x) and challenged (days 33-40) with OVA, and infected with RSV on day 35. Lung pathology is analysed at day 43 of the experimental protocol. A marked hypertrophy of mucus producing cells in bronchiolar epithelium is observed, together with perivascular infiltration of eosinophils and lymphocytes. The photo is made by Jan Dormans.

"Walk On"

The only baggage you can bring
is all that you can't leave behind

All that you fashion
All that you make
All that you build
All that you break
All that you measure
All that you steal
All this you can leave behind
All that you reason
All that you sense
All that you speak
All you dress up
All that you scheme
.....

Dedicated to Aung San Suu Kyi
Lyrics: Bono
Music: U2

Contents

Chapter 1 General introduction

1.1 Nature and characteristics of Respiratory Syncytial Virus

History

Respiratory syncytial virus (RSV) was first identified in 1956 as the agent that caused chimpanzee upper respiratory tract infection [137]. One year later, the same virus was recovered from infants with respiratory illness [35]. RSV has since been recognised as the most common cause of severe respiratory tract infection in young infants world-wide.

Virology

RSV is a member of the family of *Paramyxoviridae*, and belongs to the subfamily of pneumoviruses. Other members of this subfamily are bovine RSV (BRSV), ovine RSV (ORSV), caprine RSV (CRSV), pneumovirus of mice (PVM), and turkey rhinotracheitis virus (TRTV). Pneumoviruses are enveloped, nonsegmented single stranded negative-sense RNA viruses. The genomic RNA is packaged in a shell of proteins, called the nucleocapsid, which in turn is surrounded by an outer envelope of the virus that consists of viral glycoproteins embedded in a lipid bilayer. The lipid bilayer is derived from the host plasma membrane. The whole of genome, nucleocapsid and outer envelope together form the virion. Pneumoviruses are morphologically distinguished from other *Paramyxoviridae*, as they contain a smaller nucleocapsid. In addition, the members of the genus pneumovirus differ from other paramyxoviruses by having, as one of the envelope proteins, an unusual, mucin-like G attachment protein that is structurally distinct from the hemagglutinin-neuraminidase (HN) or hemagglutinin (H) protein of other paramyxoviruses [111]. The RSV genome contains 15,222 nucleotides, encoding the genetic information of eleven RSV proteins [40;71] Three transmembrane surface glycoproteins, the attachment protein G, the fusion protein F, and the small hydrophobic protein SH, are located on the outside of the viral envelope. These viral outer membrane proteins are organised separately into virion "spikes", which are visualised as short (11-20 nm), closely spaced projections out of the membrane (fig. 1.1). The matrix M protein is thought to form a layer on the inner envelope face. RSV has four nucleocapsid proteins packaged in the virion: the major nucleocapsid protein N, the phosphoprotein P, the antitermination factor M2-1, and the large polymerase subunit L. The M2-2 regulatory protein, situated in the matrix on the inner site of the viral envelope, down-regulates transcription and up-regulates RNA replication. In addition, RSV contains two nonstructural proteins, NS1 and NS2, which accumulate in the infected cells but are only present in small amounts in the virion [40;71].

RSV mainly infects the respiratory tract, in particular the bronchioli . The virion attaches to the epithelial cell via its G protein and subsequently penetrates the epithelial cell by fusion with the plasma membrane by the F protein. After fusion, the viral genomic material is released into the cells cytoplasma, where the virus replicates. RSV induces cell-to-cell fusion of infected cells,

thereby causing multinucleated giant cells to form. These large fused cells, syncytia, gave RSV its name [225].

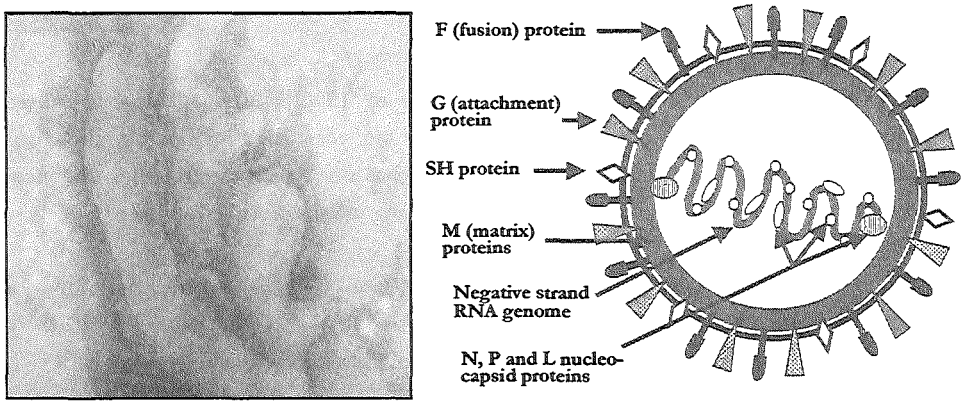

Figure 1.1. Respiratory Syncytial Virus negative contrast electron micrograph (left) and viral structure (right). The RSV virion is about 200 nm in size and consists of a nucleocapsid within a lipid envelope. The lipid bilayer is derived from the host plasma membrane and contains virally encoded transmembrane surface glycoproteins which are 11-20 nm in size and closely spaced at intervals of 6-10 nm. (The electron micrograph was kindly provided by Dr. P. Roholl).

Two major antigenic RSV subtypes are distinguished, designated A and B, with numerous strains in each group [3;141]. Distinction between the two groups is mainly based on polymorphisms of the G glycoprotein, but viruses from the two groups also differ in several other genes [3;94]. Infections with subtype A occur with a higher frequency than infections with subtype B.

Epidemiology

RSV is the most important cause of viral lower respiratory tract infection during infancy world wide [33;40;65;219]. More than 50% of infants are infected with RSV during their first year of life. In moderate climates, the virus circulates endemically each year during the winter months and is so highly contagious that 90% of children become infected before the age of 2 [40;72;127;186]. Reinfections with RSV are common and tend to have a milder or subclinical course with infections usually limited to the upper respiratory tract [78]. RSV is spread by respiratory secretions. The major mode of spreading appears to be by respiratory secretions rather than through small-particle aerosols [73;74]. Transmission requires either close contact with infected individuals or "self-infection" via contaminated hands to nasal and conjunctival mucosa [75].

Clinical features of RSV infection

RSV infection usually causes upper respiratory symptoms characterised by rhinitis, cough and a low-grade fever. However, up to 40% of primary RSV infections affect the lower respiratory tract, which may result in bronchiolitis, croup, pneumonia or wheezing [40;55;66;93;110;127]. A small proportion (up to 3%) of RSV infected children develops very severe breathing difficulties that hospitalisation is required [101;181;213]. New-borns and infants with underlying risk factors such as prematurity [145], congenital heart disease [122], or T cell immune deficiency [77].are especially at risk for a severe clinical course of RSV infection. Although several studies have shown that infection with RSV type A is associated with more severe disease, other studies have not been able to confirm this [40;93;108;130;212]. RSV infections may also induce severe illness in the elderly. During outbreaks among institutionalised elderly, RSV infections are frequently complicated by pneumonia and mortality rates up to 20% have been described [63;81].
In developing countries mortality rates for RSV infection up to 7% are reported, mortality among children in industrialised countries is 0.5 - 1% [37;186].
There is no effective antiviral therapy or an effective vaccine against RSV infection, although passive immunoprophylaxis by administration of RSV-neutralising antibodies is now available for high-risk children [69;95;155]. Numerous attempts to develop a live or inactive vaccine have failed. In the 1960s, an experimental formalin-inactivated RSV (FI-RSV) vaccine was used in infants and young children. This vaccine induced high levels of serum antibodies but failed to protect against RSV infection and RSV-induced disease. In contrast, most FI-RSV vaccinated infants developed a more severe lower respiratory tract disease upon a subsequent natural infection with RSV [104]. At this moment no licensed vaccine is available against RSV. Several strategies for save and effective vaccination, including immunisation with attenuated strains and sub-unit vaccines are at the moment under investigation.

Immunity to RSV infection

Innate immunity

Respiratory epithelial cells are the principle target for RSV infection and form also the first defence mechanism in the innate immunity to the virus. The virus infects these cells after attachment to and fusion with the cell membrane by means of the G and F glycoproteins on the viral surface [105]. Infected epithelial cells produce a range of chemokines and cytokines like interleukin (IL)-1β, IL-6, IL-8 [105;147], regulation upon activation normal T cell-expressed and secreted (RANTES) [13], and intracellular adhesion molecule (ICAM)-1 [156]. These mediators have been demonstrated to attract eosinophils, neutrophils, and $CD4^+$ T helper cells, and further initiate the immune response upon infection [48;105]. Besides the epithelial cells and natural killer (NK) cells, alveolar macrophages play an important role in the innate immune defence against RSV. Stimulated macrophages produce a wide range of

immunological mediators in response to RSV infection, among them the inflammatory cytokines TNF-α, IL-6, IL-1β and IL-8 [11;71;82]. These cytokines are secreted in the lung and reach their highest level at day 1 post infection [82]. In contrast to most other respiratory viral infections, RSV does not, or minimally, induce type 1 interferons (α and β) in nasal secretions [76;124].

Adaptive immunity

In response to RSV infection, antibodies to most RSV proteins develop, but neutralising antibodies are mainly directed against the major surface glycoproteins F and G [40]. Secretory antibodies in the respiratory tract, produced after the first contact with RSV, are important in resistance to reinfection [134;144], especially local IgA has been found to correspond with virus clearance [134;218]. Nevertheless, secretory IgA antibodies are only partially protective, and the effect of neutralising antibodies is not long-lasting because of a restricted period they are present.

RSV specific cellular immunity is believed to play the major role in resistance to RSV infection and recovery from disease [49]. This is illustrated by the fact that T cell immunodeficient children fail to clear RSV and have a high incidence of RSV infection leading to serious disease [34;40;77]. The dendritic cell (DC) has primary responsibility for antigen processing, antigen presentation and T lymphocyte activation, and thus initiates and shapes the adaptive immune response [158]. DCs present viral peptides via their MHC class II molecules to $CD4^+$ T lymphocytes. $CD4^+$ T cells are cytokine-secreting helper cells, and can be divided into type 1 helper (Th1) cells, which secrete IFN-γ and IL-2, or type 2 helper (Th2) cells, which secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 [1;53;139]. These cytokines function through a 'cytokine-network' as either positive or negative signals that orchestrate the immune response and the interaction of participating inflammatory cells (fig. 1.2). Measurements of the cellular immune memory response in peripheral blood mononuclear cells (PBMC) from normal children and adults infected with RSV showed a predominant type 1 like T cell response, characterised by the production of IFN-γ [4;21]. $CD8^+$ T lymphocytes recognise processed viral antigen in the context of MHC class I molecules. They are mainly cytotoxic killer cells and produce, among others, IFN-γ. $CD8^+$ cytotoxic T-lymphocytes (CTL) recognise the F, SH, N, M, M2, and NS2 proteins of RSV, but not the G protein [40;71;91;152;153]. RSV infection leads to the activation of both $CD4^+$ and $CD8^+$ T cell-mediated immune responses, but the majority of T lymphocytes recovered from the lung during the first 5 days of a primary RSV infection are of the $CD4^- 8^-$ (null) phenotype [153]. During elimination of the virus from the lungs (day 6-9), $CD8^+$ are the principal T-cells, although $CD4^+$ cells are also found.

Unfortunately, the role of the cellular immune response is dual. It is not only protective and helps eliminating virus from the body, but it may also induce immunopathology. In RSV-infected mice, both $CD4^+$ and $CD8^+$ T cells are required for full development of illness and pulmonary inflammation [2;28;67]. In addition, FI-RSV vaccine-enhanced disease in mice was

associated with T cell-mediated lung pathology upon RSV challenge. This pathology was related to a Th2-like immune response, characterised by the production of IL-4, IL-5 and eosinophilic inflammation [17]. In addition, experimental RSV infection of mice after immunisation with the G protein resulted in a Th2 $CD4^+$ response that was associated with aggravated pathology [97;193]. New-born babies still have an immature immune response that reacts with a Th2 cell response upon infection [52]. This Th2 milieu may lead to a more severe inflammation in these children. Indeed, the presence of type 2 cytokines in supernatants of PBMC from children who experienced a RSV infection at an very early age has been demonstrated [171].
Taken together, experimental data indicate that RSV infections may induce both type 1 and type 2 like T cell responses. Whether a skewing towards type 2 like response in some infected infants is the cause of more severe manifestations of RSV related disease, still needs to be elucidated [22].

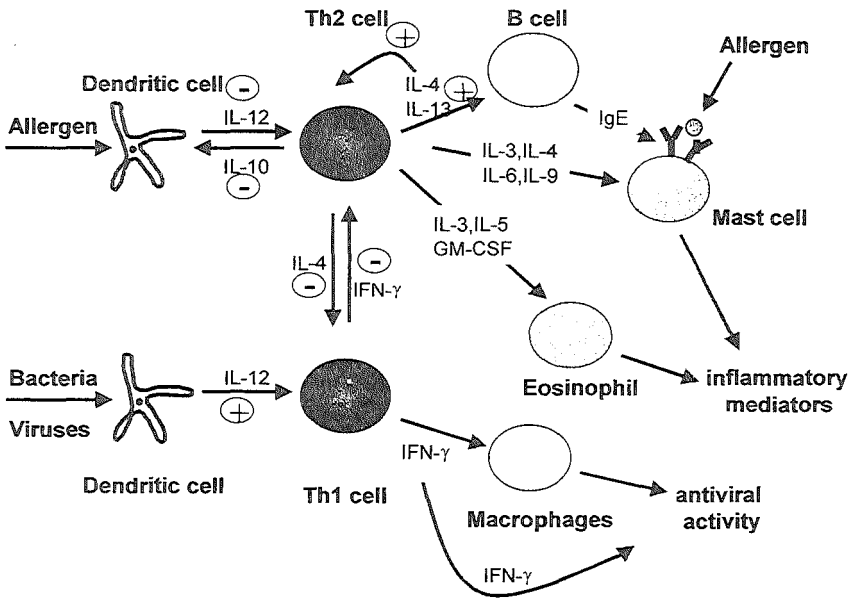

Figure 1.2. Schematic overview of T helper (Th) 1 and Th2 cellular immune response.
IL: interleukin, IFN: interferon, +: activation, -: inhibition.

Pathogenesis of RSV infection

The incubation period from RSV infection to onset of illness is 4 to 5 days. After infection, RSV primarily replicates in the epithelial cells of the nasopharynx [40]. Viral spread from the upper to the lower respiratory tract involves aspiration of nasopharyngeal secretions, spread via the respiratory epithelium, or spread from cell to cell by fusion without emergence into the extracellular fluid [40]. Indications of lower respiratory tract infection may develop 1 – 3 days

after the appearance of upper respiratory symptoms, indicating viral spread to the lower respiratory tract [40].
During severe lower respiratory tract infection, epithelial cells are destructed and cell debris is released into the bronchiolar space. In addition, a peri-bronchiolar inflammation is induced, characterised by a cellular infiltrate consisting mainly of mononuclear cells. Mucosal necrosis and mucus secretion together with swelling of submucosal tissue may induce obstruction of the bronchioli, leading to breathing problems [40]. Severe lower respiratory tract disease in RSV infection is, at least in part, caused by direct cytopathic changes in the lung tissue. However, an aberrant immunological response, occurring in severely infected children, has been suspected to contribute to the severity of the inflammatory process. After the disastrous vaccination trials with the formalin-inactivated RSV vaccine in the late sixties, T cells have become an important focus of interest in the study of RSV pathogenesis. RSV-specific T cells have been demonstrated in the circulation and in the lung of both RSV-infected laboratory animals, adult volunteers and patients with RSV bronchiolitis [2;91;106].

1.2 Allergic asthma

Characteristics of allergic inflammation

In the Netherlands allergic asthma affects approximately 5% of the adult population and 10% of the children [Netherlands asthma foundation]. The characteristic symptoms of asthma are recurrent wheezing, shortness of breath, cough, and tightness in the chest. The severity of these symptoms can vary between people, and are often worsened or prolonged by environmental triggers like house dust mite, dust, pollen, exercise, and smoke [192]. The breathing problems seen in asthma are due to increased airway hyperresponsiveness (AHR), extensive mucus secretion by goblet cells, and chronic airway inflammation [192]. It has been demonstrated that asthma is in part heritable and that several genetic loci predispose to the disease [42]. Asthma is described as an atopic disorder. The term "atopy" (from the Greek *atopos*, meaning out of place) is used to describe IgE-mediated diseases. Persons with atopy have a hereditary predisposition to produce high levels of IgE antibodies against common environmental allergens, and may have one or more atopic diseases (i.e. allergic rhinitis, asthma, and atopic eczema) [102].

Pathology of Allergic asthma

Allergic asthma is preceded by a period of sensitisation to allergens, which can occur years before the onset of clinical symptoms. During sensitisation allergen is taken up by antigen presenting cells and presented to T helper cells. These T cells secrete specific cytokines which activate allergen-specific B cells to proliferate, undergo isotype switching and produce allergen specific IgE [117]. The allergen specific IgE binds with the constant region to high affinity IgE

receptors (FcεRI) on mast cells. Upon allergen-challenge, the allergen will bind to the variable region of allergen-specific IgE on mast cells thereby cross-linking the IgE and inducing mast cell degranulation [26;164;232]. The contents of these granula (like histamine, serotonine, and several other inflammatory mediators) initiate the early asthmatic reactions which consist of smooth muscle contraction, extensive vascular leakage, and mucus hypersecretion by goblet cells [26;58]. In 50% of the asthmatic patients this early phase (acute inflammatory reaction) initiates a late phase reaction (chronic inflammation), in which infiltration of inflammatory cells in bronchial tissue, mucus hypersecretion, airway muscle hypertrophy, and thickening of the bronchial wall occur. Several of the early and late phase events can result in irreversible airway damage, and increased sensitivity of the airways for different non-allergen related stimuli [36].

T cells in allergic asthma

It is generally recognised that $CD4^+$ Th2 lymphocytes and their cytokines play a central role in allergic inflammation. The presence of Th2 cells in the airways of symptomatic patients together with the specific capacities of Th2-derived cytokines underlines their significance in this disease [26]. The major Th2 cytokines that mediate allergic inflammation are IL-4, IL-5, IL-6, IL-9 and IL-13. IL-4 is crucial for the maturation of Th2 cells and is an initiating factor IgE synthesis by B-cells through the regulation of transcription of the germline C_ε gene [138;210]. In addition, IL-4 inhibits the differentiation of Th1 cells and the production of IFN-γ [26]. IL-13 binds to the α-chain of the IL-4 receptor complex on immune cells, and has very similar effects as IL-4 [70;227]. IL-13 can induce the development of airway hyperreactivity (AHR), by mechanisms that are independent of IgE and eosinophils [227]. Besides their role in the initiation of allergic disease, Th2-derived cytokines may also play an important role in the effector phase of allergic asthma. IL-4 in combination with TNFα induces upregulation of the adhesion molecule VCAM-1 expression on vascular endothelium [30]. Increased expression of VCAM-1 facilitates both eosinophil and lymphocyte extravasation into lung tissue [30]. IL-3, IL-5 and GM-CSF are known to activate eosinophils [180;216], act as eosinophil chemoattractants [216;217] and prolong eosinophil survival by preventing their apoptosis [187]. The degranulation products of the eosinophil, like eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) can damage airway epithelial cells leading to exposure of sensory nerve endings [140]. Subsequently, these nerve endings will be more easily stimulated by inflammatory mediators and exogenous triggers leading to contraction of the airway smooth muscle cells, resulting in AHR [26].

Less is known about the role of $CD8^+$ T lymphocytes in allergic asthma. Within the $CD8^+$ T cell population, a subset of type 2 cells can be distinguished which have the potential to make IL-4 and IL-5 [60;115].

1.3 RSV infection and Allergic asthma: linking the two

Human studies

Childhood asthma is in most cases associated with sensitisation to inhaled allergens and allergic airway inflammation. This inflammation can be caused by viral lower respiratory tract infections [25]. RSV is probably the most important causative agent in inducing childhood asthma, because it is the virus most frequently isolated during wheezing episodes in infants under the age of 2 years [168].

In view of the pathological similarities between RSV bronchiolitis and asthma, one could speculate on common T cell-modulated immunopathogenic mechanisms in both diseases. In addition, the cellular immunological response in severe RSV disease, resembles that observed in allergic inflammation [105]. Several epidemiological studies have identified a close link between RSV-induced bronchiolitis and the development of childhood asthma during the first decade of life [31;136;143;148;154;167;184;188;194]. One of the most important ongoing clinical studies investigating the relationship between RSV infection with both allergy and asthma has been the work by Sigurs et al. in which they have longitudinally followed a cohort of 47 infants who suffered from severe RSV bronchiolitis during one winter season [182;184]. They found that RSV bronchiolitis that required hospitalisation was a risk factor for subsequent asthma and allergic sensitisation during childhood. The combination of family history of allergic asthma and severe RSV bronchiolitis gave the greatest risk for asthma [184]. Whether RSV bronchiolitis during infancy is a risk factor for allergic disease and asthma later in life is not clear. It seems that RSV infection during infancy does not increase the risk for developing asthma after the age of 11 [194].

Over 70% of all children are infected with RSV during their first year of life but only a small part develops allergic illness. It has therefore been suggested that if RSV can trigger the "allergic process", this will occur in subjects who developed a severe RSV infection or are predisposed either by their genetic background, or by events occurring before there first contact with RSV, that have "primed" their immune system and lungs [125;165].

Animal models

In order to investigate the underlying immunological mechanisms involved in the interplay of RSV infections, allergic airway sensitisation, and the development of obstructive airway disease and asthma, animal models have been developed. Most of our current understanding about the immunological mechanism of RSV disease and allergic inflammation is obtained from experimental work in mice.

Murine models of allergen-induced pulmonary inflammation share many features with human asthma, including antigen-induced eosinophilia, AHR, antigen-specific cellular and antibody response, increase in Th2 cytokines (IL-4, IL-5, IL-13) levels, and induction of antigen-specific IgE. Ovalbumin (OVA, a chicken egg protein serving as an allergic antigen) is the most widely

used antigen to induce airway inflammation [83;113], although the more relevant house dust mite or cockroach allergens are also used. The different models reported in the literature vary widely in the strain of mice used, the method of sensitisation to and challenge with OVA, and the time lag between allergen exposure and analysis of the different parameters [reviewed in 119]. In its simplest form, OVA is injected intraperitoneally with or without a Th2 skewing adjuvant, such as aluminium hydroxide. Sensitisation to the antigen is followed 10-20 days later by a period of OVA challenge, which is administered as aerosols. Analysis of airway inflammation is performed after challenge.

The last decade, researchers have studied animal models to test the hypothesis that RSV infection can provoke or enhance allergic inflammation, as has been reported for children. In fact, two groups can be distinguished, namely those who investigate the influence of prior RSV infection on the later development of allergic inflammation, and those who examined the influence of RSV infection on an already established allergic inflammation.

Influence of prior RSV infection on the later development of allergic inflammation.

Leibovitz et al. [116] and Freihorst et al. [64] were the first who determined in a murine model the impact of infection with RSV on subsequent sensitisation to inhaled allergens (ragweed and OVA). RSV infection resulted in increased levels of allergen-specific IgE and IgG in serum, and IgA and IgG in bronchoalveolar lavage fluid (BAL), measured at different time points after challenge with OVA aerosols. More recently, O'Donnell demonstrated that the subtype of RSV-enhanced OVA-specific IgG was IgG_1, a class of antibody which is known to mediate anaphylaxis. Prior RSV infection also increased the OVA-specific IL-4 production by cultured splenocytes [151]. In contrast to the above mentioned findings, Schwarze et al [178] did not observe increased serum levels of OVA-specific antibodies when OVA-allergic mice received RSV infection prior to OVA-sensitisation[177;179]. However, they demonstrated that RSV, given before allergen sensitisation and challenge, increased the airway hyperresponsiveness (AHR), increased IL-4 production by peribronchiolar lymph nodes (PBLN), and pulmonary infiltration of eosinophils and neutrophils [178]. Studies in IL-$5^{-/-}$ mice showed that IL-5 is necessary for the development of pulmonary eosinophilia, and that IL-5 and eosinophilia is critical for the development of RSV-enhanced AHR in response to allergen sensitisation via the airways [178]. The studies in animal models reviewed above indicate that primary RSV infection not only induces a type 1 immune response, but also a type 2 immune response in non-sensitised mice. They therefore suggest that this might be the mechanism by which RSV augments allergic inflammation that occurs with subsequent airway exposure to allergens.

Influence of RSV infection during an already established allergic inflammation

Individuals with airway allergies and asthma are prone to develop acute obstructive airway disease when infected with respiratory viruses [131]. The question arises whether RSV infection contributes to the severity and duration of established allergic airway disease. These issues have been addressed by studies in mice that investigated the impact of RSV infection on airway

inflammation and AHR in animals already sensitised to allergen [15;126;161;163]. These studies indicate that RSV infection in previously sensitised mice lead to enhanced mucus secretion into the airways [15] and increased AHR after allergen challenge that can persist for long periods of time [163]. Factors involved in this potentiation in allergic hosts could be increased production of chemokines in response to infection (eotaxin, RANTES, MIP-1α, MIP-1β) [161;223], and increased recruitment of Th2-lymphocytes resulting in enhanced production of Th2-derived cytokines such as IL-4 and IL-5. These studies suggest that pre-existing allergic inflammation is necessary for RSV to have a significant effect on the physiologic parameters that are associated with allergen-induced disease.

1.4 Aim of the thesis

For many years animal studies are performed to investigate the immunity induced by an RSV infection and the immune regulatory role of RSV infections on the development and exacerbation of respiratory allergies. Since different strategies of allergen sensitisation and challenge, moments of virus infection during allergen-sensitisation and -challenge, and timing of analysis after challenge are chosen, the precise role of RSV infection in allergic inflammation is still not clear.

The aim of this thesis is to investigate whether RSV infection modulates respiratory allergy. In addition, the immune mechanisms which determine the influence of RSV infection on respiratory allergy are investigated.

The research questions addressed in this thesis are:

- What is the influence of RSV infection on the parameters of allergic sensitisation and challenge? (chapter 2).
- What role do antiviral Th1 cytokines IFN-γ and IL-12 play in the RSV-enhanced respiratory allergy? (chapter 3).
- What is the significance of timing of infection on respiratory allergy? (chapter 4).
- Does RSV-induced immunity influences the effect of a secondary RSV infection on respiratory allergy? (chapter 4).
- Is the RSV-enhanced respiratory allergy a specific feature of RSV, or do other respiratory viruses have the same capacity? (chapter 5).
- Is the RSV-specific cellular immune response influenced by allergic inflammation? What is the mechanism of immune interaction between viral infection and allergic inflammation? (chapter 6)

The goal of these studies is to unravel the immune mechanism of RSV-enhanced respiratory allergy in mice. The knowledge obtained in these studies will be valuable for the development of clinical intervention strategies for prevention of RSV-related allergic exacerbations in children.

Chapter 2 Influence of Respiratory Syncytial Virus infection on cytokine and inflammatory responses in allergic mice.

Marion Barends, Anita Boelen, Lia de Rond, Joan Kwakkel, Theo Bestebroer, Jan Dormans, Herman Neijens, and Tjeerd Kimman.

Clinical & Experimental Allergy, 2002; 32:463-471

2.1 Abstract

T helper (Th) 2-lymphocyte responses are associated with inflammation and disease during allergic responses. Exposure to particular environmental factors during the expression of allergy could result in more pronounced Th2-like immune responses and more severe disease. One factor might be a respiratory virus infection.

The aim of our study was to investigate the influence of respiratory syncytial virus (RSV) infection on the expression of ovalbumin (OVA)-induced allergy in BALB/c mice. We therefore determined OVA-specific IgE in serum, cytokine profiles, and histopathologic lesions in lungs of OVA-allergic mice after RSV infection.

OVA sensitisation and challenge induced OVA-specific IgE in serum, Th2 cytokine mRNA expression, and mononuclear and eosinophilic inflammation in the lungs. RSV inoculation during the challenge period enhanced OVA-induced IL-4 and IL-5 mRNA expression in lung tissue. RSV further enhanced the OVA-induced hypertrophy of mucous cells and eosinophilic infiltration in lung tissue. Surprisingly, RSV infection decreased Th2 cytokine secretion and eosinophilic influx in bronchoalveolar lavage of OVA-allergic mice. Because inactivated RSV did not influence these responses, replication of RSV appeared essential for the modification of OVA-induced Th2 cytokine expression. RSV did not change OVA-specific IgE levels in serum. Furthermore, the RSV-induced IL-12 mRNA expression in lung tissue of OVA-allergic mice was diminished, but IFN-γ mRNA expression was not affected.

In conclusion, RSV infection enhanced particular OVA-induced Th2-cytokine mRNA responses and pulmonary lesions in allergic mice and thus aggravated allergic respiratory disease.

2.2 Introduction

Respiratory allergies are characterised by pronounced Th2-lymphocyte responses, including IL-4 and IL-5 cytokine expression, enhanced IgE responses, mast cell degranulation, and influx of activated eosinophils [87;88;172]. Several authors examined whether respiratory viruses influence the development or expression of respiratory allergy. For example, Rakes *et al.* [168] examined in wheezing children and controls the prevalence of respiratory viruses. They showed that the majority of childhood wheezing is associated with infection with respiratory syncytial virus (RSV) in infants before 2 years of age, and with rhinovirus in older children. Skoner *et al* [190] studied in allergic and non-allergic persons the effects of a rhinovirus infection on immune and inflammatory parameters. Their most important finding was the increased total serum IgE level in rhinovirus-infected allergic subjects compared to non-infected allergic

subjects and rhinovirus-infected non-allergic subjects. Many population studies have been performed in which the relation of RSV infection in early childhood and the development of allergy later in life were investigated. For example, Sigurs *et al.* showed in a cohort study with matched controls that RSV infection during the first year of life is an important risk factor for the development of asthma and the sensitisation to common aeroallergens at the age of 7 [184;185]. However, controversy exists about the long-term effects of RSV on the development or expression of allergic asthma. Stein *et al.* failed to confirm the relation between a childhood RSV infection and allergy in teenagers [194]. Unfortunately, nothing is known about the course of an RSV infection in persons who already developed an allergy, and about the influence of RSV infection on the expression of allergy.

A recent study of Peebles *et al.* [163] showed in mice that an RSV infection during the expression phase of ovalbumin-induced allergy enhanced airway mucus production, increased numbers of lung lymphocytes, and prolonged the period of airway hyperreactivity. These data supports the study of Blyth *et al* [15] who demonstrate an increased discharge of goblet cell mucin in the airways of allergic mice infected with RSV.

The aim of the present study was to examine whether RSV enhances Th2 cytokine responses during respiratory allergy that could explain the RSV-enhanced allergic response. Therefore we have determined the influence of an RSV infection on the expression of an OVA-induced respiratory allergy, which is characterised by cytokine responses in the lungs, pulmonary lesions, and enhanced serum IgE levels. In this study we demonstrate that RSV infection during OVA-challenge enhanced the OVA-induced Th2 cytokine response in lung tissue but not in bronchoalveolar lavage.

2.3 Methods

Virus: Human Respiratory Syncytial virus stock A2 (RSV A2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The virus was cloned by limiting dilution on HEp-2c cells (ATCC, Rockville, MD) in 24 wells plates. Stock virus was subsequently grown on Hep-2 cells in RPMI 1640 medium (Gibco BRL, Life Technologies), containing 10% heat-inactivated foetal calf serum (FCS, Hyclone Laboratories Inc., Logan, UT), 2 mM glutamine, 100 IE/ml penicillin and 100 units/ml streptomycin. Cells and media were harvested when ca. 75% cytopathic effect was observed, sonificated on ice for 3 x 5 seconds (Vibra cell Sonics & Materials model VC-40, 40W, 60% output control), and centrifuged for 10 min at 1,000g. The supernatant was divided into aliquots, and snap frozen at -80°C. The suspension contained 10^8 plaque forming units (pfu) RSV/ml. Virus was quantified according to Waris *et al.[215]*. To inactivate RSV, the original stock was divided in small aliquots and UV irradiated (302nm, dose 1.5 x 10^{-2} $\mu W/mm^2$, Transilluminator, ultra-violet products, San Gabriel, CA) for 45 minutes [14].

Animals and experimental design: Female BALB/c mice (RIVM, The Netherlands) were used at 7-9 weeks of age. The institute's committee on animal welfare approved the study. Mice were made allergic to OVA by administration of 7 intraperitoneal (i.p.) injections of 0.5 ml OVA (20 µg/ml saline, no adjuvant), every other day starting at day 0. After 3 weeks, mice were challenged by OVA aerosols (2 mg/ml saline) during 5 minutes on 8 consecutive day's [83] (fig. 2.1).

To examine the influence of RSV infection on OVA-allergy, OVA-allergic mice were inoculated intranasally with 10^7 pfu RSV according to the schedule as outlined in fig.1. These mice were compared to OVA-allergic mice sham-inoculated with an uninfected HEp-2c cell lysate (mock), non-allergic mice (sensitised and challenged with saline), and non-allergic mice infected with RSV.

To examine the influence of RSV replication, OVA-allergic mice were inoculated with UV-inactivated RSV. These mice were compared to OVA-allergic mice infected with 10^7pfu RSV.

At different time points after the last OVA-challenge, mice were anaesthetised with barbiturate/chloralhydraat i.p. before they were sacrificed. To examine if RSV replicates in the lungs of OVA-allergic mice (in our experimental setting), one experiment was performed wherein mice were sacrificed at days 4 and 6 postinfection. Lungs were homogenised and the virus titre was determined according to Waris *et al*.[215].

Each experiment was performed in duplicate and data of one representative experiment are shown.

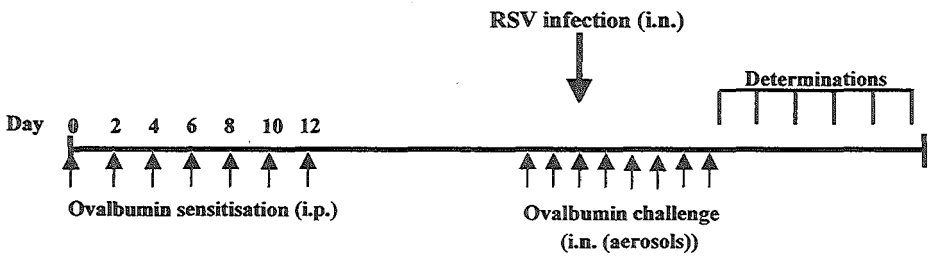

Figure 2.1. Study design

Histopathologic examination: Lungs were removed, filled intratracheally with formalin, and immersed in fixative. Lungs were embedded in paraplast (Monoject, Kildare, Ireland), transverse sections of 5 µm were stained with haematoxylin and eosin. Different lung lesions were scored semiquantitatively from absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5).

Bronchoalveolar lavage: Lungs were lavaged 3 times using 1 ml of saline (37°C) and the BAL was kept on ice until further processing. Supernatant was obtained by centrifugation, and stored at -80°C until cytokines were determined by ELISA. Cytospin preparations were stained with May-

Giemsa/Grünwald. In each cytospin at least 400 cells were differentiated into mononuclear cells, neutrophils and eosinophils.

RNA isolation and RT-PCR: Total cellular RNA was isolated from frozen lung samples using the SV Total RNA isolation system (Promega, Madison, USA) according to the manufacturer's protocol. cDNA was made by reverse transcriptase (RT) reaction using poly(dT) primers. cDNA was amplified by PCR using specific sense and anti-sense primers (IFN-γ, IL-4, IL-5, IL-12, IL-13 and eotaxin; final concentration 2 ng/ml) [20]. The PCR reactions and analysis were performed as described by Boelen *et al.* [17]. Every PCR of all samples of one experiment were done in the same reaction and run on the same agarose gel for quantification. All results were corrected to the sample's mRNA content using β-actin mRNA, which was amplified according to the manufacturer's instructions (mouse control amplifier set, Clontech, Palo Alto, CA).

Cytokine ELISAs: The concentrations of IFNγ, IL-4, IL-5, and IL-13 in the BAL were assessed by ELISA according to the protocol described by Boelen *et al.*[17]. Coating antibodies were rat anti-mouse cytokine antibody IgG (IFNγ-IgG_1, clone R4-6A2; IL-4-IgG_1, clone 11B11; IL-5-IgG_1, clone TRFK-5 (all derived from Pharmingen, San Diego, CA), and IL-13-IgG_{2b}, clone 35213.11 (R&D Systems, Minneapolis, USA)). Detection antibodies were biotin-conjugated monoclonal rat-anti-mouse cytokine IgG (IFNγ-IgG_1, clone XMG1.2; IL-4-IgG_1, clone BVD6-24G2; IL-5-IgG_{2a}, clone TRFK-4; all derived from Pharmingen, San Diego, CA). IL-13 was detected using biotin-conjugated goat anti-mouse IL-13-IgG (R&D Systems, Minneapolis, USA). The detection limit of the assays were, IFN-γ: 30 pg/ml, IL-4: 7 pg/ml, IL-5: 30 pg/ml, IL-13: 15 pg/ml. All samples of one experiment were measured within the same assay.

IgE antibodies in serum: Both total and OVA-specific IgE antibodies were determined in serum using a modified capture ELISA according to the protocol of Van Halteren *et al.* [207], using rat anti-mouse IgE monoclonal antibody EM-95 [5] as coating antibody. As detecting antibody for total IgE, biotinylated rat-anti-mouse IgE was used (Pharmingen, San Diego, USA). OVA-IgE was detected with digoxigenin-coupled ovalbumin. Total IgE concentrations were calculated by interpolation from a standard titration curve with known concentrations of recombinant mouse IgE_3 (Pharmingen, San Diego, CA). The detection limit of the total IgE assay was 900 pg/ml. OVA-specific IgE levels were expressed as the optical density at 490 nm of a 1/32 diluted serum.

Data analysis: Comparisons between experimental groups on one single time point were made using the Student's *t* test. Comparisons of histological scores were made using the nonparametric Wilcoxon's test. Series of time points were analysed by analysis of variance (ANOVA) with two grouping factors (time and treatment) using Excel (Excel, Microsoft Corporation, USA).

2.4 Results

Characterisation of the OVA-induced allergy

To characterise the OVA-induced allergy we determined total and OVA-specific IgE, eosinophil influx in lungs and bronchoalveolar lavage, hypertrophy of the mucous cells in the bronchiolar epithelium resulting in the production of mucus, and the production of Th2 cytokines. These parameters were assessed one day after the last OVA-challenge.

IgE antibody production in serum: Total serum IgE levels were tenfold increased in OVA-allergic mice compared to non-allergic mice. Furthermore, OVA-specific IgE was only demonstrated in OVA-allergic mice and not in controls.

Cytokine mRNA expression in lung: We observed approximately 3 fold increased IL-4, 2 fold increased IL-5, and 4 fold increased IL-13 mRNA expression in the lungs of OVA-allergic mice compared to the background levels in non-allergic mice. No IFN-γ mRNA expression above background level was observed.

Inflammation of the lungs: We observed an increased influx of eosinophils in BAL of OVA-allergic mice compared to non-allergic mice ($2.1x10^4$ versus $2.9x10^2$ cells/ml, $p < 0.01$). OVA-allergy induced a moderate hypertrophy of the mucous epithelial cells. This hypertrophy was not present in untreated controls.

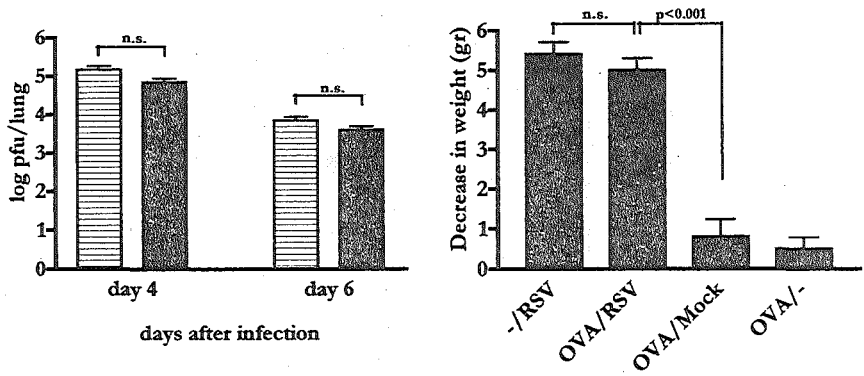

Figure 2.2. Left: RSV titres in lung homogenates ([10]log pfu/lung) of OVA-allergic (black bars) and non-allergic (striated bars) mice on 4 and 6 days post infection. Right: Weight changes between day 35 and 41 of OVA-allergic and non-allergic mice with or without RSV infection. The decrease in weight is shown. In both figures mean values ± SEM (n=6) are depicted. Comparisons between indicated groups were made using Student's *t* test, n.s.: not significant.

The influence of RSV infection on OVA-allergy

Subsequently, we examined the influence of RSV infection on the expression of OVA-induced allergy. Therefore, mice were inoculated during the OVA-challenge-phase (fig 2.1).

The kinetics of RSV-infection in OVA-allergic and non-allergic mice: Virus titres in the lungs were determined at days 4 and 6 postinoculation. No difference in virus recovery from the lungs was observed between the allergic and non-allergic mice. The severity of illness was determined by weight loss. Mice were weighted before and 6 days after RSV inoculation. RSV infection induced severe weight loss, but no difference in weight loss was observed between RSV-infected allergic and non-allergic mice. (fig 2.2). Nevertheless, these data do not rule out that weight loss at other time points could have shown differences between those two groups.

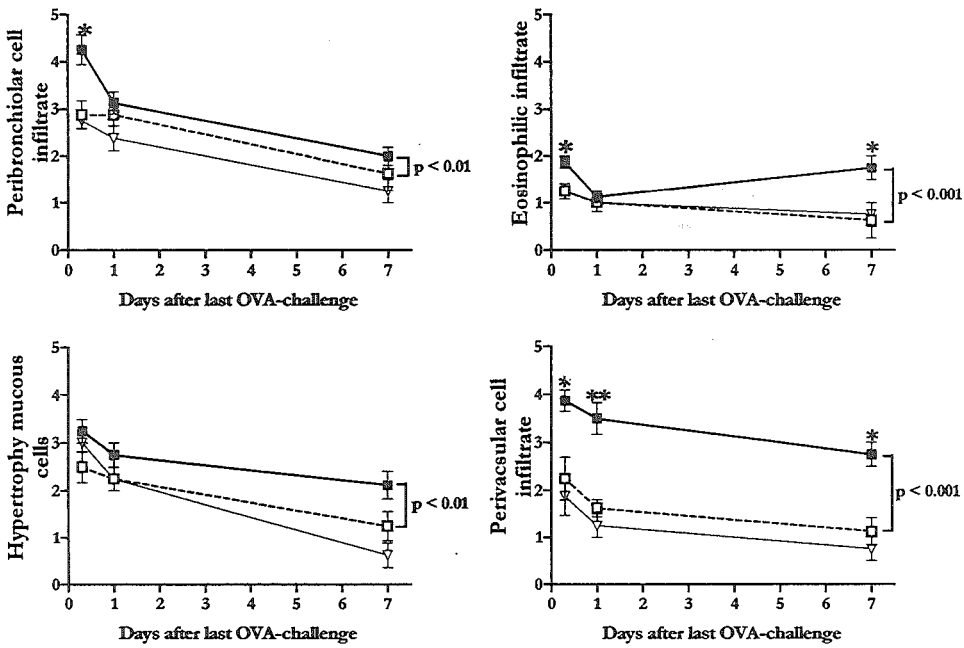

Figure 2.3. Histopathological changes in lungs of OVA-allergic mice infected with RSV (■-■), inoculated with mock (□-□), or uninfected (▽-▽) determined at 1 hour, 1 day and 1 week after the last OVA-challenge. Pulmonary lesions are scored from minimal (1) to severe (5). Scores are depicted as mean value ± SEM (n=8). Only the statistical differences between OVA/RSV and OVA/Mock are shown. Series of time points were analysed by analysis of variance in accordance with Fishers least significant difference strategy. Statistical difference on one single time point between OVA/RSV and OVA/Mock is analysed using the nonparametric Wilcoxon's test, differences are indicated by *: $p < 0.05$, **: $p < 0.01$.

Influence of RSV on histopathologic lesions in lungs of OVA-allergic mice: Fig2.3 summarises the pulmonary lesions induced in OVA-allergic mice 1 hour, 1 day and 1 week after the last OVA-challenge. RSV infection enhanced peribronchiolitis in OVA-allergic mice (mononuclear cells: $p < 0.01$, eosinophils $p < 0.001$). In addition, RSV infection in OVA-allergic mice enhanced hypertrophy of the bronchiolar mucous epithelial cells and mucus production, compared to mock-inoculated allergic mice ($p < 0.01$). Moderate to marked perivascular infiltrates were predominantly observed in RSV-infected allergic mice ($p < 0.01$).

Influence of RSV on cytokine mRNA expression in lungs of OVA-allergic mice: We subsequently examined the influence of RSV on the OVA-induced Th2 cytokine responses in lung tissue at several time points after the last OVA-challenge. The results are presented in fig 2.4. RSV enhanced the IL-4 and IL-5 mRNA expression in the lungs of allergic mice compared to mock-inoculated allergic mice ($p < 0.001$ and $p < 0.01$ respectively). In contrast, no significant differences in IL-13 and eotaxin mRNA expression were observed.

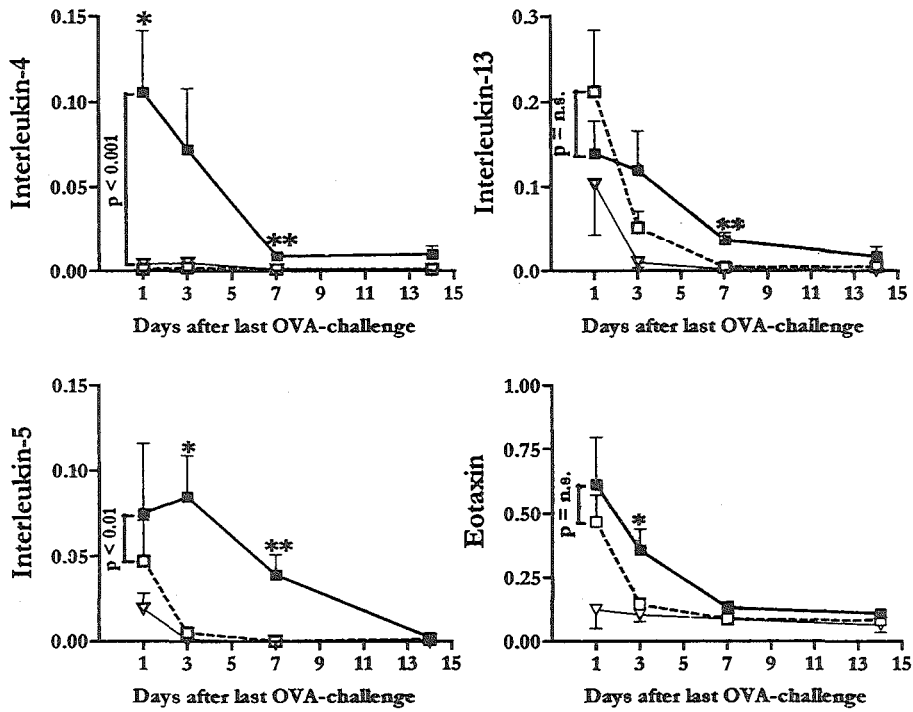

Figure 2.4. Relative expression (against β-actin) of lung IL-4, IL-5, IL-13 and eotaxin mRNA in OVA-allergic mice infected with RSV (■-■), inoculated with Mock (□-□) or uninfected (▽-▽) determined at 1, 3, 7 and 14 days after the last OVA-challenge. Mean values ± SEM ($n = 6$) are depicted. Only statistical differences between OVA/RSV and OVA/Mock are shown. Series of time points were analysed by analysis of variance in accordance with Fishers least significant difference strategy and P values are depicted left in the graphs. Statistical difference on one single time point between OVA/RSV and OVA/Mock is analysed using the Student's *t* test, differences are indicated by *: $p < 0.05$, **: $p < 0.01$.

Influence of RSV infection on cytokine content and cell composition of bronchoalveolar lavage in OVA-allergic mice: At day one after the last OVA-challenge, the IFN-γ concentration in BAL was higher in the allergic mice infected with RSV compared to those inoculated with mock. We measured decreased IL-4, IL-5 and IL-13 concentrations in the BAL of RSV-infected allergic mice compared with mock-inoculated allergic mice. However, only the decreased IL-5 concentration was statistically significant different between those two groups ($p < 0.05$) (fig. 2.5).
In contrast with the enhanced eosinophil influx in the peribronchiolar space in lung tissue, RSV infection decreased the influx of eosinophils in BAL of allergic mice compared to mock-inoculated allergic mice ($3.7x10^3$eosinophils/ml versus $3.8x10^4$eosinophils/ml, $p < 0.001$) (fig. 2.5).
An increase of neutrophils and lymphocytes in BAL was only observed in RSV-infected mice. No difference was found in neutrophil and lymphocyte numbers in BAL of RSV-infected allergic mice compared to RSV-infected non-allergic mice (fig. 2.5). The majority of cells in the BAL of all groups were macrophages and no differences in macrophage numbers were found between the different treatments (data not shown).

IgE antibody responses in serum: Total IgE production in serum is increased in OVA-allergic mice and OVA-specific IgE is only detected in OVA-allergic mice. In our experiments, RSV infection in allergic mice did not affect both total IgE and OVA-specific IgE levels in serum from mice sacrificed at different time points during 3 weeks after the last OVA-challenge.

Influence of RSV replication on OVA-allergy; comparison of live versus UV-inactivated RSV

To determine whether the effects of an RSV infection on OVA-induced allergy were dependent of virus replication in bronchiolar epithelium or were induced by the presence of non-replicating viral antigens, we inoculated OVA-allergic mice with live RSV or with UV-inactivated RSV (UV-RSV).

Influence of UV-inactivated RSV on cytokine mRNA expression in lungs of allergic mice: We observed no IFN-γ mRNA expression in lung tissue of mice inoculated with UV-RSV, while high levels of IFN-γ mRNA were detected in RSV-infected allergic and non-allergic mice. As mentioned, RSV enhanced the OVA-induced IL-4 mRNA expression, but UV-RSV did not ($p < 0.05$) (fig. 2.6).

Cytokine and cell composition of bronchoalveolar lavage: In contrast with live RSV, UV-RSV did not induce the production of IFN-γ in BAL of both OVA-allergic and non-allergic mice ($p < 0.001$). In addition, while RSV infection diminished IL-4, IL-5 and IL-13 production in BAL of OVA-allergic mice, UV-RSV did not ($p < 0.01$) (fig. 2.5). No significant differences were

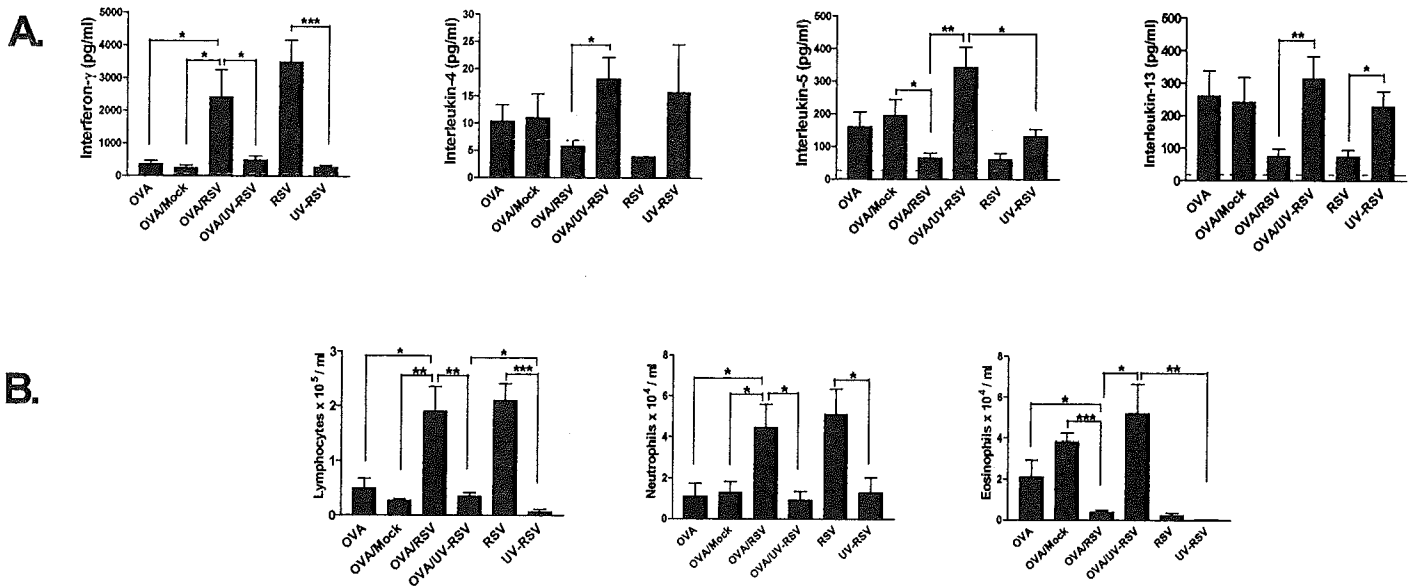

Figure 2.5. A: IFN-γ, IL-4, IL-5, and IL-13 concentrations in BAL, and B: Composition of cell count from BAL of allergic and non-allergic mice with different treatments (X-axis) determined at 1 day after the last OVA-challenge. Mean values ± SEM (n = 6) are depicted. Statistical differences between indicated individual groups are determined using Student's *t* test, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Detection limit of the cytokine ELISA assays is presented as a dotted line in the graph.

measured between allergic mice inoculated with mock compared to those inoculated with UV-RSV. So, UV-RSV did not influence the OVA-induced Th2 cytokine response in BAL.
In contrast to live RSV, which decreased the influx of eosinophils into BAL, no difference was found in eosinophil influx between allergic mice inoculated with UV-RSV or mock (fig. 2.5).

Influence of OVA-allergy on cytokine response during RSV infection

We previously demonstrated that RSV infection in non-allergic mice resulted in a dominant Th1 response [17]. Confirming previous results, we observed no IL-4 and IL-5 mRNA expression in lungs of RSV-infected non-allergic mice. At day 6 post infection, no difference in IFN-γ mRNA expression was observed between RSV-infected allergic and non-allergic mice. However, less expression of IL-12 mRNA ($p < 0.001$) was found in RSV-infected allergic mice compared to RSV-infected non-allergic mice (fig. 2.6). Thus OVA-allergy diminished RSV-induced IL-12 mRNA expression.

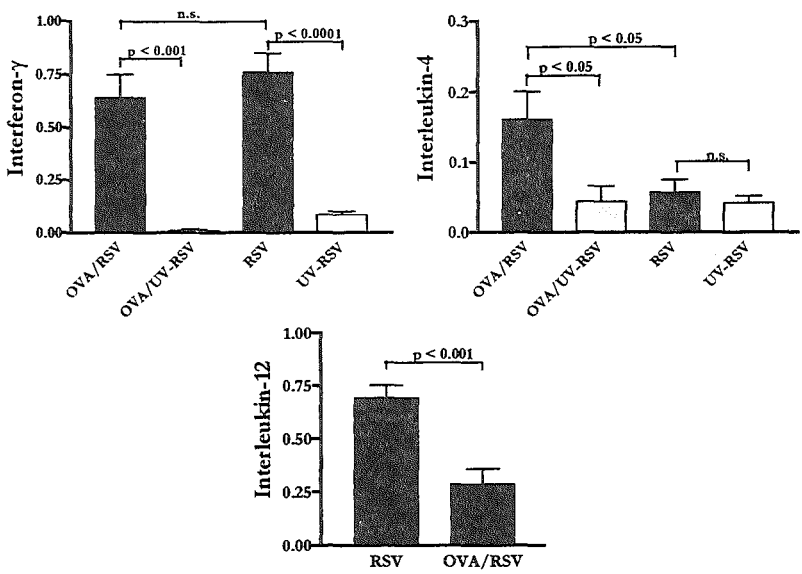

Figure 2.6. A: Relative expression (against β-actin) of lung IFN-γ, IL-4 and IL-12 mRNA in allergic and non-allergic mice inoculated with live RSV (black bars) or UV-inactivated RSV (open bars) determined at 1 day after the last OVA-challenge. Mean values ± SEM (n = 6-8) are depicted. Comparisons between indicated groups were made using Student's *t* test.

2.5 Discussion

In this study we investigated the influence of RSV infection on a pre-existing ovalbumin (OVA)-induced allergy in BALB/c mice. OVA sensitisation and challenge induced strong Th2 cytokine mRNA expression in the lungs, OVA-specific IgE production in serum, and pulmonary lesions (increased mucus production and eosinophilic influx).

We hypothesised that RSV infection induced more pronounced Th2 cytokine profiles in the lungs of OVA-allergic mice. In our animal model we indeed showed that RSV infection enhanced the OVA-allergy-associated cytokine mRNA responses in the lung. IL-4 and IL-5 mRNA expression was specifically increased after RSV infection, and a raised eosinophilic influx in lung tissue and mucus production in the bronchioles was observed.

Several authors investigated the role of RSV on OVA-induced allergy in mice. In contrast to our experimental protocol, most authors examined the influence of RSV when given before the OVA-sensitisation period [151;178]. For example, O'Donnell et al. [151] demonstrated an acute collapse in RSV- and influenza virus-infected mice after OVA-sensitisation during the active phase of viral infection. They observed increased numbers of IL-4-positive splenocytes in RSV-infected mice sensitised with OVA. However, no lung cytokines were determined. In a comparable study, Schwarze et al. [178] observed increased airway hyperreactivity (AHR) and raised eosinophil influx in the lungs of RSV-infected and OVA-sensitised mice compared to OVA-sensitised mice only. In the peribronchial lymph nodes they observed increased IL-4 production together with a decrease of IFN-γ. This decrease was rather striking because in our experiments we observed raised IFN-γ mRNA expression despite increased IL-4.

As far as the authors are aware only Peebles *et al.* [161;163] used the same study protocol in mice as we did, i.e. RSV infection during OVA-challenge. They observed increased airway hyperreactivity when OVA-allergic mice were infected with RSV during the challenge period. However, they did not find increased Th2 cytokine levels in lung tissue, determined both by mRNA expression level and protein concentrations. This is in contrast to our results because we do see increased Th2 cytokine mRNA expression in lung tissue of RSV infected OVA-allergic mice compared to OVA/Mock mice. Possible explanations for the discrepant results could be the different time points after infection and challenge on which the cytokine levels were determined, and a different method of mRNA detection.

In our experiments IL-4 and IL-5 are the key cytokines affected by RSV in OVA-allergic mice. The enhancement of IL-5 mRNA expression in the lungs correlated with the increased eosinophilic influx in lung tissue. Interestingly, Schwarze *et al.* showed in IL-5 deficient mice that RSV-induced IL-5 production during active infection is essential for the influx of eosinophils in lungs and the subsequent development of airway hyperreactivity (AHR) after OVA-challenge [175;177;178].

IL-4 is a central cytokine promoting B cells to switch to IgE antibody production. However, we observed that RSV enhanced IL-4 mRNA expression in lung tissue of OVA-allergic mice without increasing allergen-specific or total IgE. A similar observation was done by Schwarze *et*

al. [178], although they studied the influence of an RSV infection on subsequent allergic-sensitisation. IL-13, independent of IL-4, is crucial in inducing allergic asthma in animal models and is involved in IgE production by B cells [38;59;70]. In our experiments, RSV did not further increase the OVA-induced IL-13 mRNA expression, which could be a reason for the unaffected serum IgE levels. In a recent study, Tekkanat *et al* showed that RSV induces IL-13-dependent airway hyperreactivity and mucus production in BAL and they suggested that IL-13 and not IL-4 might be an important cytokine in RSV-induced disease [200]. We did not observe increases in the Th2 cytokines IL-4 and IL-5 after primary RSV infection, but the level of IL-13 mRNA in lung tissue was increased (data not shown). However, we did not measure increased IL-13 protein concentration in BAL. IL-13 mRNA expression in OVA-allergic mice, with or without RSV infection, was far more increased than in RSV-infected non-allergic mice.

We and others previously demonstrated that primary RSV infection induced a predominant Th1 cytokine response, characterised by high levels of IFN-γ and low levels of IL-4 and IL-5 mRNA expression in the lung [17;90;202]. In allergic mice we found a relative decrease in RSV-induced IL-12, but IFN-γ expression was unaffected, despite high levels of IL-4. IL-12 is primarily produced by antigen-presenting cells and has a critical role as a regulator of Th1-driven immune responses. The production of IL-12 is inhibited by IL-10, but also IL-4 has a critical role in the regulation of the production of IL-12 [201]. The enhanced IL-4 expression might therefore explain the decrease in IL-12 mRNA expression. The unchanged IFN-γ mRNA expression is interestingly because of its reported inhibitory function of the Th2 immune response. However, a recent study in atopic children showed that a strong Th2 response might develop despite high levels of IFN-γ [206]. In fact, studies in mice indicated that Th1 lymphocytes (producing IFN-γ) may be essential for the migration of Th2 lymphocytes to the airways [169]. It is therefore tempting to speculate that RSV-induced IFN-γ producing cells are essential in the enhancement of the OVA-induced Th2 response.

The RSV-induced enhancement of the OVA-induced Th2 cytokine response and eosinophil influx in lung tissue seem in conflict with the results we obtained in BAL. In BAL we observed decreased IL-4, IL-5 and IL-13 protein concentrations and decreased influx of eosinophils in allergic mice after RSV infection, compared to mock- and UV-RSV-inoculated allergic mice. The airway and lung tissue results, however, may vary because RSV replicates in respiratory epithelium and also in alveolar macrophages. Alveolar macrophages are the majority of cells recovered in BAL and these cells may be activated by RSV [12;132;209]. These macrophages produce upon stimulation different mediators, among whom the cytokines IL-12, IL-1, TNF-α and IFN-γ. The synergistic effect of these cytokines could result in stimulation of the Th1 response and inhibition of the Th2 response. Whatever the mechanism, our results indicate that the outcome of studies on the cytokine and inflammation responses may differ depending on the sites studied.

The pathogenesis of RSV-induced enhancement of allergy is still not clear. Virus-induced immune responses and allergic airway inflammation probably interact to produce altered responses to both virus and allergen [163]. One possible mechanism responsible for the RSV-

induced enhancement of OVA-allergy could be increased permeability of the respiratory epithelial barrier during RSV infection, which makes it easier for OVA to enter the tissue [64]. In addition, whether other viruses are able to modulate allergic responses similarly as RSV needs to be investigated.

In conclusion, we showed that RSV infection in already allergic mice enhanced the allergy-associated IL-4 and IL-5 cytokine mRNA responses in lung tissue, and increased eosinophilic influx in the lung tissue and mucus production in the bronchioles. However, RSV did not enhance serum IgE levels. Replication of RSV in the lung appears essential for the enhanced allergic responses. Our mice model may allow further mechanistic studies on the influence of RSV on immune and inflammatory responses during childhood allergy.

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Chapter 3 Respiratory syncytial virus enhances respiratory allergy in mice despite the inhibitory effect of virus-induced interferon-γ.

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3.1 Abstract

In mice, respiratory syncytial virus (RSV) infection during allergic provocation aggravates the allergic Th2 immune response, characterised by production of interleukin (IL)-4, IL-5, and IL-13, and eosinophilic inflammation. This enhancement of the Th2 response occurs simultaneously with a strong RSV-induced Th1 cytokine response (IL-12 and IFN-γ). The present study investigated whether IFN-γ and IL-12 are critically involved in this RSV-enhanced OVA-allergy. Therefore, IFN-γR- and IL-12-deficient mice (both on a 129/Sv/Ev background) were sensitised and challenged with ovalbumin (OVA) and infected with RSV during the OVA-challenge period. Neither gene deletion affected the development of ovalbumin-induced allergic inflammation in mice. However, when OVA-allergic IFN-γR deficient mice were infected with RSV, an increased pulmonary eosinophilic infiltrate and increased IL-4 and IL-13 mRNA expression in lung tissue were observed compared to identically treated wild type mice. In contrast, deficiency of IL-12 did not aggravate the Th2 immune and inflammatory response in OVA/RSV-treated mice compared to the wild type. In conclusion, the virus-induced IFN-γ response diminishes the Th2 inflammatory response during OVA-allergy, but fails to totally prevent the enhancement of the OVA-allergy by RSV. In contrast, IL-12 is not involved in inhibiting nor increasing the RSV-enhanced allergy in 129/Sv/Ev mice.

3.2 Introduction

Respiratory syncytial virus (RSV, a member of the pneumovirinae subfamily of the *Paramyxoviridae*) is a frequent cause of lung disease in infants and young children. The symptoms of RSV infection vary from mild upper respiratory tract illness to severe bronchiolitis and pneumonia [40]. Vaccine trials using formalin-inactivated RSV showed devastating effects, causing more severe lung disease and higher mortality after subsequent RSV infection [104]. In mice, this vaccine-enhanced disease is related with a T helper (Th) 2-like immune response (characterised by the production of interleukin (IL)-4, IL-5 and eosinophilic inflammation), while primary RSV infection resulted in a dominant Th1 immune response (characterised by the production of IL-12 and interferon-γ (IFN-γ)) [17;152;208].

Allergic asthma is associated with Th2 immune responses [174]. Because severe RSV infection may induce asthma-like symptoms (among others wheezing, airway hyperreactivity), epidemiological studies have been carried out to investigate the role of RSV infections in respiratory allergy. Some researchers reported an association between RSV infections with development and aggravation of allergic disease in children [184;195]. However, others have not replicated such findings [194]. Recently, we showed in a mouse model of allergic asthma,

that RSV infection aggravates the consequences of airway sensitisation and challenge with ovalbumin (OVA), resulting in enhanced pulmonary eosinophilic influx and Th2 cytokine expression in lung tissue. In addition, the RSV-induced Th1 response, characterised by IFN-γ and IL-12, occurred simultaneously with enhancement of the OVA-induced Th2 response. Thus, although IFN-γ is known to inhibit the Th2 response, the virus induced IFN-γ appeared insufficient in modulating the allergic Th2 response [7].

IL-12 is important in the initial phase of bacterial, parasitic, and viral infections, and stimulates the development of the Th1 type immune response, while inhibiting differentiation of Th2 cells [201;227]. Th1 cells produce, among others, IFN-γ, which has direct antiviral activity *in vitro* and is also a strong inhibitor of the Th2 cytokine response [16].

Boelen et al.[18] already delineated the role of IFN-γ and IL-12 in a primary RSV infection model, and concluded that IFN-γ receptor signalling is required for a pronounced Th1 response after RSV infection and suppression of the Th2 immune response, while IL-12 is not. Because the roles of IFN-γ and IL-12 are still unravelled in the mechanism of RSV-induced enhancement of the OVA-allergic responses, the present article further delineated the role of these cytokines in the OVA-RSV model. Based on previous observations, i.e. RSV-enhanced allergy despite the presence of IFN-γ, we hypothesised that IFN-γ and IL-12 do not limit the RSV-enhanced Th2 response in OVA-allergy. Therefore, mice that are genetically deficient in the IFNγ receptor (R) or IL-12 were sensitised and challenged with OVA, and infected with RSV during the OVA challenge period. The pulmonary eosinophilic inflammation, serum IgE, and lung cytokine mRNA responses were determined and the results were compared with identically treated corresponding wild type mice. The results indicate that the virus-induced IFN-γ response diminished the Th2 inflammatory response during OVA-allergy, but this is insufficient to prevent the allergy enhancement by RSV.

3.3 Methods

Virus: Human Respiratory Syncytial virus stock A2 (RSV A2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The virus was grown and quantified on HEp-2 cells and a stock was prepared as described previously [18].

Animals: Female knockout mice and matching wild type controls were used at 6-10 weeks of age. IL-12p40/35$^{-/-}$ mice (on a 129/Sv/Ev background) were generated by Magram et al. [123]. IFN-γR$^{-/-}$ mice (on a 129/Sv/Ev background) were generated by Huang et al. [89]. Wild type 129/Sv/Ev and BALB/c mice were obtained from the breeding colony of our own institute (RIVM, Bilthoven, The Netherlands). The mice were kept in a 12-hour light/dark cycle, in a temperature-controlled room (22°C), and received food and water *ad libitum*. A week before the

experiments started, mice were housed in groups according to the experimental set-up. The study was approved by the Institute's committee on animal welfare.

Experimental Design: Mice were sensitised to OVA by intraperitoneal (i.p.) administration of 0.5 ml OVA (20 μg/ml saline, no adjuvant), for 7 times on every other day starting at day 0. From day 33 till day 40, mice were intranasally challenged by OVA aerosols (2 mg/ml saline) during 5 minutes on 8 consecutive days [7;83].
To examine the influence of RSV infection on OVA-allergy in the cytokine knock-out and wild type mice, OVA-sensitised mice were inoculated intranasally with 10^7 plaque forming units (in 50 μl) RSV on day 35, during the OVA-challenge period (= OVA/RSV). These mice were compared to OVA-allergic mice inoculated with uninfected culture lysate (mock) (= OVA/Mock), and non-allergic mice infected with RSV (= -/RSV). Before inoculation, mice were anaesthetised with halothane. At days 41 and 43 of the experimental protocol (days 6 and 8 post RSV inoculation), mice were anaesthetised with barbiturate/chloralhydrate i.p. and sacrificed. Two different time points for determination were chosen in order to detect a prolonged inflammatory response. Blood was collected via orbital puncture. The lungs were removed. The right lung was snap-frozen in liquid nitrogen and stored till it was further processed. The left lung was fixed intratracheally with formalin and immersed in fixative. Each experiment was performed in duplicate. Data of one experiment are shown.

Histopathologic examination: Formalin-fixed lungs were embedded in paraplast (Monoject, Kildare, Ireland). Transverse sections of 5 μm were stained with haematoxylin and eosin. Various lung lesions were scored semiquantitatively in a from absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5). Histological scoring was done blind by an independent observer.

RNA isolation and RT-PCR: Total cellular RNA was isolated from frozen lung samples using Trizol Reagens (GibcoBRL, Life technologies, Rockville, USA) according to the manufacturer's protocol with modifications as described by Kodavanti et al. [109]. 10 μl of total lung RNA was reverse transcribed and cytokine PCR reactions (IL-4, IL-5, IL-13, IFN-γ and IL12) on the cDNA were carried out as described previously [17]. To prevent inter-assay variation, PCRs of all samples from one experiment were done simultaneously and loaded on the same agarose gel for quantification. Band intensities were analysed using the Molecular Analyst software system (Bio-Rad Laboratories, Hercules, CA). All results were corrected for mRNA content of the sample using β-actin mRNA as a standard. β-Actin mRNA was amplified using the mouse control amplifier set (Clontech, Palo Alto, CA) according to the manufacturer's protocol.

IgE antibodies in serum: Total and OVA-specific IgE antibodies in serum were determined using a modified capture ELISA according to the protocol of Van Halteren et al. [207]. As coating antibody, rat anti-mouse IgE monoclonal antibody EM-95 was used [5]. Total IgE was detected

using biotinylated rat-anti-mouse IgE (Pharmingen, San Diego, USA). OVA-IgE was detected with digoxigenin-coupled ovalbumin with recombinant mouse IgE as a standard (Pharmingen, San Diego, CA). Total IgE concentrations were calculated by interpolation from a standard titration curve with known concentrations of recombinant mouse IgE (Pharmingen, San Diego, CA). The detection limit of the total IgE assay was 900 pg/ml. OVA-specific IgE levels were expressed as the optical density at 490 nm of a 1/32 diluted serum.

Statistical analysis: Data are presented as mean ± Standard Error of the Mean (SEM). Statistical significance for differences in cytokine ratios and IgE concentrations was determined using the Student's *t* test (Excel, Microsoft Corporation, Redmond, USA), and for histological scores using the nonparametric Wilcoxon's test (SAS, SAS Institute Inc., USA).

3.4 Results

Development of OVA allergy in 129/Sv/Ev wild type mice

Because the IFN-γR$^{-/-}$ and IL-12$^{-/-}$ mutations were generated in 129/Sv/Ev mice, the development of OVA-allergy in these mice was examined first and results were compared with those obtained in BALB/c mice. Our previous study showed that OVA-treatment induced eosinophilic influx and increased Th2 cytokine (IL-4, IL-5, IL-13) mRNA expression in lung tissue of BALB/c mice compared to saline-treated BALB/c mice [7]. In contrast, OVA-treatment in 129/Sv/Ev mice caused only minimal eosinophilic infiltration and Th2 cytokine response. Total-IgE production was almost 10 times higher in 129/Sv/Ev compared to BALB/c mice. In both strains, OVA-specific IgE was only produced after OVA-treatment, but the OVA-specific IgE response was 5 times higher in OVA-treated BALB/c mice compared to the response in OVA-treated 129/Sv/Ev mice (data not shown). Thus, expression of allergy, as determined by histopathology, IgE responses, and cytokine expression levels, was less in 129/Sv/Ev mice than in BALB/c mice, and was confined to the production of OVA-specific IgE.

RSV-enhanced OVA-allergy in 129/Sv/Ev mice

To investigate if RSV infection was able to enhance the allergic response in 129/Sv/Ev mice (similarly as in BALB/c mice [7]), the immune and inflammatory responses after OVA/RSV-treatment were determined and the results were compared with those obtained in OVA/Mock-treated 129/Sv/Ev mice. RSV infection in OVA-treated mice did not change the OVA-specific IgE nor total-IgE levels in serum (table 1). However, OVA/RSV-treatment induced a marked to severe hypertrophy of the mucus-producing bronchiolar cells in contrast to the slight response after OVA-treatment alone. RSV did not affect the eosinophilic influx in the

peribronchiolar space (data not shown). Furthermore, OVA/RSV-treated 129/Sv/Ev mice showed statistically significant enhanced IL-4, IL-5, and IL-13 mRNA expression in the lungs, compared to OVA-treatment alone (data not shown). In addition, the cytokine expression was prolonged. Thus, despite the poor expression of OVA-induced allergy in 129/Sv/Ev mice, RSV enhanced and prolonged the Th2 cytokine expression patterns in the lungs of allergic 129/Sv/Ev mice.

Table 3.1. Influence of IL-12 and IFN-γR deficiency on IgE responses in serum of ovalbumin sensitised/challenged mice, inoculated with RSV (OVA/RSV) or Mock (OVA/Mock).

	Total IgE (μg/ml ± SEM)			OVA-IgE (OD490 ± SEM)		
Treatment	WT	IL-12$^{-/-}$	IFN-γR$^{-/-}$	WT	IL-12$^{-/-}$	IFN-γR$^{-/-}$
OVA/RSV	2.08 ± 0.43	3.03 ± 0.82	1.36 ± 0.20	0.385 ± 0.102	0.567 ± 0.119	0.206 ± 0.047
OVA/Mock	1.72 ± 0.28	2.19 ± 0.31	1.42 ± 0.28	0.251 ± 0.045	0.411 ± 0.059#	0.188 ± 0.045

Serum concentrations are measured at day 43 of the experimental protocol (day 8 post RSV infection). Mean values ± SEM are shown (n=6). Statistical differences ($p < 0.05$) are indicated with; #: between wild type (WT; 129/Sv/Ev) and knockout mice.

Development of OVA-induced allergy in IFNγReceptor and IL-12 deficient 129/Sv/Ev mice.

Subsequently the development of OVA-allergy in IFN-γR$^{-/-}$ and IL-12$^{-/-}$ mice compared to identically treated 129/Sv/Ev wild type mice was studied. The only difference observed between OVA-treated knock-out and wild type mice was an increased concentration of OVA-specific IgE in serum of IL-12$^{-/-}$ mice compared to the wild type (table 3.1). However, the cytokine mRNA expression and histopathologic lesions in lung tissue were similar in OVA-treated IFN-γR$^{-/-}$, IL-12$^{-/-}$, and wild type mice (table 3.2).

RSV-enhanced OVA-allergy in IFNγReceptor and IL-12 deficient mice.

Finally, the effect of IFNγR and IL-12 deficiency on the immunologic and inflammatory response after RSV infection of OVA-allergic mice was examined.

Serum total- and OVA-specific IgE: Serum concentrations of total-IgE and OVA-specific IgE were similar in OVA/RSV-treated IFN-γR$^{-/-}$ and IL-12$^{-/-}$ mice, compared to identically treated wild type mice (table 3.1).

Histopathological pulmonary lesions (fig. 3.1): OVA/RSV-treatment in wild type mice induced a moderate alveolitis and peribronchiolitis, marked hypertrophy of the mucous bronchiolar cells, and minimal eosinophilic inflammation. These pulmonary lesions were not different in OVA/RSV-treated IL-12$^{-/-}$ mice. In contrast, an increased influx (from minimal to moderate) of eosinophils in the lung tissue was observed in IFNγR$^{-/-}$ mice compared to wild type mice (on day 41: $p < 0.01$, on day 43: $p < 0.001$). Also an increase in hypertrophy of the mucus-producing epithelial cells was observed in IFNγR$^{-/-}$ mice compared to wild type mice ($p < 0.01$) on day 43 of the experimental protocol.

Table 3.2 Allergic responses in wild type, IFN-γR$^{-/-}$ and IL-12$^{-/-}$ mice.

		WT	IFN-γR$^{-/-}$	IL-12$^{-/-}$
IL-4	Exp. A	0.040 ± 0.016	0.006 ± 0.002	
	Exp. B	0.066 ± 0.032		0.059 ± 0.024
IL-5	Exp. A	0.481 ± 0.205	0.279 ± 0.044	
	Exp. B	0.264 ± 0.016		0.394 ± 0.119
Eosinophilic influx		1.1 ± 0.3	1.3 ± 0.5	0.5 ± 0.3
Hypertrophy mucous bronchiolar cells		2.3 ± 0.3	2.5 ± 0.3	2.6 ± 0.4

Cytokine mRNA expression ratios (against β-actin) (n=6) and histopathological scores (n=10-16) in lung tissue of ovalbumin sensitised/challenged and Mock inoculated (OVA/Mock) 129/Sv/Ev wild type (WT), IFN-γR$^{-/-}$ and IL-12$^{-/-}$ mice, determined at day 43 of the experimental protocol. Histopathological data originate from several identical experiments. Cytokine data originate from one representative experiment. Mean values ± SEM are shown.

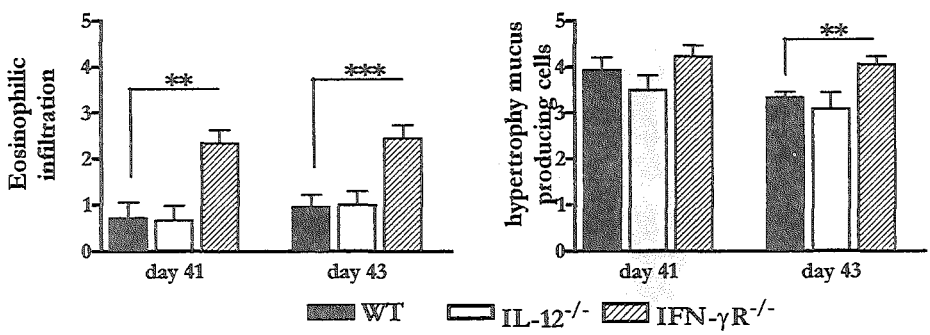

Figure 3.1. Pulmonary eosinophilic infiltration and hypertrophy of mucous epithelial cells in lungs of ovalbumin sensitised/challenged and RSV infected (OVA/RSV) wild type 129/Sv/Ev mice (black bars), IL-12$^{-/-}$ (grey bars), and IFN-γR$^{-/-}$ mice (dashed bars). Lung pathology was analysed on day 41 and 43 of the experimental protocol, and scored semiquantitatively from minimal (1), slight (2), moderate (3), marked (4), to severe (5). Histological data are originating from several identical experiments. Mean values ± SEM (n = 12-24 per group) are depicted. Statistical differences between wild type and knock-out mice are indicated; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Pulmonary cytokine mRNA expression: RSV infection induced a two-fold increase in expression of IL-4 and IL-13 mRNA (both $p < 0.001$) in OVA/RSV-treated IFN-γR$^{-/-}$ mice on day 43 compared to wild type mice, but not on day 41 of the experimental protocol (fig. 3.2). Thus, IL-4 and IL-13 cytokine responses induced by OVA/RSV-treatment were prolonged in IFN-γR$^{-/-}$ mice compared to wild type mice, in contrast to the IL-5 mRNA expression which did not differ (fig. 3.2).

No differences in IL-4 and IL-5 mRNA expression were observed in OVA/RSV-treated IL-12$^{-/-}$ mice compared to the corresponding wild type mice. However, a significant decrease in IL-13 mRNA expression was observed in OVA/RSV-treated IL-12$^{-/-}$ mice on day 43 compared to wild type mice ($p < 0.05$) (fig. 3.3).

Moreover, the Th1 cytokine mRNA response was affected in OVA/RSV-treated IL-12$^{-/-}$ mice. A decrease in IFNγ mRNA expression was observed in OVA/RSV-treated IL-12$^{-/-}$ mice compared to wild type mice on both day 41 and 43 (fig. 3.3).

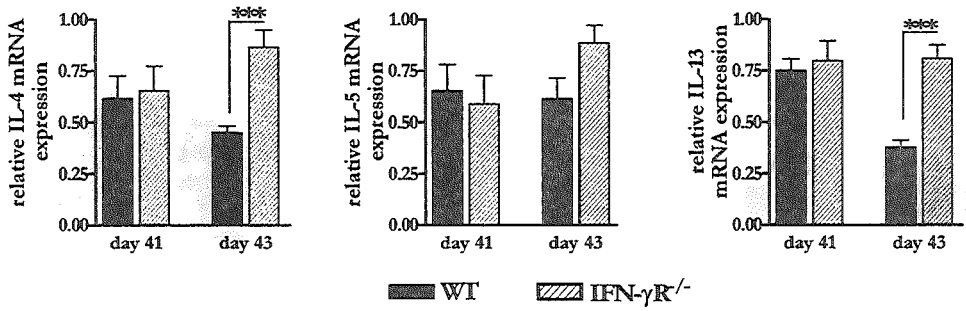

Figure 3.2. Relative expression (against β-actin) of lung IL-4, IL-5 and IL-13 mRNA in ovalbumin sensitised/challenged and RSV infected (OVA/RSV) IFN-γR$^{-/-}$ mice (dashed bars) compared to the wild type (black bars). Analysis were performed at day 41 and 43 of the experimental protocol (is 6 and 8 days post RSV-infection). Mean values ± SEM ($n = 6$ per group) are depicted. Statistical differences between wild type and knock-out mice are indicated; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.5 Discussion

RSV infection during the provocation of an allergic inflammation resulted in a more pronounced Th2-like immune and inflammatory response in BALB/c mice, together with a virus-induced IFN-γ and IL-12 mRNA expression in lungs [7]. Simultaneous occurrence of an enhanced Th2 response and increased IFN-γ mRNA suggested that IFN-γ has a minor role in inhibiting the RSV-enhanced OVA-allergy. In order to further unravel the role of IFN-γ and IL-12 in the RSV-enhanced OVA-allergy, the cytokine and inflammatory responses in IFN-γR and IL-12 knock out mice were investigated. IL-12 is an important cytokine in the development

of a Th1 immune response by stimulating IFN-γ production of Th1 and NK cells [201]. IFN-γ stimulates the Th1 response and suppresses the Th2 response by inhibiting IL-4 secretion [16]. Since IFN-γ and IL-12 both are involved in immune regulation of the Th1/Th2 balance, one would expect that OVA-sensitisation and challenge might result in a more pronounced Th2 immune and inflammatory response in IFN-γR$^{-/-}$ or IL-12$^{-/-}$ mice [46]. However, in this study

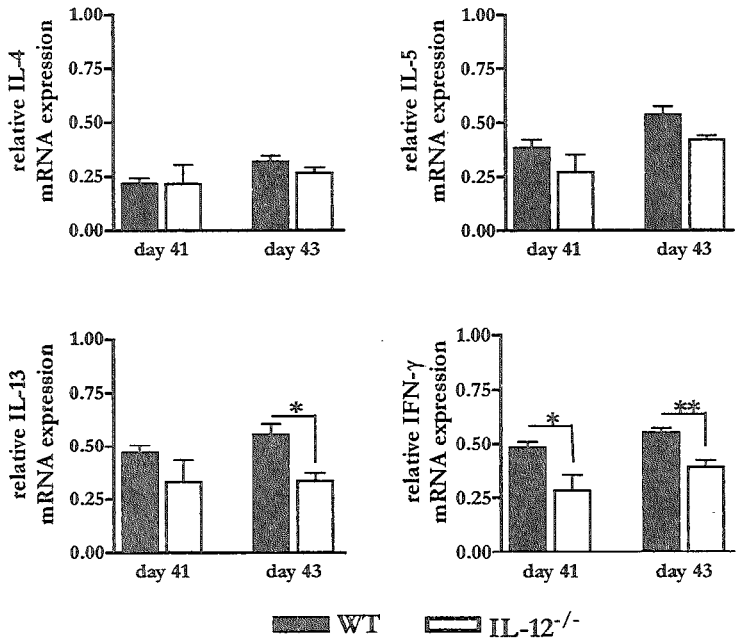

Figure 3.3. Relative expression (against β-actin) of lung IL-4, IL-5, IL-13 and IFN-γ mRNA in ovalbumin sensitised/challenged and RSV infected (OVA/RSV) IL-12$^{-/-}$ mice (grey bars) compared to the wild type (black bars). Analysis were performed at day 41 and 43 of the experimental protocol (is 6 and 8 days post RSV-infection). Mean values ± SEM (n = 6 per group) are depicted. Statistical differences between wild type and knock-out mice are indicated; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

OVA-treatment did not result in differences in Th2 responses between IFN-γR$^{-/-}$, IL-12$^{-/-}$ and wild type mice, indicating that IFN-γ and IL-12 do not play a major regulatory role in the OVA-allergic response itself. Bruselle et al. also demonstrated in a murine model of allergic asthma that IFNγR deficiency (on a 129/Sv x C57bl/6 background) did not influence the development of the Th2 immune responses [23]. It seems obvious therefore that IFN-γ skews the balance towards Th1 only when the IFN-γ signalling pathway is induced. Evidently, when the IFN-γ pathway is not activated, no effects may be expected from IFN-γR inactivation.
RSV infection enhanced the pulmonary eosinophilic inflammation and hypertrophy of mucus producing cells in OVA-treated IFN-γR deficient mice, compared to wild type mice. Also a

prolonged expression of lung IL-4 and IL-13 mRNA was observed compared to the wild type mice. These results indicate that the immune response in OVA/RSV-treated IFN-γR$^{-/-}$ mice is more Th2 skewed compared to the wild type mice. Hence, RSV-induced IFN-γ diminished IL-4 and IL-13 production and histopathologic changes.

Schwarze et al. [177] also investigated the effect of RSV-enhanced allergic sensitisation in IFN-γ$^{-/-}$ mice. In conflict with our results, they did not observe increased pulmonary eosinophilic infiltration in OVA/RSV-treated IFN-γ$^{-/-}$ mice, compared to the wild type (BALB/c). However, it is difficult to compare these data with those presented in our paper, because of the different genetic background of the mice. Furthermore, Schwarze et al. used IFN-γ$^{-/-}$ mice instead of IFN-γR$^{-/-}$ mice, and these authors sensitised mice to OVA after recovery from acute RSV infection instead of infection during OVA-challenge.

Because IFN-γ is an inhibitor of the Th2 immune response [16], lack of IFN-γR may result in an unrestrained Th2 response [198], at least when the IFN-γ signalling pathway is activated (see above). Boelen et al. [18] already showed in a primary RSV infection model that the absence of IFN-γR resulted in a shift towards a Th2 immune response, demonstrating that IFN-γ inhibits the development of a Th2 response upon RSV infection. In the series of experiments presented in this article also a non-allergic group only infected with RSV was incorporated. As reported previously, in IFN-γR$^{-/-}$ mice, RSV infection alone resulted also in a shift towards a Th2 immune response. However, the OVA/RSV treated animals always displayed a more pronounced Th2 response (data not shown). Nonetheless, in this article we hypothesised that IFN-γ has only a minimal role in the RSV-enhanced allergic Th2 response, because similar expression of IFN-γ mRNA was observed in allergic and non-allergic mice infected with RSV [7]. This indicated that an RSV-enhanced allergic Th2 response could occur despite the presence of IFN-γ. Interestingly, the results in the present study show that the absence of IFN-γ signalling resulted in an enhanced Th2 response. Therefore it appears that the virus infection has a dual role in influencing respiratory allergy. The RSV-induced IFN-γ response restricts the Th2 response, but this response appears insufficient in completely inhibiting the RSV-enhanced respiratory allergy. Thus, RSV enhances the allergic inflammatory and Th2 cytokine responses by an IFN-γ independent pathway. Possible mechanisms responsible for the IFN-γ-independent effects of RSV on the allergic responses could be the interaction of RSV with the respiratory epithelial cells resulting in enhanced production of β-chemokines (a.o. MIP-1α, eotaxin, RANTES) [221] and expression of ICAM-1 [214], both involved in allergic inflammation. Furthermore, RSV infection may increase the permeability of the epithelial barrier, which makes it easier for OVA to enter the tissue [64].

In contrast to IFN-γR deficiency, deficiency of IL-12 did not aggravate the Th2 immune and inflammatory response in OVA/RSV-treated mice compared to wild type mice, although some diminishing effect on the IFN-γ response was observed. The same observation was done by Jankovic and colleagues who infected IL-12 deficient mice with *Mycobacterium avium*, *Toxoplasma gondii*, or *Schistosoma mansoni*. These authors also found no increased Th2 immune response in IL-12 deficient mice compared to the wildtype mice (BALB/c) [92]. One explanation for the

unaffected IL-4 and IL-5 responses in IL-12$^{-/-}$ mice is that another cytokine took over the role of IL-12 to compensate its absence. A likely candidate is IL-18, because IL-18 is, like IL-12, able to induce IFN-γ production by NK cells [129]. Apparently, IFN-γ has a pivotal effect on the Th2 immune response, and no compensatory mechanism is able to take over its function.
In these experiments 129/Sv/Ev mice responded less sensitive to OVA-sensitisation and challenge compared to BALB/c mice. Host genetic determinants thus appears to play a role in this mouse model of allergic sensitisation. A similar genetic influence is also described by De Sanctis and Drazen [50], who measured airway hyperreactivity in different mouse strains and showed clear differences between various inbred strains. Furthermore, Hussell et al. [90] reported a relationship between the genetic background of mice in MHC haplotype ($H2^d$ for BALB/c and $H2^b$ for 129/Sv/Ev mice) and RSV disease severity. Taken together, our results show that host genetic background, virus infection and the IFN-γ pathway determine the outcome of allergic inflammation.
In summary, RSV enhances OVA-allergy in BALB/c mice, as well as in 129/Sv/Ev mice. Both IFN-γR and IL-12 are not involved directly in the development of OVA-allergy in 129/Sv/Ev mice, which appears a purely Th2-regulated disease. However, IFN-γR deficiency further aggravated RSV-enhanced OVA-allergy, while absence of IL-12 did not. In conclusion, the virus-induced IFN-γ response diminished the Th2 inflammatory response during OVA-allergy, but this was not sufficient to prevent the allergy enhancement by the viral infection. Thus, RSV-infection, in addition to down-regulating Th2 responses by IFN-γ, enhances OVA-allergy by an IFN-γ independent pathway.

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Chapter 4 Respiratory Syncytial Virus-enhanced allergic inflammation depends on timing of infection and is affected by prior RSV immunisation.

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4.1 Abstract

Respiratory syncytial virus (RSV) infection has been shown to be a risk factor for the development of allergy in human and mice. The allergy-enhancing property of RSV may be dependent on atopic background and RSV infection history of an individual. We examined the influence of moment of infection and prior RSV immunisation in a mice model of allergic asthma. Mice were sensitised and challenged with ovalbumin (OVA) and inoculated with RSV before or during the sensitisation or challenge period. One group of mice received RSV both before OVA-sensitisation and during OVA-challenge. Only when OVA-sensitised mice were inoculated with RSV shortly before or during OVA challenge, increased pulmonary IL-4, IL-5 and IL-13 mRNA expression, and aggravated alveolitis and hypertrophy of mucus-producing cells were observed. Despite protection against virus replication, prior RSV infection did not abrogate the RSV-enhanced OVA-induced Th2 cytokine expression in the lung. In conclusion, RSV infection only enhanced allergic disease when the immune system has already been Th2 primed by the allergen (OVA). This RSV-enhanced allergy is not completely abrogated by previous RSV infection.

4.2 Introduction

Respiratory Syncytial Virus (RSV, member of the family *Paramyxoviridae*, genus *Pneumovirinae*) is the most common cause of respiratory tract infections in young children [62]. The clinical presentation can vary from mild upper respiratory tract illness to severe bronchiolitis and pneumonia [40]. Epidemiological data show that almost all children become infected with RSV in their first or second year of life, and that the incidence of RSV-bronchiolitis reaches a maximum at the age of two months [103]. Because severe RSV infection induces asthma-like symptoms (among others wheezing and airway hyperreactivity), studies have been performed to investigate the impact of RSV bronchiolitis on the development of respiratory allergy and asthma in children. So far, controversial results have been reported [31;167;184;191;194;224]. Consequently the precise role of RSV infection in the development of allergic disease in children is not clear.

Allergic asthma is a T-helper 2 (Th2)-associated airway inflammation, characterised by the infiltration of eosinophils, and the production of interleukin (IL)-4, IL-5, and IL-13 by T lymphocytes in response to certain antigens [26;174]. In contrast, RSV infection predominantly induces a Th1 immune response, characterised by the production of IL-12 and interferon-γ (IFN-γ) [17;202]. We [7] and others [163] previously demonstrated in a mouse model of allergic asthma, that RSV infection, given during the provocation phase of allergen exposure, aggravates the consequences of airway sensitisation and challenge with ovalbumin (OVA), resulting in enhanced pulmonary Th2 cytokine expression, allergy-associated lung pathology,

and airway hyperresponsiveness. In addition, the RSV-induced Th1 response remains unchanged [7].
Several studies suggested that the time interval between infection and allergen exposure may be critical in determining whether viral [203;229] or bacterial [39] infection will influence the development of respiratory allergy in mice. To gain more insight into the mechanisms of RSV-enhanced OVA-allergy, we investigated whether the moment of RSV infection, i.e. before or during the period of allergen sensitisation and challenge, determines the outcome of allergic disease in mice.
As mentioned before, most children become infected with RSV in their first or second year of life [103]. Since immunity against RSV is only partial, re-infections occur throughout life [40]. This means that an RSV infection shortly before or during allergen-challenge might have been preceded by prior RSV infection episodes, for example before allergen-sensitisation. The RSV infection history could modulate virus replication and the immune response during a second RSV infection, thus influencing its potential effect on respiratory allergy. Whether prior RSV immunisation enhances, or protects against RSV-enhanced OVA-allergy needs to be investigated. In the present study we therefore inoculated mice with RSV prior to OVA-sensitisation and -challenge and re-infected them during the OVA-challenge. The cytokine and inflammatory data obtained from the experiments presented in the current article lead to a better understanding of the mechanism by which RSV affects respiratory allergy.

4.3 Methods

Virus: Human respiratory syncytial virus stock A2 (RSV A2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The virus was cultured on HEp-2 cells (ATCC, Rockville, MD) in medium (RPMI 1640, Gibco BRL, Life Technologies, Rockville, MD) containing 10% heat-inactivated foetal calf serum (FCS, Greiner, Frickenhausen, Germany), 2mM glutamine, 100 IE/ml penicillin, and 100 U/ml streptomycin, as described before[18] The virus inoculum contained 3 x 10^6 plaque forming units (pfu) RSV/ml as assessed by quantitative plaque-forming assay [18].

Animals: Female BALB/c mice were obtained from Harlan Olac (Harlan, Horst, The Netherlands) and were used at 6-10 weeks of age. A week before the experiments started, mice were housed per group according to the experimental set-up in a temperature-controlled animal room. Mice were kept in a 12-hour light/dark cycle under pathogen free conditions, and received food and water *ad libitum*. The institute's committee on animal welfare approved the study.

Experimental Design: Mice were sensitised to OVA by intraperitoneal (i.p.) administration of 0.5 ml OVA (20 μg/ml OVA (Grade II, Sigma-Aldrich, Steinheim, Germany) in saline) for 7 times on every other day starting at day 0. From day 33 till day 40, mice were challenged with OVA aerosols (2 mg/ml saline) during 5 minutes on 8 consecutive days [7;83].

To examine the influence of the time point of RSV infection during the sensitisation / challenge protocol, OVA-sensitised mice were inoculated intranasally with RSV ($1.5x10^7$ plaque forming units (pfu) in 50 μl) at different time points according to the schedule in fig. 4.1A. The infected mice were compared to OVA-allergic mice inoculated with uninfected culture lysate (mock) at the same time point. To investigate the influence of prior RSV immunisation on RSV-enhanced allergy, one group received $1.5x10^7$ pfu RSV on both day 0 and day 35 of the experimental protocol (fig. 4.1B). Before inoculation, mice were anaesthetised with halothane. RSV-enhanced lesions are best analysed at day 43 of the experimental protocol (day 3 after the last OVA-challenge) [7], therefore mice were anaesthetised at that day with Ketamine Rompun (=xylazine) Atropine (KRA) i.p. and sacrificed. Blood was collected via orbital punction. The lungs were removed. The right lung was snap-frozen in liquid nitrogen and stored till further process. The left lung was intratracheally fixed with formalin and immersed in fixative.

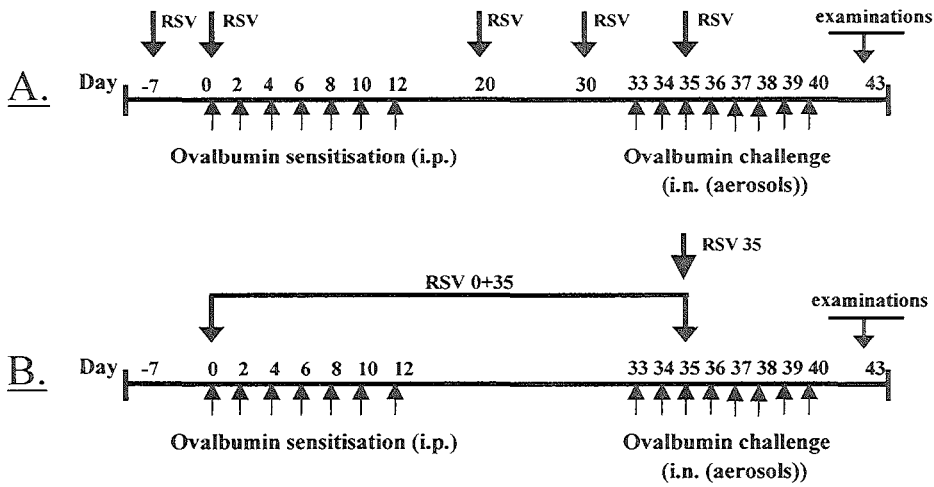

Figure 4.1. Study design.
A: To investigate the influence of RSV infection time point on RSV-enhanced OVA-allergy, ovalbumin (OVA)-sensitised and challenged BALB/c mice received 10^7 pfu RSV on either day – 7, 0, 20, 30 or 35 (upper arrows).
B: To examine the influence of prior RSV infection on RSV-enhanced OVA-allergy, OVA sensitised and challenged mice received $1,5x10^7$ pfu RSV on both day 0 and day 35

Histopathologic examination: Formalin-fixed lungs were embedded in paraplast (Monoject, Kildare, Ireland). Transverse sections of 5 μm were stained with haematoxylin and eosin. An independent observer examined the slides in a blinded fashion for: peribronchiolitis (infiltration

of inflammatory cells in the peribronchiolar space), alveolitis (infiltration of inflammatory cells in the alveolar wall), perivasculitis (infiltration of inflammatory cells in the perivascular space), hypertrophy of mucous producing glands, and eosinophilia. Lung lesions were scored semiquantitatively from absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5).

RNA isolation and RT-PCR: Total cellular RNA was isolated from frozen lung samples using Trizol Reagent (GibcoBRL, Life Technologies, Rockville, USA). 10 µl of total lung RNA was reverse transcribed and cytokine PCR reactions (IL-4, IL-5, IL-13, IFN-γ and IL12) on the cDNA were carried out as described previously [17]. Eotaxin RT-PCR was carried out according to the same protocol as used for the cytokine RT-PCR reactions using sense (CCCCAACACACTACTGAAGA) and antisense (CTACATGAAGCCAAGTCCTT) primers. To prevent inter-assay variation, PCRs of all samples from one experiment were done simultaneously and loaded on the same agarose gel for quantification. Band intensities were analysed using the Molecular Analyst software system (Bio-Rad Laboratories, Hercules, CA). All results were corrected for mRNA content of the sample using β-actin mRNA as a standard. β-Actin mRNA was amplified using the mouse control amplifier set (Clontech, Palo Alto, CA) according to the manufacturer's protocol. For quantification of the RSV-load in lung tissue, RT-PCR was performed using RSV F-protein specific primers (upper primer: TTA ACC AGC AAA GTG TTA GA, lower primer: TTT GTT ATA GGC ATA TCA TTG) as previously described by Paton et al. [157]. The PCR product was analysed by detecting the band intensities on the agarose gel using the Molecular Analyst software system (Bio-Rad Laboratories, Hercules, CA).

IgE antibodies in serum: Total and OVA-specific IgE antibodies in serum were determined using a modified capture ELISA according to the protocol of Van Halteren et al. [207]. As coating antibody, rat anti-mouse IgE monoclonal antibody EM-95 was used [5]. Total IgE was detected using biotinylated rat-anti-mouse IgE (Pharmingen, San Diego, USA). Total IgE concentrations were calculated by interpolation from a standard titration curve with known concentrations of recombinant mouse IgE (Pharmingen, San Diego, CA). The detection limit of the total IgE assay was 900 pg/ml. OVA-IgE was detected with digoxigenin-coupled ovalbumin. OVA-specific IgE levels were expressed as the optical density at 490 nm of a 1/32 diluted serum.

Statistical analysis: Data are presented as mean ± Standard Error of the Mean (SEM). Statistical significance for differences in cytokine ratios and IgE concentrations was determined using the Student's *t* test (Excel, Microsoft Corporation, Redmond, USA), and for histological scores using the nonparametric Wilcoxon's test (SAS, SAS Institute Inc., USA).

4.4 Results

Influence of RSV-infection time point on respiratory allergy

We previously observed that RSV, when inoculated during OVA-challenge, enhances OVA-induced pulmonary infiltration of inflammatory cells and mRNA expression of Th2 cytokines IL-4, IL-5 and IL13 [7]. In the present study, we examined whether the timing of infection during allergen sensitisation and challenge is critical. Mice were therefore inoculated with RSV before or during OVA-sensitisation, and before or during OVA-challenge (fig. 4.1A).

As shown in figure 4.2, RSV enhanced the mRNA expression of IL-4 and IL-13 in lung tissue of OVA-allergic mice only when infection is given shortly before (day 30) or during (day 35) the OVA-challenge, compared to their corresponding OVA/mock-treated controls. Additionally, RSV-inoculation on all 3 time points after OVA-sensitisation (day 20, 30, and 35) enhanced pulmonary IL-5 mRNA expression in OVA-allergic mice. Simultaneously with the enhanced Th2 cytokine mRNA expression, RSV infection given after OVA-sensitisation (day 20, 30, or 35) induced enhanced Th1 cytokine mRNA expression (IFN-γ and IL-12) in OVA-allergic mice, compared to the corresponding OVA/mock-treated mice (fig. 4.2). In addition, pulmonary IL-4, IL-13 and IFN-γ mRNA expression was significantly higher in allergic mice inoculated on day 30 or 35 compared to the other time points of RSV inoculation.

In parallel with the increased Th2 cytokine mRNA expression, RSV only enhanced hypertrophy of mucus producing epithelial cells when inoculation was given shortly before (day 30) or during (day 35) the OVA-challenge period, compared to the corresponding OVA/mock-treated mice. Likewise, the alveolar inflammation was only significantly increased compared to OVA/mock-treated mice when RSV-inoculation was given on day 20, 30 or 35. In addition, alveolitis and hypertrophy of mucus producing epithelial cells were significantly higher in lungs of allergic mice inoculated with RSV on day 35, compared to the other time points of virus inoculation. In contrast, no relation between increased levels of Th2 cytokines and peribronchiolar and perivascular inflammation was observed. Finally, pulmonary eosinophilic infiltration could only be observed in OVA-allergic mice inoculated with RSV on day 35. However, there was no difference between the OVA/mock and OVA/RSV groups, indicating that RSV inoculation did not affect the OVA-induced eosinophilia in this study. The lung pathology of OVA-sensitised and challenged mice inoculated with RSV on day 35 is illustrated in figure 4.3 (Appendix A).

To further elucidate the influence of RSV infection on OVA-allergy, total and OVA-specific IgE levels were determined in serum at day 43 of the experimental protocol. OVA sensitisation and challenge resulted in the production of OVA-specific IgE in serum. As previously demonstrated [7], RSV infection during the OVA-challenge period did not increase the production of total- and OVA-specific IgE, compared to the corresponding OVA/mock-treated mice (data not shown). However, RSV inoculation on day 35 was associated with significantly higher levels of both total (2,10±0,45 μg/ml) and OVA-specific IgE (1,284±0,092

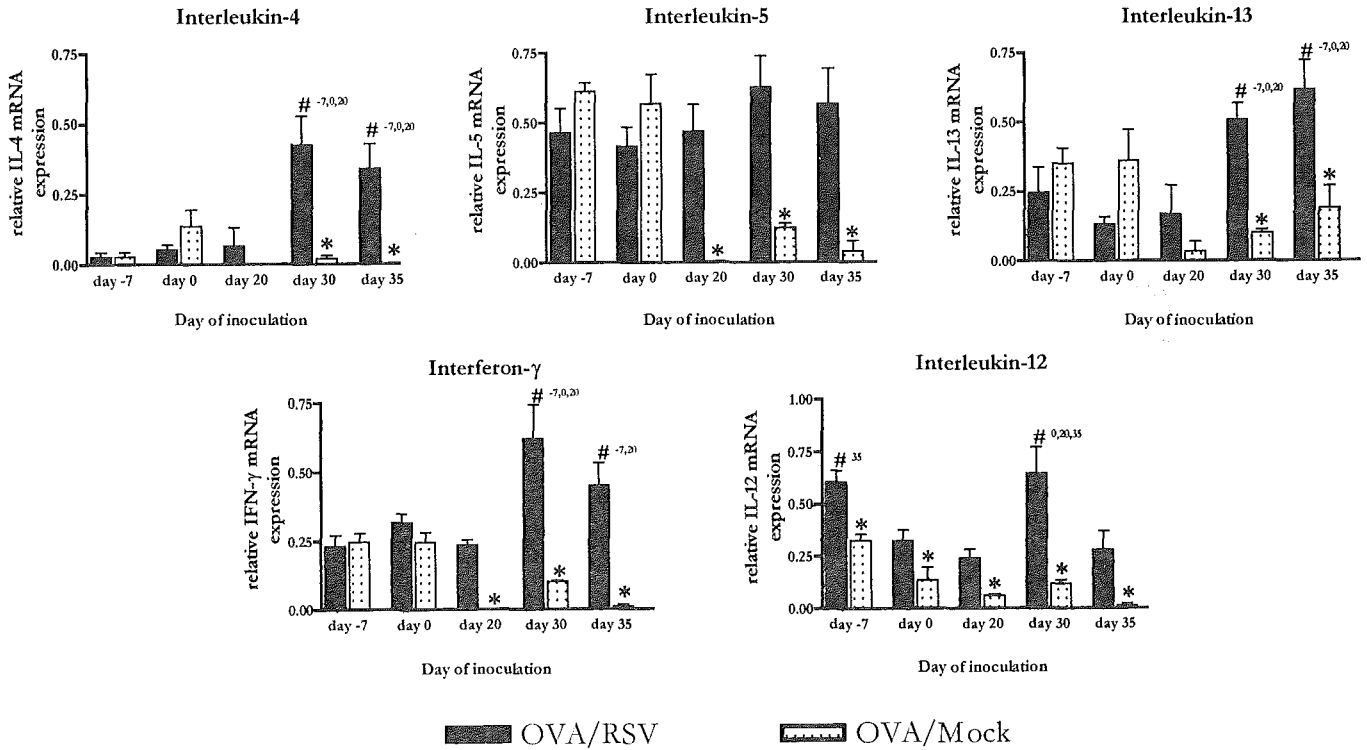

Figure 4.2. Relative expression (against β-actin) of lung IL-4, IL-5, IL-13, IFN-γ and IL-12 mRNA determined at day 43 of the experimental protocol (i.e. day 3 after the last OVA-challenge). Mice were inoculated with RSV on different time points during OVA sensitisation and challenge (OVA/RSV, black bars). Control mice were inoculated with mock on the corresponding time points (OVA/mock, hatched bars).

Mean values ± SEM (n = 6 per group) are depicted. Statistical difference between corresponding OVA/RSV and OVA/mock-treated mice are indicated by *: $P < 0.05$. Statistical difference between OVA-treated mice receiving RSV infection on different time points is indicated by #: $P<0.05$.

OD_{490}) compared to inoculation on day 0 (total IgE: 0,66±0,12 μg/ml, OVA-IgE: 0,698±0.176 OD_{450}) or day 30 (total IgE: 0,77±0,21 μg/ml, OVA-IgE: 0,559±0,134 OD_{450}). However, the same time dependency was observed after mock inoculation (data not shown).

Influence of prior RSV infection on RSV-enhanced OVA-allergy

We subsequently examined whether prior immunisation with RSV could diminish the effects of RSV on OVA allergy. We therefore compared the cytokine and inflammatory responses of OVA-allergic mice inoculated on day 35 (during the OVA-challenge) to identically treated mice that were immunised with live RSV intranasally on day 0 of the experimental protocol (fig. 4.1B). To examine if prior RSV immunisation influences virus replication after re-infection, the viral load in lung tissue was examined at day 43 of the experimental protocol (i.e. day 8 post inoculation) using RT-PCR. We observed that prior RSV immunisation on day 0 significantly decreased the viral load. The viral load was 8 times lower in previously immunised mice, indicating that prior RSV immunisation diminished virus replication upon re-infection (data not shown).

As demonstrated before, RSV infection during allergen challenge enhanced the expression of the Th2 cytokines in lungs of OVA-allergic mice (fig. 4.2). However, prior RSV immunisation did not abrogate this effect (fig. 4.4 left). In addition, prior RSV immunisation did not significantly diminish the pulmonary expression of Th1 cytokine mRNA (IFN-γ and IL-12) in OVA/RSV-treated mice (fig. 4.4 left).

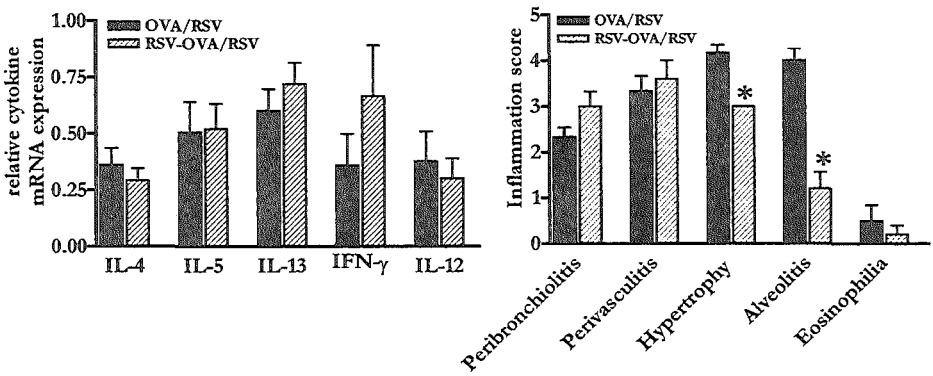

Figure 4.4 Relative expression (against β-actin) of lung IL-4, IL-5, IL-13, IFN-γ and IL-12 mRNA (left graph) and severity scores of pulmonary lesions (right graph), examined at day 43 of the experimental protocol (is 3 after the last OVA-challenge). OVA sensitised and challenged mice received RSV infection on day 35 during the OVA-challenge period and were (RSV-OVA/RSV, hatched bars) or were not (OVA/RSV, black bars) immunised with RSV on day 0 of the experimental protocol. Indicated pulmonary lesions are scored from absent (0) to severe (5). Mean values ± SEM (n = 6 per group) are depicted. Statistical differences between differently treated groups is analysed using the Students *t* test for mRNA expression data or the non-parametric Wilcoxon's test for histopathological scores. Differences are indicated by *: $P<0.05$.

In contrast, prior RSV immunisation diminished the alveolitis (from marked to minimal) and hypertrophy of mucus-producing epithelial cells (from marked to moderate) in lungs of OVA/RSV-treated mice (fig. 4.4 right). However, peribronchiolar and perivascular inflammation in OVA/RSV-treated mice was not influenced by prior RSV infection on day 0 (fig. 4.4 right). Also no significant reduction of the eosinophilic infiltration was observed (fig. 4.4 right).
As mentioned before, no change in production of total- or OVA-specific IgE was measured in OVA-allergic mice infected with RSV on day 35 compared to their corresponding controls. Nevertheless, prior RSV immunisation decreased the concentration of both total- ($p<0.05$) and OVA-specific IgE ($p<0.001$) in serum of OVA/RSV-treated mice, to approximately one third of the concentration in serum of OVA/RSV-treated mice that were not immunised with RSV before (data not shown).

4.5 Discussion

Results from different epidemiological studies in which the association between RSV infection in infancy and the development of respiratory allergy later in life is investigated have been inconclusive [184;194]. In addition to epidemiological studies, mice models are used to study the mechanisms through which RSV infection influences the allergic immune response. We previously demonstrated in a mouse model of allergic asthma that RSV infection exacerbates the allergic Th2 cytokine response and lung pathology [7], while RSV-infection in non-allergic mice did not induce a significant pulmonary Th2 cytokine response [7;17]. We now show that the timing of RSV infection is critical for RSV-enhanced allergic responses. RSV infection only enhanced the pulmonary Th2 cytokine response when mice were inoculated after OVA-sensitisation. The increased Th2 cytokine mRNA responses coincided with increased alveolitis and hypertrophy of mucus-producing epithelial cells. Since both alveolitis and pulmonary IFN-γ mRNA expression are specifically enhanced by RSV infection [17]. The enhanced alveolitis is likely due to the effect of RSV alone, but not by (RSV-enhanced) allergy. Therefore, only the hypertrophy of mucus-producing cells seems to be related to increases in Th2 cytokine expression. In contrast to the other parameters of allergic inflammation, perivasculitis and peribronchiolitis were not dependent on timing of RSV inoculation, indicating that they were likely not correlated with elevated Th2 cytokine mRNA expression levels in lungs. Since IL-5 has been identified as an eosinophil attractant [79];[175], and we observed high levels of IL-5 mRNA expression in OVA-treated mice inoculated with RSV on day 35, we expected to see increased eosinophilia. A possible explanation for the absence of aggravated eosinophilia could be that the moment of examination was too late and eosinophilia was already in decline [85]. Furthermore, Hessel et al. observed in an identical murine model of allergic asthma that airway hyperreactivity (one of the key characteristics of asthma) can develop without the presence of

eosinophils in the lung [83;84]. We did not measure hyperreactivity of the airways, but we observed a moderate hypertrophy of mucus producing glands in allergic mice, which was even enhanced after RSV infection. Increased mucus production into the airways is reported to contribute to the development of AHR [15].

[15]In contrast to the observation that RSV infection before OVA-sensitisation and challenge did not influence the allergic responses in lung tissue, Peebles et al. demonstrated a decreased airway hyperreactivity (AHR) and pulmonary IL-13 protein production in mice receiving a comparable treatment [159]. Since Peebles also found that AHR and Th2 cytokine production are not always correlated, these different observations do not have to be in conflict [161]. Possibly other factors than Th2 cytokines could explain such observations.

A possible explanation for the time dependent effect of RSV-infection on OVA-allergy, as observed in our study, may be the OVA-induced cytokine milieu. Among antigen-presenting cells, dendritic cells (DC) have a central role in regulating the cellular immune response and T cell polarisation [150]. The outcome of T cell polarisation by DC can be either Th1 or Th2, depending on the microenvironment and the character of pathogen-induced inflammatory reactions [114]. In the OVA-sensitisation and challenge model, a Th2 cytokine environment is induced by OVA-sensitisation in lung tissue of mice. Presentation of RSV by DC's to T cells in this environment might stimulate T cells to become Th2 cells, aggravating the Th2 response. Coyle et al [45] demonstrated *in vivo* (mice) and *in vitro* that IL-4 production by OVA-specific $CD4^+$ T cells switches the virus-specific $CD8^+$ T cells in the lung to produce IL-5. These results indicate that the virus-specific immune response could be modified by a local, allergen (OVA)-induced Th2 immune response. When RSV is given before or during allergen-sensitisation, no Th2 cytokine environment is present yet, which might result in a failure to enhance the Th2 response.

Besides the influence of the time of infection on the allergic inflammation, a high background for IL-5 and IL-13 mRNA expression was observed in the lungs of mice receiving mock- or RSV-inoculation prior to OVA-sensitisation. The reason for this high background is unclear. Nevertheless, because the RSV and mock inocula differ only in RSV content (no RSV in mock), significant differences between the RSV and mock groups must be specifically induced by RSV.

Epidemiological data show that almost all children become infected with RSV in their first or second year of life [103], and that re-infections with RSV occur throughout life. This means that a RSV infection shortly before or during OVA-challenge might have been preceded by an earlier RSV infection before allergen sensitisation. We demonstrated that prior RSV immunisation did not change the pulmonary cytokine mRNA expression of allergic mice infected with RSV during OVA challenge. Like Th2 cytokine mRNA expression, peribronchiolar and perivascular inflammation, and eosinophilia are not altered by prior RSV immunisation. These results are in accordance with those of Peebles et al. who observed that RSV immunisation before allergen sensitisation has no effect on lung IL-4 and IL-5 protein levels in OVA/RSV-treated mice [162]. However, they found that RSV immunisation protected

against RSV-enhanced AHR. In our study, prior RSV immunisation diminished hypertrophy of mucus producing cells in OVA-allergic mice infected with RSV. Taken together this suggests that increased hypertrophy of mucus producing cells is related to enhanced AHR. In correspondence to previously published data [17;162], prior RSV immunisation dramatically diminished the viral load after secondary infection, indicating protection against viral replication. Prior RSV immunisation also diminished alveolitis after secondary infection. This decreased alveolitis is probably due to the absence of RSV replication after prior immunisation, since alveolitis is specifically induced by RSV infection [17]. Which factors are responsible for RSV-enhanced allergy still remains unresolved. However, the lack of correlation between viral load and enhancement of allergy indicates that the anti-viral inflammatory response, rather than virus-induced lesions is responsible for allergy-enhancement.

In summary, timing of RSV infection during OVA-sensitisation and challenge is critical for the development of allergic responses in mice. Increased pulmonary Th2 cytokine mRNA expression, and hypertrophy of mucus producing cells are only observed when mice received RSV infection after OVA-sensitisation. Despite strongly reduced virus replication, previous infection with RSV could not abrogate the RSV-enhanced OVA-induced Th2 cytokine expression in lungs. These data provide evidence that a pre-existing Th2 cytokine milieu leads to RSV-enhanced respiratory allergy. In addition, prior RSV immunisation is only partial beneficial for later RSV-enhanced respiratory allergy.

Acknowledgements

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Chapter 5 Respiratory Syncytial Virus, Pneumonia Virus of Mice, and Influenza A virus differently affect respiratory allergy in mice.

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5.1 Abstract

Background: Respiratory viral infections in early childhood may interact with the immune system and modify allergen sensitisation and/or allergic manifestations. In mice, respiratory syncytial virus (RSV) infection during allergic provocation aggravates the allergic T helper (Th) 2 immune response, characterised by the production of interleukin (IL)-4, IL-5 and IL-13, and inflammatory infiltrates. However, it is unclear whether the RSV-enhanced respiratory allergic response is a result of non-specific virus-induced damage of the lung, or virus-specific immune responses.

Objective: In the present study we investigated whether RSV, pneumonia virus of mice (PVM) and influenza A virus similarly affect the allergic response.

Methods: BALB/c mice were sensitised and challenged with ovalbumin (OVA), and inoculated with virus during the challenge period. Pulmonary inflammation, lung cytokine mRNA responses, and IgE production in serum were assessed after the last OVA-challenge.

Results: Like RSV, PVM enhanced the OVA-induced pulmonary IL-4, IL-5, and IL-13 mRNA expression, which was associated with enhanced perivascular inflammation. In addition, PVM increased the influx of eosinophils in lung tissue. In contrast, influenza virus decreased the Th2 cytokine mRNA expression in the lungs. However, like PVM, influenza virus enhanced the pulmonary eosinophilic infiltration in OVA-allergic mice.

Conclusion: The Paramyxoviruses RSV and PVM both are able to enhance the allergic Th2-cytokine response and perivascular inflammation in BALB/c mice, while the Orthomyxovirus influenza A is not.

5.2 Introduction

Childhood asthma is a serious global public health problem. According to the World Health Organisation [228], asthma is the most common chronic disease in children. Allergic asthma is a T-helper 2 (Th2)-associated airway inflammation, characterised by the infiltration of eosinophils, and production of interleukin (IL)-4, IL-5, and IL-13 by Th2 lymphocytes in response to certain antigens [26;174]. Although no single specific cause of asthma has been found, a number of predisposing factors has been identified that are targets of ongoing clinical investigation. One potential risk factor is viral respiratory tract infection in infancy, especially respiratory syncytial virus (RSV) [143;184].

RSV (a member of the pneumovirinae subfamily of the *Paramyxoviridae*) is the most frequent cause of bronchiolitis and pneumonia in infants and young children. The symptoms vary from mild upper respiratory tract illness to severe bronchiolitis and pneumonia [40]. In mice, RSV infection enhances the pulmonary Th2 cytokine expression and eosinophilic infiltration induced by airway sensitisation and challenge with ovalbumin (OVA) [7;163]. Recently, we

showed that the RSV-induced Th1 response, characterised by IFN-γ and IL-12 production, occurred simultaneously with enhancement of the OVA-induced Th2 response [7]. However, in the absence of virus-induced IFN-γ the Th2 enhancement was much stronger [6]. It is unclear whether the RSV-enhanced respiratory allergic responses in mice are a result of non-specific or virus-specific stimuli. For this reason, we investigated the influence of other respiratory viruses on the allergic immune response.

The natural rodent pathogen, pneumonia virus of mice (PVM), is also a member of the *Pneumovirinae* subfamily of the *Paramyxoviridae,* and is related to human RSV and the recently identified human metapneumovirus (hMPV) [205]. Molecular cloning has shown that the organisation of the PVM genome is similar to that of (human) RSV [32]. Restricted serological cross-reactivity between the nucleocapsid protein (N) and the phosphoprotein (P) of PVM and RSV has been described, but none of the external proteins show cross-reactivity, and the viruses can be distinguished from each other in neutralisation assays [118].

Like RSV, influenza virus (a member of the *Orthomyxoviridae* virus family) can induce inflammation in humans throughout the respiratory tract [142]. Mouse-adapted influenza virus can induce a lower respiratory tract infection, and the pathology in mice is comparable with that in humans [142]. Experimental studies in mice demonstrated that influenza virus infection enhanced the sensitisation for allergic responses later in life [151;197]. Infection with Influenza A virus is also associated with asthma exacerbation in humans [61;199]. Here we study the effect of influenza virus infection on an already existing respiratory allergy in mice. We thus investigated whether the RSV-enhanced respiratory allergy is restricted to RSV, or is also induced by other paramyxoviruses and non-paramyxoviruses, such as the *Orthomyxoviridae.* We therefore sensitised and challenged mice with ovalbumin (OVA) and infected them during the OVA challenge period with RSV, PVM, or influenza A virus. Subsequently, we determined the pulmonary inflammation, serum IgE, and lung cytokine mRNA responses. The results indicate that in our allergy model the paramyxoviruses RSV and PVM enhance allergic Th2 cytokine responses while the orthomyxovirus influenza A does not.

5.3 Methods

Viruses: Human respiratory syncytial virus type A2 (RSV A2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The virus was cultured on HEp-2 cells (ATCC, Rockville, MD) in medium (RPMI 1640 Gibco BRL, Life Technologies, Rockville, MD), containing 10% heat-inactivated foetal calf serum (FCS, Greiner, Frickenhausen, Germany), 2mM glutamine, 100 IE/ml penicillin, and 100 U/ml streptomycin. After 40-44 hr of culture, the cells were sonificated and centrifuged for 10 min. at 1,000*g*, 4°C. The supernatant was collected and 50% PEG_{6000} (Merck, Darmstadt, Germany) in 150 mM NaCl, 50 mM Tris, 1 mM EDTA, pH 7.5, was added to a final concentration of 10%. After incubation for 1.5 hr at

4°C (while stirring), the suspension was centrifuged for 15 min at 3,200*g*, 4°C. The pellet was resuspended in culture-medium in 10% of the original volume. The suspension was aliquotted, snap-frozen, and stored at -80°C. The virus stock contained 3 x 10^8 plaque forming units (pfu) RSV/ml as assessed by quantitative plaque-forming assay [18].
Pneumonia virus of mice (PVM, a gift of Dr. Elzinga-Gholizadea, Bilthoven, The Netherlands) was grown and quantified by virus titration on BHK-21 C_{13} cells in medium (Eagle MEM, containing Hanks salts, 10 mM $NaHCO_3$, 10% FCS, 2mM glutamine, 20 mM Hepes, 100 IE/ml penicillin, and 100 U/ml streptomycin).
Influenza A virus (mouse adapted strain A/PR/8/34 (H1N1) was grown and quantified by virus titration on MDCK-1 cells in medium (Eagle MEM, containing Hanks salts, 10 mM $NaHCO_3$, 2mM glutamine, 13 mM Hepes, 100 IE/ml penicillin, 100 U/ml streptomycin, 10^{-5}% trypsin (Merck), and 10% PGR-Albumin (containing 10 gr. Pepton, 10 gr. Glucose and 10 gr. Bovine albumin per 800 ml Eagle MEM). The influenza A and PVM virus stocks were prepared as described for RSV (see above).

Animals: Female SPF BALB/c mice were obtained from Harlan Olac (Horst, The Netherlands) and were used at 6-10 weeks of age. A week before the experiments started, mice were housed per group according to the experimental set-up, and cages were placed in a temperature-controlled isolator (22°C) to prevent infection of control animals and animal caretakers. Mice were kept in a 12-hour light/dark cycle, and received food and water *ad libitum*. The study was approved by the Institute's committee on animal welfare.

Experimental Design: Mice were sensitised to OVA by intraperitoneal (i.p.) administration of 0.5 ml OVA (20 µg/ml saline, no adjuvant (Sigma-Aldrich, Steinheim, Germany)) for 7 times on every other day starting at day 0. From day 33 till day 40, mice were intranasally challenged with OVA aerosols (2 mg/ml saline) during 5 minutes on 8 consecutive days (Fig. 5.1).
To examine the influence of the different viral infections on a pre-existing allergic inflammation, OVA-sensitised mice were inoculated intranasally with 50 µl RSV ($1{,}5x10^7$ plaque forming units per dose ~ 8.10^6 $TCID_{50}$), 50 µl PVM (20 $TCID_{50}$ per dose), or 50 µl influenza virus (50 $TCID_{50}$ per dose), on day 35 during the OVA-challenge period (= OVA/RSV, OVA/PVM, or OVA/Influenza). A pilot study was performed in which day 35 was determined to be the optimal day for inoculation during OVA-challenge (data not shown). The lowest infection dose, that still caused clinical symptoms, was chosen (data not shown). Virus-infected OVA-allergic mice were compared to OVA-allergic mice inoculated with their respective uninfected culture lysate (mock) (= OVA/Mock-r, OVA/Mock-p, OVA/Mock-i), and non-allergic mice infected with virus only (= -/RSV, -/PVM, -/Influenza). Before inoculation, mice were i.p. anaesthetised with Ketamine, Rompun (=xylazine), and Atropine (KRA). At days 41, 43, and 47 of the experimental protocol (days 6, 8 and 12 days post virus inoculation), mice were anaesthetised with KRA i.p. and sacrificed. Three different time points for sampling were chosen in order to detect a prolonged inflammatory response. Blood was

collected via orbital punction. The lungs were removed. The right lung was snap-frozen in liquid nitrogen and stored till further process. The left lung was fixed intratracheally with formalin, and subsequently immersed in fixative. Each experiment was performed in duplicate, and gave similar results. Data of one experiment are shown.

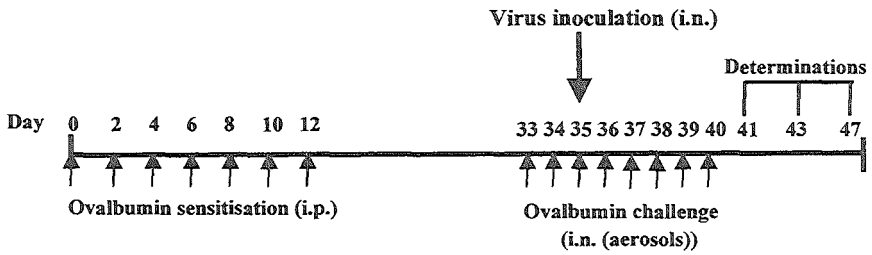

Figure 5.1. Study design

Histopathologic examination: Formalin-fixed lungs were embedded in paraplast (Monoject, Kildare, Ireland). Transverse sections of 5 µm were stained with haematoxylin and eosin. Different lung lesions were scored semiquantitatively from absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5).

Immunohistochemistry: Immunostaining of PMV, RSV and influenza virus was done on paraffin sections of lung. Deparaffinized sections were incubated with respectively rabbit anti-PVM antibody (gift from G. Drost, RIVM), goat anti-RSV (Biodesign International, Saco, Maine, US), and mouse anti-HB-65 antibody, (mIgG2a, directed against nucleoprotein of influenza virus, gift from Dr. A. Meyer, RIVM). The following peroxidase labelled secondary antibodies were used: swine anti-rabbit IgG (DAKO Diagnostics, Glostrup, Danmark), donkey anti-goat IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA, USA), and goat anti-mouse IgG2a, (Southern Biotechnology Associates, Birmingham, Alabama, USA). Primary and secondary antibodies were diluted in PBS containing 1% Bovine Serum Albumin (Sigma, Zwijndrecht, The Netherlands) and used in an optimal dilution. Sections were washed in PBS containing 0.5% Tween 20 (Merck, Darmstadt, Germany). Detection of peroxidase occurred with the substrate 3,3-diaminobenzidine (Sigma, Zwijndrecht, The Netherlands) with ammonium nickel sulphate (Fluka Chemie GmbH, Buchs, Switzerland). Cells were counterstained with nuclear fast red (Sigma, Zwijndrecht, The Netherlands).

RNA isolation and RT-PCR: Total cellular RNA was isolated from frozen lung samples using Trizol Reagent (GibcoBRL, Life technologies, Rockville, USA). 5 µl of total lung RNA was reverse transcribed, cytokine PCR reactions (IL-4, IL-5, IL-13, IFN-γ and IL12) on the cDNA, and analysis of PCR products were carried out as described previously [6;18]. Eotaxin RT-PCR

was performed according to the same protocol using sense (CCCCAACACACTACTGAAGA) and antisense (CTACATGAAGCCAAGTCCTT) primers. Macrophage inflammatory protein-1α (MIP-1α) RT-PCR was carried out as described by Su et al. [196].

IgE antibodies in serum: Total and OVA-specific IgE antibodies in serum were determined using a modified capture ELISA as described previously [6].

Statistical analysis: Data are presented as mean ± Standard Error of the Mean (SEM). Statistical significance for differences in cytokine ratios and IgE concentrations was determined using the Student's *t* test (Excel, Microsoft Corporation, Redmond, USA), and for histological scores using the nonparametric Wilcoxon's test (SAS, SAS Institute Inc., USA).

5.4 Results

Kinetics of virus replication

Differences in replication kinetics between RSV, PVM and influenza virus might have consequences for their effect on respiratory allergy in mice. We therefore determined the virus titers of RSV, PVM and influenza A virus in lung tissue of non-allergic mice on days 4, 5 and 6 post inoculation (fig. 5.2). RSV reached a peak titer on day 4 post inoculation, influenza virus on day 5 post inoculation, and the PVM titer was still increasing at day 6 post inoculation. The peak titers of influenza and PVM were approximately 1-2 log higher compared to that of RSV.

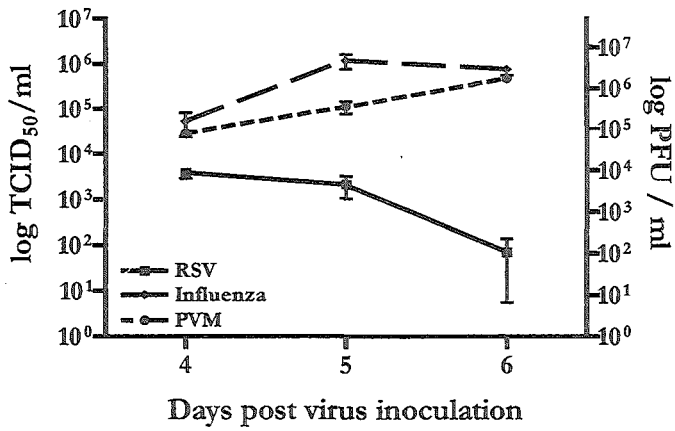

Figure 5.2. Respiratory syncytial virus (RSV), Pneumonia virus of mice (PVM), and influenza A virus titres in lung homogenates of BALB/c mice on day 4, 5 and 6 post inoculation. The virus titres are expressed as log 10 $TCID_{50}$/lung for PVM and influenza A (left Y-axis), and log 10 pfu/lung for RSV (right y-axis). Mean values ± SEM (n=6) are shown.

Pulmonary histopathology

In mice, OVA-sensitisation and -challenge induced significant peribronchiolar (data not shown) and perivascular (fig. 5.3A/B/C) infiltration of eosinophilic and mononuclear cells. In addition, OVA-allergy induced a marked hypertrophy of the mucus-producing epithelial cells.

RSV infection in non-sensitised/challenged mice resulted in a slight peribronchiolar infiltration of mononuclear cells and hypertrophy of the mucus-producing cells, together with marked perivasculitis and alveolitis (fig. 5.3A). PVM and influenza virus induced similar lesions in lungs of non-sensitised/challenged mice as RSV (fig. 5.3B/C). However, the pathological changes in PVM and influenza virus infected mice were still marked on day 12 post inoculation (is day 47 of the experimental protocol), while those in RSV infected mice were already reduced to slight lesions. None of the virus-infected non-allergic mice showed eosinophilic inflammation in the lungs (fig. 5.3).

The histopathologic changes in lungs of OVA-allergic mice infected with RSV, PVM or influenza virus were compared with changes in the corresponding OVA/mock group (fig. 5.3). PVM and RSV infection increased the perivasculitis in OVA-allergic mice. Influenza virus did not further enhance perivasculitis in OVA-allergic mice. In fact, perivasculitis was similar in allergic and non-allergic mice, infected with influenza virus. Influenza virus and PVM enhanced the hypertrophy of the mucus producing cells in OVA-allergic mice on day 41 and 43, while RSV did not enhance it at any time point (fig. 5.3). PVM and influenza virus significantly increased the influx of eosinophils in allergic mice on day 41 (1 day after the last OVA-challenge) (fig. 5.3). Remarkably, RSV did not significantly enhance the influx of eosinophils in OVA-sensitised/challenged mice as we have seen before [7].

Localisation of RSV, PVM, and Influenza virus antigen in lung tissue.

RSV was detected mainly in the cytoplasm of alveolar epithelium and to a lesser extend in alveolar macrophages at day 6 post inoculation (day 41 of the experimental protocol) (fig. 5.4A Appendix). In the epithelium both alveolar epithelial cells of type I (characterised by thin long cytoplasmic extensions) and type II (cubic cells) stained positive for RSV. OVA-allergy induced no difference in cell types staining positive for RSV at day 6 post inoculation (fig. 5.4A/B Appendix). At day 8, only in OVA-allergic mice, a few alveolar macrophages were still RSV-positive and no epithelial cells stained positive, while at day 12 no RSV-positive cells were present at all, both in OVA-allergic and non-allergic mice (data not shown).

PVM was detected mainly in the cytoplasm of bronchiolar and alveolar epithelial cells of both type I and type II, as well as in alveolar macrophages at day 6 post inoculation (fig. 5.4C Appendix). OVA-allergy induced no differences in cellular localisation of PVM (fig. 5.4C/D Appendix). At day 8 post PVM-inoculation, the number of PVM-positive cells in the OVA-allergic mice was lower than on day 6, while in the non-allergic mice PVM-positive cells were nearly absent (data not shown). At day 12 post inoculation, no PVM-positive cells were observed in both OVA-allergic as non-allergic mice (data not shown).

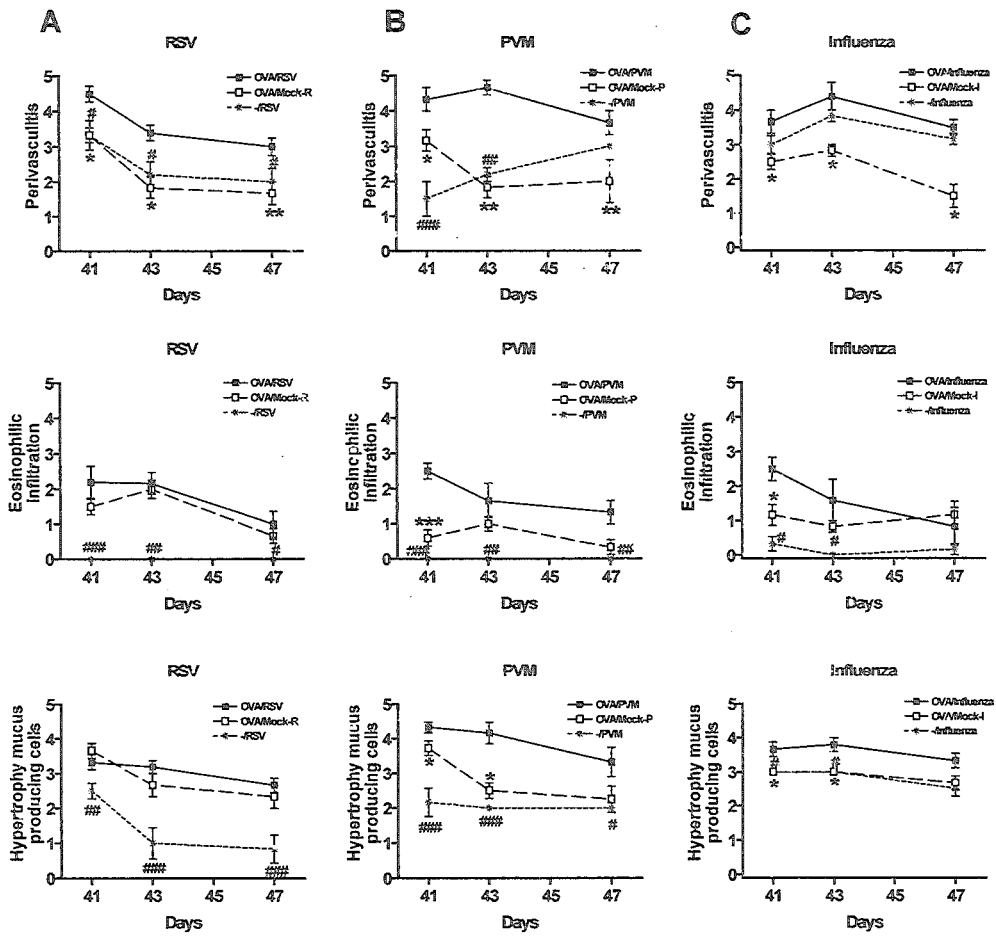

Figure 5.3. Histopathologic changes in lungs examined on day 41, 43 and 47 (is 1, 3 and 7 days after the last ovalbumin (OVA)-challenge). A: OVA-allergic mice infected with RSV (OVA/RSV), inoculated with mock (OVA/mock-r) or non-allergic mice infected with RSV (-/RSV). B: OVA-allergic mice infected with PVM (OVA/PVM), inoculated with mock (OVA/mock-p) or non-allergic mice infected with PVM (-/PVM). C: OVA-allergic mice infected with influenza A (OVA/influenza), inoculated with mock (OVA/mock-i) or non-allergic mice infected with influenza A (-/influenza). Pulmonary lesions are scored from minimal (1) to severe (5). Histological scores are depicted as mean value ± SEM (n=6). Statistical difference on one single time-point between differently treated groups is analysed using the non-parametric Wilcoxon's test; differences are indicated by *: $P < 0.05$, **: $P<0.01$ between OVA/virus and OVA/mock, #: $P<0.05$, ##: $P<0.01$, ###: $P<0.001$ between OVA/virus and -/virus.

Influenza virus was only detected in some solitary bronchiolar cells (non-ciliated, non-mucus-secreting epithelial cells) and alveolar macrophages, but not in alveolar epithelial cell, as examined on day 6 post inoculation (fig. 5.4E Appendix). No difference in cell-types staining positive for influenza was noticed between OVA-allergic and non-allergic mice (fig. 5.4E/F Appendix). At days 8 and 12 post inoculation no influenza-positive cells could be demonstrated in both OVA-allergic as non-allergic mice (data not shown).

Th2 cytokines in lung tissue

OVA sensitisation and challenge induced a predominant Th2 cytokine milieu in lung tissue, characterised by significant increases in IL-4, IL-5, and IL-13 mRNA expression on day 41 (1 day after the last OVA-challenge) when compared with non-allergic mice (fig. 5). As shown before [7], RSV in non-allergic mice induced no or minimal mRNA expression of the Th2 cytokines IL-4, IL-5 and IL-13 (fig. 5A). Also PVM and influenza virus infection in non-allergic mice did not induce mRNA expression of these Th2 cytokines (fig. 5.5B/C).

RSV infection during OVA-challenge significantly increased the OVA-induced pulmonary IL-4 mRNA expression till at least day 47, and the IL-5 and IL-13 mRNA expression on day 43 of the experimental protocol (fig 5.5). Like RSV, PVM infection during OVA-challenge strongly enhanced the pulmonary IL-4, IL-5, and IL-13 mRNA expression when compared to OVA/Mock-p mice (fig. 5.5). In contrast, influenza A virus infection did not enhance mRNA expression of IL-4, IL-5 and IL-13 in OVA-allergic mice (fig. 5.5C). Influenza virus infection even down-regulated mRNA expression on day 41 of all three Th2 cytokines tested compared to OVA/mock-i mice.

Chemokine expression in lung tissue

The decrease in pulmonary IL-4, IL-5, and IL-13 mRNA expression in OVA/influenza mice on day 41 compared to OVA/mock-i mice, seems in contrast with the influenza virus enhanced eosinophilic inflammation, since IL-5 is considered important for eosinophilic infiltration. Besides IL-5, the β-chemokines eotaxin and MIP-1α are also involved in eosinophilic inflammation [26]. MIP-1α can induce infiltration of eosinophils in lungs independent of IL-5 [56]. OVA-sensitisation/challenge without virus infection resulted in increased expression of pulmonary eotaxin mRNA, but had no effect on MIP-1α mRNA expression, compared to non-infected non-allergic controls. RSV, PVM and influenza virus infection of non-allergic mice also induced strong expression of eotaxin and MIP-1α mRNA (fig. 5.6). When OVA-allergic mice were infected with one of the viruses, only PVM enhanced the expression of eotaxin mRNA, while influenza virus even down-regulated eotaxin mRNA expression on day 41. The high expression of pulmonary MIP-1α mRNA in RSV-, PVM- or influenza virus infected mice was not altered by OVA-sensitisation/challenge (fig. 5.6).

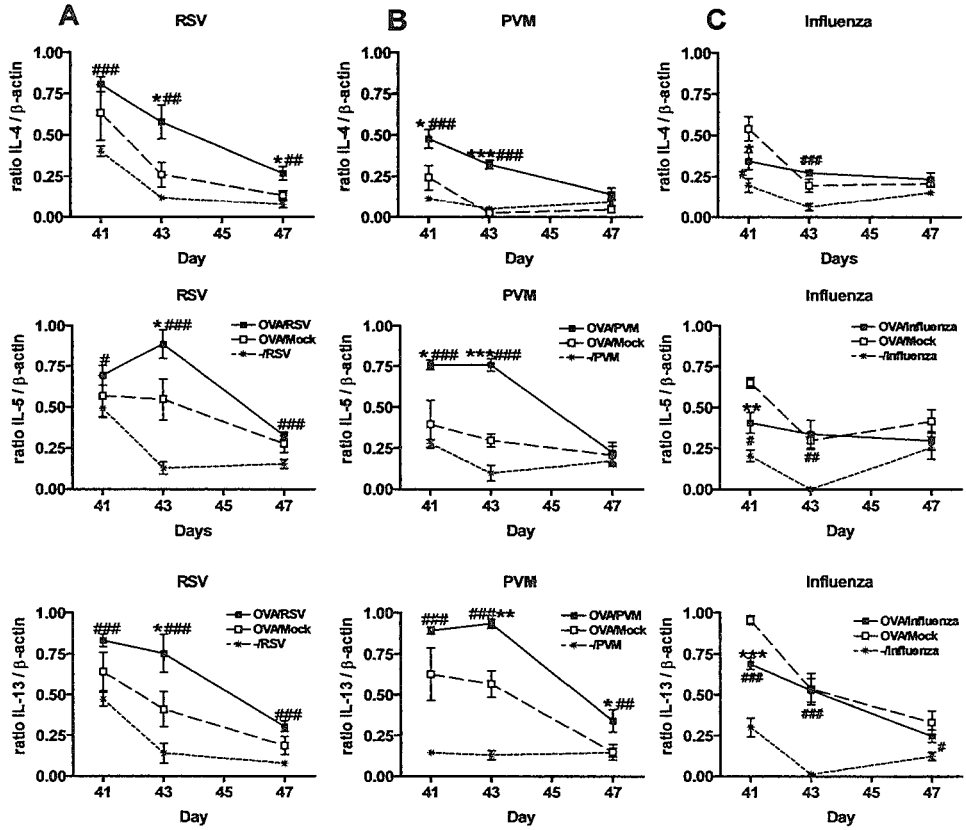

Figure 5.5 Relative expression (against β-actin) of lung IL-4, IL-5 and IL-13 mRNA determined at days 41, 43 and 47 of the experimental protocol (is 1, 3 and 7 days after the last OVA-challenge). A: OVA-allergic mice infected with RSV (OVA/RSV), inoculated with mock (OVA/mock-r) or non-allergic mice infected with RSV (-/RSV). B: OVA-allergic mice infected with PVM (OVA/PVM), inoculated with mock (OVA/mock-p) or non-allergic mice infected with PVM (-/PVM). C: OVA-allergic mice infected with influenza A (OVA/influenza), inoculated with mock (OVA/mock-i) or non-allergic mice infected with influenza A (-/influenza). Mean values ± SEM (n = 6 per group) are depicted. Statistical differences are indicated by *: $P < 0.05$, **: $P<0.01$, ***: $P<0.001$ between OVA/virus and OVA/mock, #: $P<0.05$, ##: $P<0.01$, ###: $P<0.001$ between OVA/virus and -/virus.

Th1 cytokine mRNA expression

IFN-γ and IL-12 are the major Th1 cytokines. Both are associated with antiviral activity and reduce Th2-like responses. OVA-treatment without viral infection did not result in the expression of Th1 cytokines. RSV, PVM and influenza virus infection induced high expression levels of IFN-γ and IL-12 mRNA in lung tissue up to at least 12 days post inoculation (day 47 of the experimental protocol) (data not shown). The expression of IFN-γ and IL-12 induced by RSV, PVM or influenza virus was not reduced by OVA-sensitisation/challenge (data not shown). PVM infection in OVA-sensitised/challenged mice even enhanced expression of IL-12 on day 43 compared to non-sensitised/challenged mice infected with PVM.

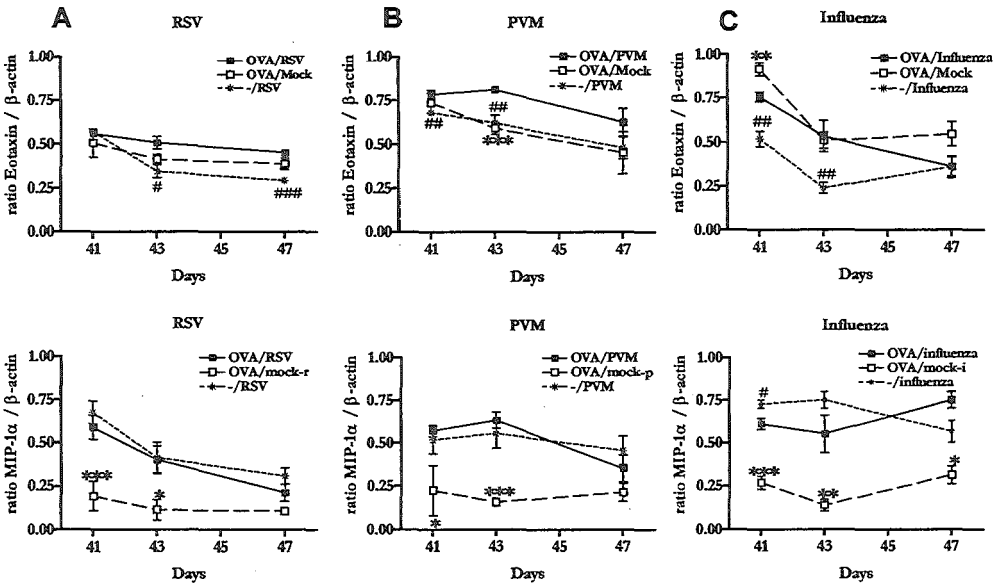

Figure 5.6. Relative expression (against β-actin) of lung eotaxin and MIP-1α mRNA determined at day 41, 43 and 47 of the experimental protocol (is 1, 3 and 7 days after the last OVA-challenge). A: OVA-allergic mice infected with RSV (OVA/RSV), inoculated with mock (OVA/mock-r) or non-allergic mice infected with RSV (-/RSV). B: OVA-allergic mice infected with PVM (OVA/PVM), inoculated with mock (OVA/mock-p) or non-allergic mice infected with PVM (-/PVM). C: OVA-allergic mice infected with influenza A (OVA/influenza), inoculated with mock (OVA/mock-i) or non-allergic mice infected with influenza A (-/influenza). Mean values ± SEM (n = 6 per group) are depicted. Statistical differences are indicated by *: $P < 0.05$, **: $P<0.01$, ***: $P<0.001$ between OVA/virus and OVA/mock, #: $P<0.05$, ##: $P<0.01$, ###: $P<0.001$ between OVA/virus and -/virus

IgE in serum

We previously reported that OVA sensitisation and challenge resulted in the production of OVA-specific IgE in serum, measured after the OVA-challenge period. In addition, RSV infection during the OVA-challenge period did not affect titers of OVA-specific IgE and total-IgE in serum [7]. In the present series of experiments none of the virus infections influenced the total- and OVA-specific IgE production in OVA-sensitised/challenged mice, at least till day 47 of the experimental protocol (data not shown). In serum of non-allergic mice infected with PVM, RSV or influenza virus no detectable levels of total-IgE could be measured.

5.5 Discussion

In mice, RSV infection aggravates the effects of allergic sensitisation and challenge, predominantly by an increase in Th2 cytokine mRNA expression in the lungs [7] and hyperreactivity of the airways [163]. However, it is not clear how RSV enhances the allergic response and whether RSV is unique in its ability to enhance the inflammatory and cytokine responses in allergic mice. We therefore studied the influence of infection with two other respiratory viruses, PVM and influenza A virus.

PVM is closely related to RSV and belongs to the same virus family; the *Pneumovirinae*. Because RSV is not a murine pathogen, large doses are required to infect mice and infection results in little clinical disease [27;40]. In contrast, mice are more sensitive to PVM than to RSV infection [this article, [57]. Therefore, PVM has been used as a model to study the pathology and immune responses of RSV in humans [19;41]. The data presented in this article indicate that PVM infection of allergic mice is an even better model to study the effects of respiratory virus infection on allergy than RSV.

Like RSV, influenza A virus infection is associated with asthmatic exacerbations in humans [61;199] and enhanced allergic sensitisation in mice [151;197].

RSV, PVM and influenza A virus infection of mice result in a dominant Th1 like cytokine response in lungs, together with alveolar, peribronchiolar and perivascular inflammation. OVA-sensitisation/challenge did not affect the virus-induced Th1-cytokine expression. The virus-induced lung pathology and expression of pulmonary IFN-γ and IL-12 mRNA decreased more rapidly in RSV-infected, than in PVM and influenza-infected mice. These differences could be due to differences in virus-replication kinetics. Like RSV, PVM infection of allergic mice increased the expression of pulmonary IL-4, IL-5 and IL-13 mRNA and enhanced the perivasculitis and infiltration of eosinophils in the peribronchiolar space. In contrast, influenza virus infection did not enhance the pulmonary Th2 cytokine mRNA expression in OVA-allergic mice, but even down-regulated the IL-4, IL-5, and IL-13 mRNA response on day 41. These results appear in contrast to other published data, which demonstrate that influenza virus infection enhances the Th2 inflammatory response in allergen-sensitised mice [151;197]. However, these studies examined the influence of infection on later development of allergic sensitisation rather than the effects of infection on a pre-existing allergy, like in our study. We cannot exclude that influenza virus infection on another moment during the OVA-sensitisation and –challenge protocol in our mice model may influence the allergic Th2 immune response.

The key question is why infection with RSV and PVM enhance the OVA-induced Th2 cytokine response, while that with influenza virus does not. The difference in Th2-enhancement by the three viruses might be explained by the difference in virus-structure between the involved virus families. A characteristic outer membrane protein of orthomyxoviruses is the HA protein, which is involved in attachment of virus to host cells and hemagglutination [112]. For attachment to host cells PVM and RSV are equipped with the G-protein [40]. G is extensively glycosylated, and lacks homology to the HA protein or to any other known paramyxovirus

protein [68]. A possible mechanism for influencing the OVA-induced Th2 cytokine response could be the immune-modulating capacity of the pneumovirus G protein. Immunisation with purified G protein or vaccinia virus vectors expressing G, stimulate a Th2 immune response [80;96;193]. The OVA-induced pulmonary inflammation and Th2 cytokine milieu in mice might favour the immune response to G and further stimulate the Th2-inflammatory response [68]. In contrast, influenza virus seems to lack a Th2-stimulating protein. This hypothesis can be investigated by inoculation with G protein only and study the OVA-allergic Th2 immune response.

A second possible mechanism that may explain the difference between the pneumoviruses and influenza virus in enhancing the OVA-induced Th2 cytokine response could be the site of virus replication in lung tissue. As demonstrated, PVM and RSV were mainly located in the epithelial cells lining the alveoli and bronchi, while influenza virus was predominantly observed in the alveolar macrophages. In addition, PVM and RSV were detected during a longer period of time in lungs of allergic mice than non-allergic mice, while influenza virus was not. The absence of influenza virus replication in alveolar epithelial cells is in contrast to previously published data [133;230]. However, in the present study mice are inoculated with the A/PR/8/34 H1N1 influenza strain while in most other studies the H3N2 [151;203;229] or A/WSN/33 H1N1 [30] influenza strain is used. Replication of RSV and PVM in the epithelium allows OVA to penetrate the barrier of the respiratory mucosa, and promoting allergen sensitisation [64]. Furthermore, RSV and PVM infection of epithelial cells could enhance the production of β-chemokines (a.o. MIP-1α, eotaxin, RANTES) [221] and expression of ICAM-1 [214] by epithelial cells, which are both involved in allergic inflammation. Influenza virus is predominantly located in macrophages, which produce, upon activation, different mediators, among them the Th1 cytokines IL-12, IL-1, TNF-α and IFN-γ, promoting cell-mediated immunity [226]. The synergistic effect of these cytokines could result in a stronger Th1-mediated inhibition of the Th2 cytokine response.

Eosinophilic leukocytes play prominent roles in promoting pathophysiologic conditions such as respiratory allergy and airway hyperreactivity [26]. Influenza virus and PVM infection increased significantly the infiltration of eosinophils in lungs of OVA-allergic mice. Thus, influenza virus infection aggravated eosinophilia despite the unaltered expression of Th2 cytokines and eotaxin. However, Domachowske et al. demonstrated that IL-5 is not a crucial mediator of pulmonary eosinophilia in response to respiratory virus infection [56]. In addition, eosinophilic infiltration in lungs of allergic mice has been reported independently of IL-4, IL-5, and IL-13 [166], suggesting a novel mechanism for eosinophilia. Macrophage inflammatory protein-1α (MIP-1α) has been characterised as an eosinophil chemoattractant independent of IL-5, RANTES and eotaxin [26]. We demonstrated that pulmonary MIP-1α levels were similar in RSV, PVM or influenza virus infected OVA-allergic and non-allergic mice, while no eosinophils were detected in lungs of infected non-allergic mice. We therefore conclude that MIP-1α is likely not responsible for the enhanced pulmonary influx of eosinophils in virus-

infected allergic mice. It therefore remains unknown which factor in responsible for the enhanced eosinophilic infiltration in influenza virus-infected OVA-allergic mice.
In summary, the results presented in this article show that the paramyxoviruses RSV and PVM both are able to enhance the allergy-induced Th2 immune response in BALB/c mice, in contrast to the orthomyxovirus influenza A. Despite the absence of enhanced Th2 cytokine and β-chemokine expression, influenza virus increased the influx of pulmonary eosinophils in OVA-allergic mice. The different effects of these viruses could be due to variation in site of replication in the lung, or to differences in the capacity of viral proteins (especially the G protein) to modulate the allergic immune response.

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Chapter 6 Mutual enhancement of RSV- and allergen-specific Th2, but not Th1 systemic cellular immune responses in a mouse model of respiratory allergy.

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Submitted for publication

6.1 Abstract

Respiratory viral infections in early childhood may interact with the immune system and modify allergen sensitisation and/or allergic manifestations. In a pre-existing allergic mice model, respiratory syncytial virus (RSV) and pneumonia virus of mice (PVM) aggravated the consequences of airway sensitisation and challenge with ovalbumin (OVA), while influenza A virus did not. The immune cell requirements for the virus-enhanced respiratory allergy are however not well defined. We therefore investigated which virus-induced immune mechanisms could be responsible for the enhanced allergic inflammation, and examined the OVA- and virus-specific cellular immune response in spleens of OVA-sensitised and –challenged mice infected with RSV, PVM or influenza virus.

In contrast to influenza virus, RSV and PVM infection of allergic mice enhanced the Th2 cytokine production by OVA-specific splenocyte. The RSV-enhanced OVA-specific IL-4 production was mainly secreted by OVA-specific $CD4^+$ cells, OVA-specific $CD8^+$ cells were not affected by RSV. In addition, virus-specific stimulation of splenocytes obtained from the same RSV, PVM or influenza virus inoculated mice resulted predominantly in IFN-γ production, mainly secreted by virus-specific $CD8^+$ cells. The virus-specific IFN-γ production was not affected by OVA-sensitisation and -challenge. Interestingly, an increased number of RSV-specific $CD8^+$ cells produced IL-4 when RSV-inoculated mice were sensitised and challenged with OVA. Influenza-specific $CD8^+$ cells did not produce IL-4.

We conclude that the pre-existing Th2 cytokine milieu, induced by OVA, modulates the RSV-specific $CD8^+$ T cells to produce IL-4. RSV infection in turn stimulates the OVA-specific $CD4^+$ T cells to enhance IL-4 production. These results provide a mechanism that may explain the link between RSV infection and enhancement of allergic asthma.

6.2 Introduction

Respiratory syncytial virus (RSV, a member of the pneumovirus subfamily of the *Paramyxoviridae*) is the most common cause of viral lower respiratory tract infections in children [40]. Although very often associated with relatively mild upper respiratory tract infection, RSV can cause severe pneumonia and bronchiolitis in infants, in older immunodeficient children, and in the elderly [29;63;186]. In moderate climate zones, RSV causes yearly epidemics during the winter season, and at the age of two all children have been infected at least once [186]. Because severe RSV infections can result in prolonged wheezing and airway hyperreactivity, an association with increased risk of developing respiratory allergy later in life is assumed [184;191;194]. However the precise role of RSV in the development of allergic disease in children is not clear. We [7] and others [159] previously demonstrated in a mouse model of allergic asthma, that RSV infection aggravated the consequences of airway sensitisation and

challenge with ovalbumin (OVA), resulting in enhanced pulmonary Th2 cytokine expression, allergy-associated lung pathology, and airway hyperresponsiveness. RSV infection thus enhanced allergic disease when the immune system was already Th2 primed by the allergen (OVA) [9;159]. This finding suggested that the allergic Th2 immune environment could also modulate the anti-viral immune response induced by RSV. Additionally, prior RSV immunisation did not abrogate the RSV-enhanced pulmonary Th2 cytokine response in OVA-allergic mice, indicating that also a secondary anti-viral response may enhance respiratory allergy [9].

Mature T cells that express α/β T cell receptors can be divided into cells expressing either CD4 or CD8 surface antigen [53]. $CD8^+$ cells are mainly cytotoxic cells and produce, among others, IFN-γ. $CD4^+$ T cells are mainly cytokine-secreting helper cells and can be divided into T helper 1 (Th1) or Th2 cells, depending on the cytokines produced [53;139]. RSV infection leads to activation of both $CD4^+$ and $CD8^+$ Th1-like T cell mediated immune responses, characterised by the production of IFN-γ [67;152]. For allergic asthma it was shown that pulmonary $CD4^+$ Th2 lymphocytes [102], and long-lived $CD4^+$ Th2 memory cells in the spleen and lungs are involved in inducing inflammation [135]. In addition to $CD4^+$ T cells, type 1 and type 2 cytokine-producing $CD8^+$ T cells have been demonstrated in the lung during allergic inflammation [60], suggesting that these cells also play a role in allergic asthma [115]. Experimental studies showed that a Th2 cytokine environment can transform virus-specific $CD8^+$ T cells from cytotoxic cells into non-cytotoxic, IL-4- and IL-5-producing cells *in vitro* [60] and *in vivo* [45]. These type 2 cytokine producing $CD8^+$ T cells were able to induce airway eosinophilia. Since allergic asthma is characterised by increased Th2 cytokine production, this may, after subsequent virus infection, stimulate the virus-specific $CD8^+$ T cells to switch to Th2 cytokine production and thus aggravate respiratory allergy.

We have demonstrated recently [8] that infection with the natural rodent pathogen pneumonia virus of mice (PVM, like RSV a member of the *Pneumovirinae* subfamily of the *Paramyxoviridae*), resulted in a similar enhancement of the OVA-allergy as induced by RSV. In contrast to RSV and PVM, influenza A virus (a member of the *Orthomyxoviridae* virus family) failed to enhance the pulmonary Th2 allergic response.

In the present study we investigated which virus-induced immune mechanisms could be responsible for the enhanced allergic inflammation in BALB/c mice. We therefore examined the allergen (OVA)- and virus-specific systemic cellular immune response in spleens of OVA-sensitised and –challenged mice infected with RSV, PVM or influenza virus.

6.3 Methods

Viruses: Human respiratory syncytial virus type A2 (RSV A2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). RSV was grown and quantified by virus titration on Hep2 cells, as described before [8].
Pneumonia virus of mice (PVM) was grown and quantified by virus titration on BHK-21 C_{13} cells in Eagle's MEM medium, containing Hank's salts, 10 mM $NaHCO_3$, 10% FCS, 2mM glutamine, 20 mM Hepes, 100 IE/ml penicillin, and 100 U/ml streptomycin.
Influenza A virus (mouse-adapted strain A/PR/8/34 (H1N1)) was grown and quantified by virus titration on MDCK-1 cells in Eagle's MEM medium, containing Hank's salts, 10 mM $NaHCO_3$, 2mM glutamine, 13 mM Hepes, 100 IE/ml penicillin, 100 U/ml streptomycin, 1.10^{-5}% trypsin (Merck), and 10% PGR-Albumin (containing 10 g pepton, 10 g glucose and 10 g bovine albumin per 800 ml Eagle MEM). The Influenza A and PVM virus stocks were prepared as described for RSV [8].

Animals: Female Specified Pathogen Free BALB/c mice were obtained from Harlan Olac (Horst, The Netherlands) and were used at 6-10 weeks of age. A week before the experiments started, mice were housed per group according to the experimental set-up, and cages were placed in a temperature-controlled isolator (22°C) to prevent infection of control animals and animal caretakers. Mice were kept in a 12-hour light/dark cycle, and received food and water *ad libitum*. The study was approved by the Institute's committee on animal welfare.

Experimental Design: Mice were sensitised to OVA by intraperitoneal (i.p.) administration of 0.5 ml OVA (20 µg/ml saline (Sigma-Aldrich, Steinheim, Germany) no adjuvant) for 7 times on every other day starting at day 0. From day 33 till day 40, mice were intranasally challenged with OVA aerosols (2 mg/ml saline) during 5 minutes on 8 consecutive days. OVA-sensitised mice were inoculated intranasally with 50 µl RSV ($1,5x10^7$ plaque forming units per dose ~ 8.10^6 $TCID_{50}$), 50 µl PVM (20 $TCID_{50}$ per dose), or 50 µl influenza virus (50 $TCID_{50}$ per dose), on day 35 during the OVA-challenge period (= OVA/RSV, OVA/PVM, or OVA/Influenza). Infection doses were determined in dose response experiments in mice. The lowest infection dose that still caused clinical symptoms, was chosen (data not shown). Virus-infected OVA-allergic mice were compared to OVA-allergic mice inoculated with their respective uninfected cell culture lysate (mock) (= OVA/Mock-r, OVA/Mock-p, OVA/Mock-i), and non-allergic mice infected with virus only (= -/RSV, -/PVM, -/Influenza). Before inoculation, mice were i.p. anaesthetised with Ketamine, Rompun (=xylazine), Atropine (KRA). At day 47 of the experimental protocol (12 days post virus inoculation), mice were anaesthetised with KRA i.p. and sacrificed. Spleens were aseptically removed. Experiments were performed in duplicate. Data of one representative experiment are shown.

In vitro splenocyte cultures: Single-cell suspensions were prepared from the spleen of each mice as previously described [17]. Splenocytes (1.5 x 10^5 cells/well) were cultured (37°C, 5% CO_2) in 96-well tissue culture plates (Nunc, Denmark) in culture medium (RPMI 1640, Gibco BRL, Life Technologies, Rockville, MD), supplemented with 10% FCS (Greiner, Frickenhausen, Germany), 2mM glutamine, 100 IE/ml penicillin, 100 U/ml streptomycin, and 0.05mM β-mercapthoethanol in the presence of 600 µg/ml ovalbumin (Sigma-Aldrich, Steinheim, Germany), RSV A2, PVM or influenza virus (all 1.10^6pfu/ml, UV-inactivated). After 72 hours of culture, supernatants were collected, aliquotted, and stored at -80°C until analysed for cytokine content.

Cytokine ELISAs: The concentrations of IFN-γ, IL-4, IL-5, and IL-13 in splenocyte culture supernatants were assessed by ELISA according to the protocol described by Boelen et al [17]. Coating antibodies were rat anti-mouse cytokine antibody IgG (anti-IFNγ-IgG_1, clone R4-6A2; anti-IL-4-IgG_1, clone 11B11; anti-IL-5-IgG_1, clone TRFK-5 (all obtained from BD Biosciences Pharmingen, San Diego, CA), and anti-IL-13-IgG_{2b}, clone 35213.11 (R&D Systems, Minneapolis, USA)). Detection antibodies were biotin-conjugated monoclonal rat-anti-mouse cytokine IgG (anti-IFNγ-IgG_1, clone XMG1.2; anti-IL-4-IgG_1, clone BVD6-24G2; anti-IL-5-IgG_{2a}, clone TRFK-4 (all obtained from BD Biosciences Pharmingen). IL-13 was detected using biotin-conjugated goat anti-mouse IL-13-IgG (R&D Systems, Minneapolis, USA). The detection limit of the assays were: IFN-γ: 7.8 pg/ml, IL-4: 7.8 pg/ml, IL-5: 7.8 pg/ml, IL-13: 62 pg/ml.

Intracellular cytokine staining and flow cytometric analysis: For detection of intracellular cytokines using four colour flow-cytometry at the single cell level, splenocytes were re-stimulated (after 72 hours of culturing with OVA, RSV, or influenza virus) for 6 hours on round bottom 96 well plates coated with anti-CD3 mAb (clone 145-2C11) in the presence of anti-CD28 mAb (clone 37.15), to provide co-stimulation [Harding et al., 1992], and monensin (1/1500 diluted Golgistop®, BD Biosciences Pharmingen) to block cytokine secretion. After washing, cells were incubated with anti-FcRII/III mAb 2.4G2 to prevent non-specific binding, followed by surface staining for CD4-FITC (clone L3T4gk1.5) and CD8-PeCy5 (clone Ly-2 (53-6.7)). Fixation, permeabilisation and staining for intracellular cytokines using anti-IFNγ-APC (clone XMG1.2), and anti-IL-4-PE (clone 11B11) were performed according to the manufacturer's protocol. All mAbs were purchased from BD Biosciences Pharmingen. Samples were analysed on a dual laser FACS callibur flow cytometer (Becton Dickenson).

Statistical analysis: Data are presented as mean ± Standard Error of the Mean (SEM). Statistical significance for differences between indicated groups were determined using the Student's *t* test (Excel, Microsoft Corporation, Redmond, USA).

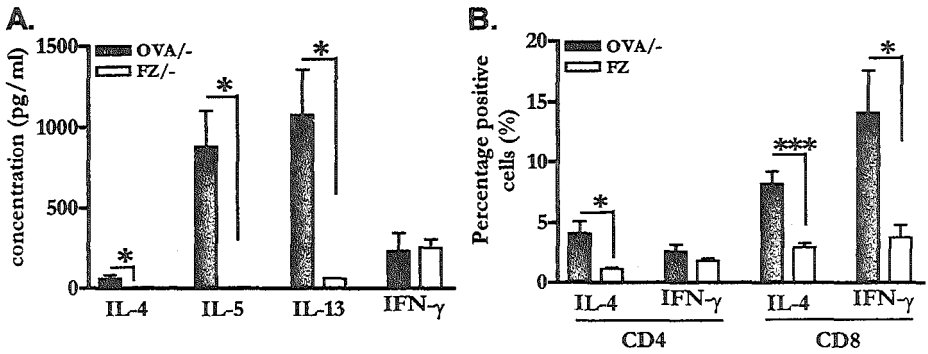

Figure 6.1. A: IL-4, IL-5, IL-13 and IFN-γ concentrations produced by splenic cells stimulated *in vitro* by ovalbumin (left). Splenocytes were obtained at day 47 of the experimental protocol (is day 7 after the last OVA-challenge) from OVA-allergic mice (OVA/-) of non-allergic mice (FZ/-). Mean values ± SEM (n = 6 per group) are depicted. Statistical differences between groups are indicated by *: $P < 0.05$. B: Intracellular IL-4 and IFN-γ staining of $CD4^+$ and $CD8^+$ splenocytes after *in vitro* stimulation by ovalbumin. Data are depicted as percent positive cells (%) and mean values ± SEM (n=6) are shown. Statistical differences between indicated groups are indicated by *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

6.4 Results

OVA-specific immune response

To investigate the OVA-specific cellular immune response, splenocytes were stimulated *in vitro* with OVA, and cytokine concentrations were measured in the culture supernatant after 72 hours. The same splenocytes were then re-stimulated, stained for intracellular cytokines, and examined by FACS analysis.

In vitro OVA-stimulation of splenocytes from OVA-sensitised and –challenged mice resulted in secretion of the Th2 cytokines IL-4, IL-5, and IL-13 in the culture supernatant (fig. 6.1 A). Intracellular cytokine staining of these splenocytes demonstrated that increased percentages of both $CD4^+$ and $CD8^+$ T lymphocytes obtained from OVA-allergic mice contained IL-4 (fig. 1 B). In addition, an increased percentage of OVA-specific $CD8^+$ cells were positive for IFN-γ (fig. 6.1B), and were not positive for IL-4. Despite an increase of IFN-γ containing OVA-specific $CD8^+$ cells according to FACS analysis, no increase of IFN-γ could be detected in culture supernatant of OVA-stimulated splenocytes from OVA-allergic mice (fig. 6.1 A).

We subsequently investigated the influence of respiratory virus infections on the OVA-specific systemic immune response. OVA-stimulation of splenocytes obtained from both RSV (fig. 6.2 left) and PVM (fig. 6.3 left) inoculated OVA-allergic mice, resulted in significantly enhanced production of IL-4, IL-5, and IL-13, compared to their corresponding mock-inoculated controls. In contrast, OVA-stimulated splenocytes from influenza virus-inoculated OVA-

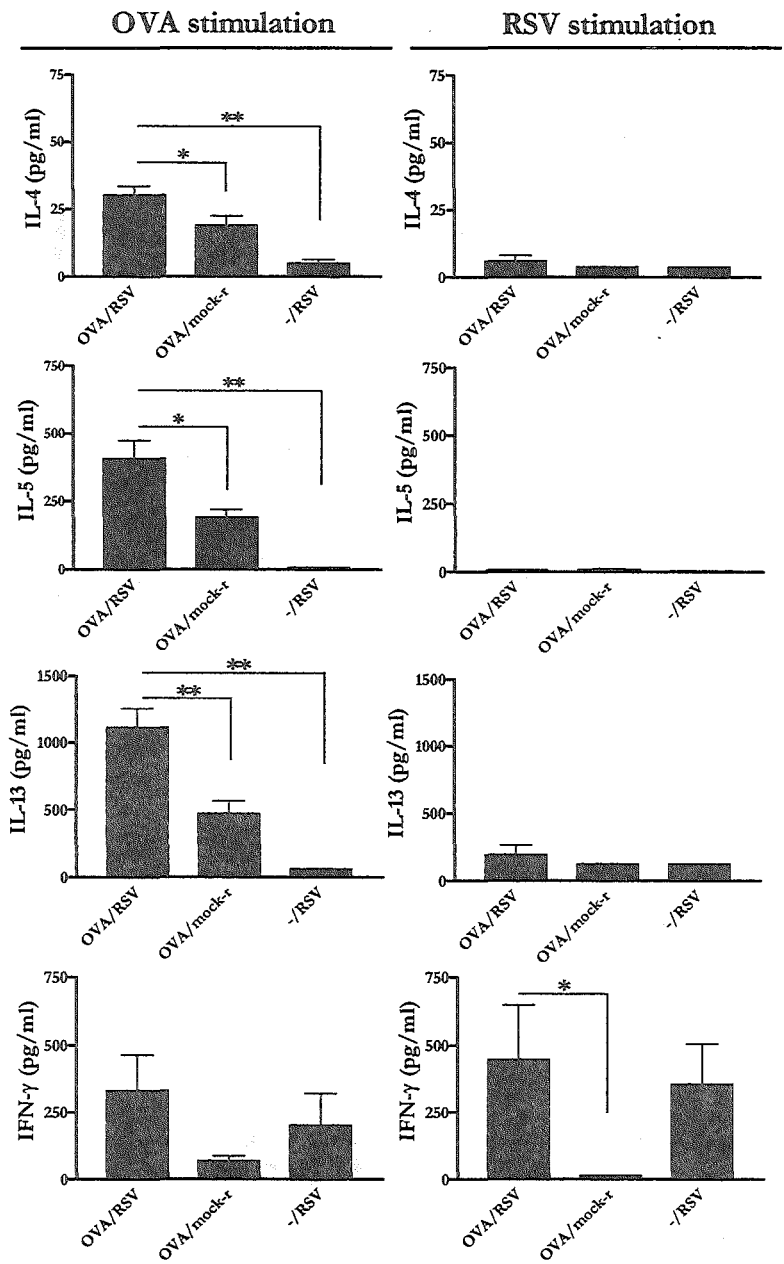

Figure 6.2. IL-4, IL-5, IL-13 and IFN-γ concentrations produced by splenic cells stimulated *in vitro* by ovalbumin (left) or UV-inactivated RSV (right). Splenocytes were obtained at day 47 of the experimental protocol (is day 7 after the last OVA-challenge) from OVA-allergic mice infected with RSV (OVA/RSV), inoculated with mock (OVA/mock-r) or non-allergic mice infected with RSV (-/RSV). Mean values ± SEM (n = 6 per group) are depicted. Statistical differences between indicated groups are indicated by *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

allergic mice did not secrete increased levels of IL-4, IL-5, and IL-13 into the culture supernatants, compared to their corresponding mock-inoculated controls (fig. 6.4 left).
Next we investigated the phenotype of the IFN-γ- and IL-4-producing splenocytes from virus-infected allergic mice. Intracellular cytokine staining demonstrated that an increased percentage of OVA-specific $CD4^+$ T cells produced IL-4, when allergic mice were inoculated with RSV (fig. 6.5A). The IL-4 production of OVA-specific $CD8^+$ T cells was not affected by RSV-inoculation of allergic mice. In contrast to RSV, influenza virus inoculation of allergic mice did not increase the number of IL-4 producing OVA-specific $CD4^+$ and $CD8^+$ T cells (fig. 6.5C), thus in accordance with the IL-4 concentrations measured in splenocyte culture supernatants (fig. 6.4 left).
OVA-stimulation of splenocytes not only induced the production of Th2 cytokines, but also resulted in production of IFN-γ by OVA-specific $CD8^+$ T cells. Virus infection of allergic mice did not significantly enhance the production of IFN-γ, as measured in splenocyte cultures (fig. 6.2, 6.3, 6.4 left), and by intracellular cytokine staining of $CD4^+$ and $CD8^+$ T cells (fig. 6.5 A,C). This indicates that RSV and PVM only affected the OVA-specific Th2 response, but not the OVA-specific Th1 response.

RSV-specific immune response

Parallel to the OVA-specific immune response, we examined the RSV-specific cellular immune response in the spleen of the same mice. *In vitro* RSV-stimulation of splenocytes obtained from RSV-infected non-allergic mice resulted in the production of IFN-γ, but not the production of IL-4, IL-5, and IL-13 (fig. 6.2 right). A similar pattern of cytokine production was observed after *in vitro* stimulation with PVM (fig. 6.3, right) or influenza virus (fig. 6.4, right) of splenocytes obtained from PVM- or influenza virus-infected mice respectively. The virus-specific IFN-γ production was not affected by OVA-sensitisation and -challenge (fig. 6.2, 6.3, 6.4 right).
RSV stimulation of splenocytes from RSV-infected allergic and non-allergic mice mainly resulted in increased percentages of IFN-γ producing $CD8^+$ cells, and a small percentage of RSV-specific $CD4^+$ cells also produced IFN-γ (fig. 6.5B). In addition, an increased number of RSV-specific $CD8^+$ cells produced IL-4 when RSV-inoculated mice were sensitised and challenged with OVA (fig. 6.5B). When non-allergic mice were infected with RSV, no RSV-specific IL-4 producing $CD8^+$ cells were observed. No RSV-specific $CD8^+$ cells producing both IL-4 and IFN-γ were detected.
Influenza virus stimulation of splenocytes from influenza-virus infected allergic and non-allergic mice resulted predominantly in increased percentages of IFN-γ producing $CD8^+$ cells (fig. 6.5D), with only few influenza-specific $CD4^+$ cells producing IFN-γ. In contrast to RSV, influenza-specific $CD8^+$ cells did not produce IL-4.

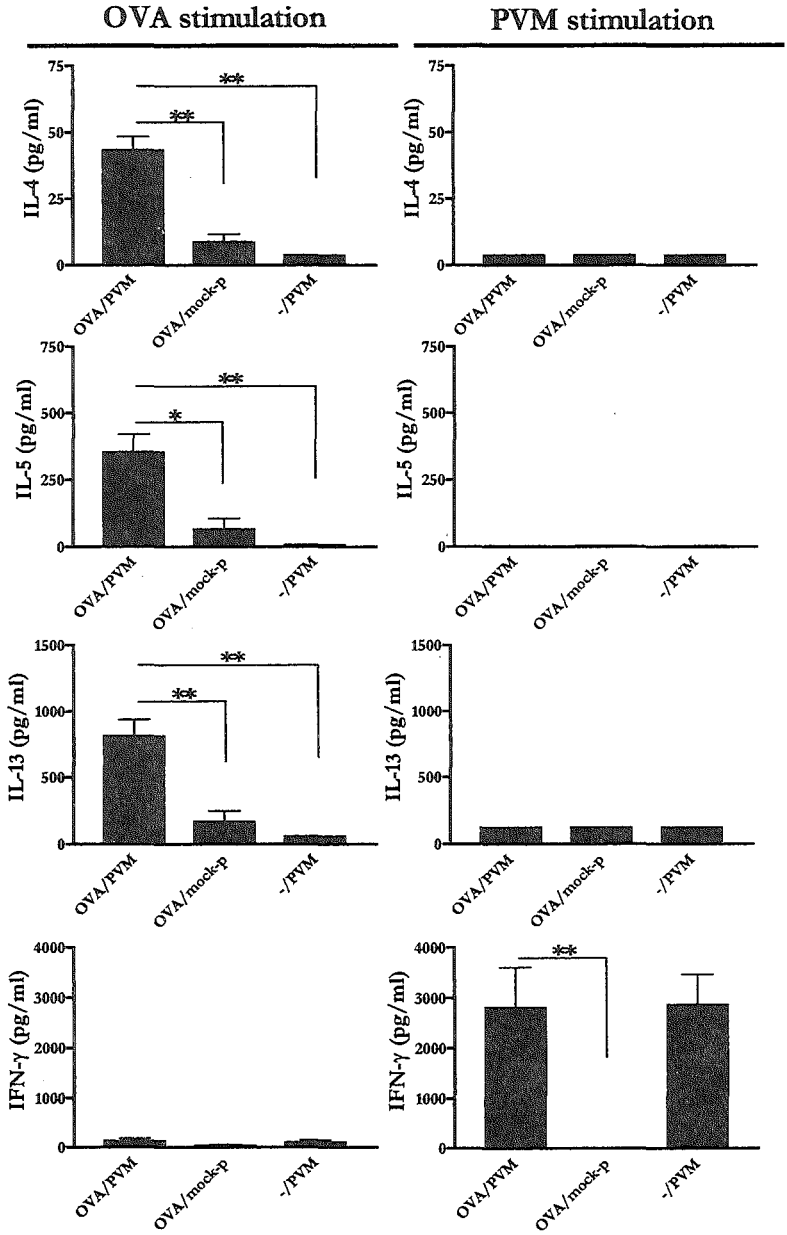

Figure 6.3. IL-4, IL-5, IL-13 and IFN-γ concentrations produced by splenic cells stimulated *in vitro* by ovalbumin (left) or UV-inactivated PVM (right). Splenocytes were obtained at day 47 of the experimental protocol (is day 7 after the last OVA-challenge) from OVA-allergic mice infected with PVM (OVA/PVM), inoculated with mock (OVA/mock-p) or non-allergic mice infected with PVM (-/PVM). Mean values ± SEM (n = 6 per group) are depicted. Statistical differences between indicated groups are indicated by *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

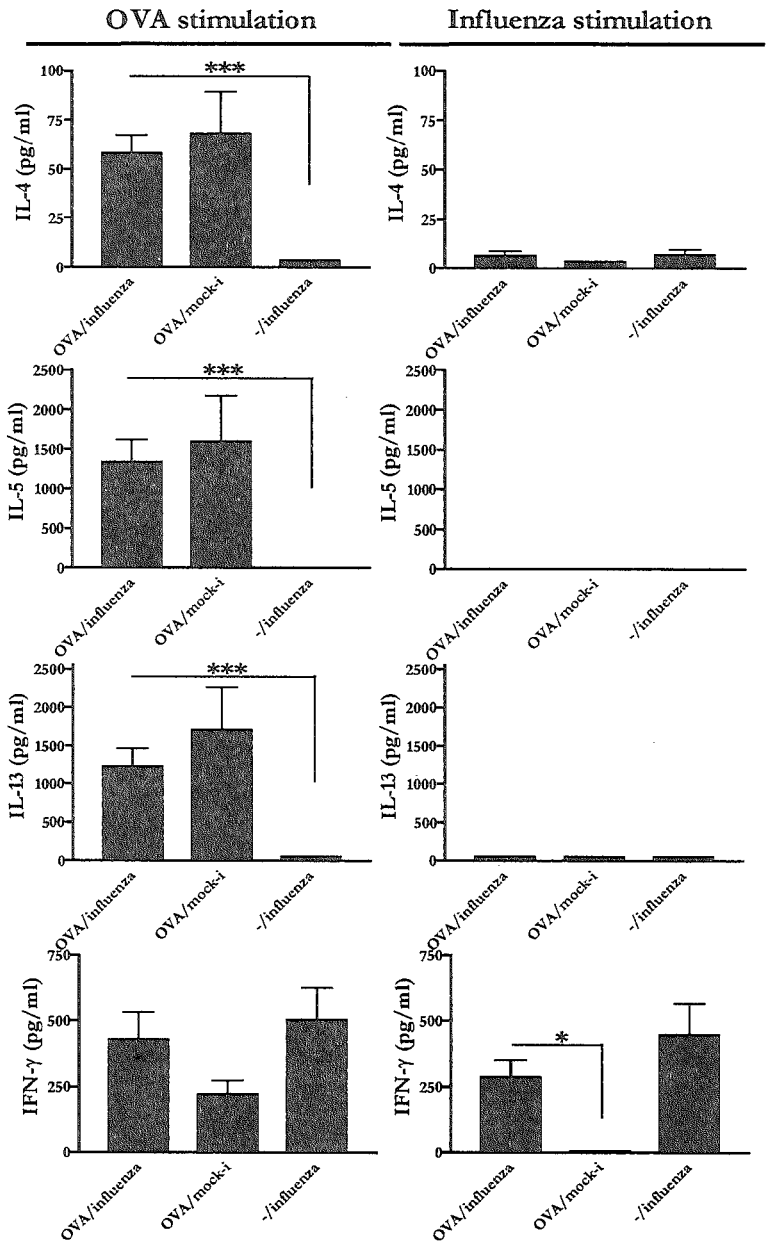

Figure 4.5. IL-4, IL-5, IL-13 and IFN-γ concentrations produced by splenic cells stimulated *in vitro* by ovalbumin (left) or UV-inactivated RSV (right). Splenocytes were obtained at day 47 of the experimental protocol (is day 7 after the last OVA-challenge) from OVA-allergic mice infected with influenza virus (OVA/influenza), inoculated with mock (OVA/mock-i) or non-allergic mice infected with influenza virus (-/influenza). Mean values ± SEM (n = 6 per group) are depicted. Statistical differences between indicated groups are indicated by *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

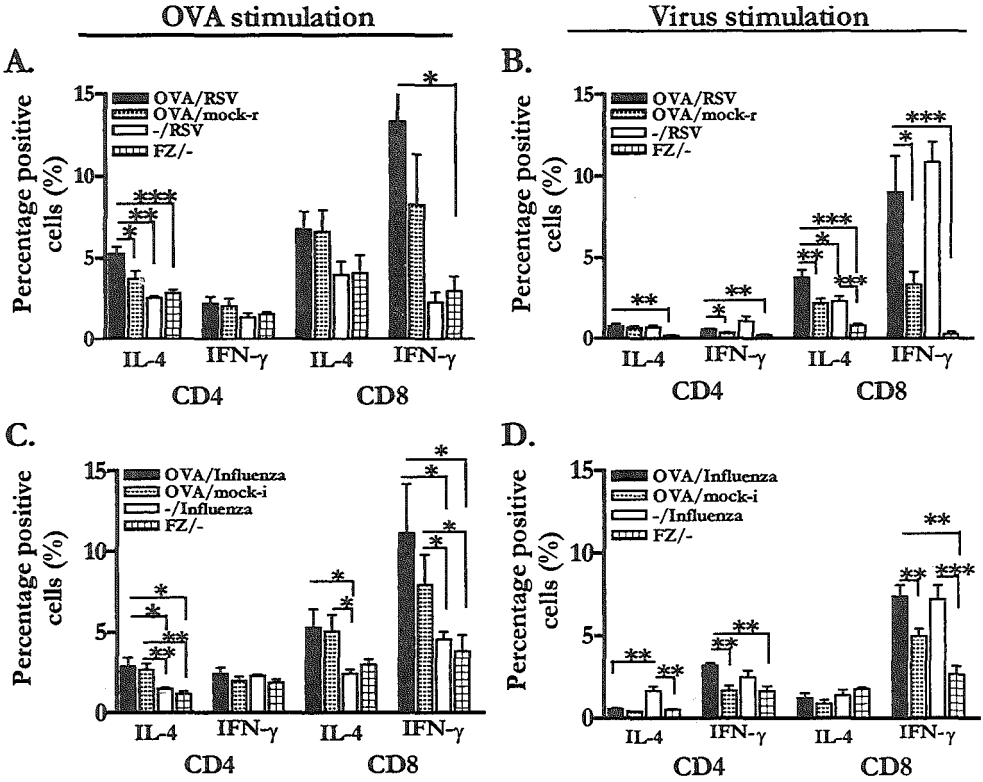

Figure 6.5. Intracellular IL-4 and IFN-γ staining of $CD4^+$ and $CD8^+$ splenocytes after *in vitro* stimulation by OVA (A and C), RSV (B) or influenza (D) stimulation. Splenocytes were obtained at day 47 of the experimental protocol (is day 7 after the last OVA-challenge) from differentially treated mice. A and B: OVA-allergic mice infected with RSV (OVA/RSV), inoculated with mock (OVA/mock-r), non-allergic mice infected with RSV (-/RSV), or non-allergic mice without virus inoculation (FZ/-). C and D: OVA-allergic mice infected with influenza (OVA/influenza), inoculated with mock (OVA/mock-i, non-allergic mice infected with influenza (-/influenza), or non-allergic mice without virus inoculation (FZ/-). Data are depicted as percent positive cells (%) and mean values ± SEM (n=6) are shown. Statistical differences between indicated groups are indicated by *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

6.5 Discussion

In the present study we investigated the OVA- and virus-specific systemic immune response in respiratory virus infected allergic mice. In addition, the mutual influence of allergen- and virus-specific immune responses was examined.

The memory T cell response in the spleen of OVA-sensitised mice appears essential for the generation of a Th2 allergic response in lungs, upon OVA re-exposure [135]. In accordance with previously reported results [135;151], we showed that splenocytes from OVA-sensitised and –challenged mice produced high levels of IL-4, IL-5, and IL-13 upon OVA-stimulation *in vitro*. IL-4 was produced by both $CD4^+$ and $CD8^+$ T cells. In addition, OVA-specific $CD8^+$ T cells other than the IL-4 producing cells, produced IFN-γ, indicating that besides a Th2 response also a Th1 response was induced. It has been shown that increased amounts of $CD8^+$ T cells producing IFN-γ are recovered from the airways of OVA-allergic mice [59;113]. We now demonstrate that also systemic $CD8^+$ cells produce IFN-γ in allergic mice. Despite the evidence for a protective role of Th1 cytokines in allergic inflammation [102], these IFN-γ producing cells are suggested to play a role in the establishment of airway hyperreactivity (AHR) following OVA-challenge in OVA-sensitised mice [84].

RSV and PVM inoculation of OVA-allergic mice during allergen challenge enhanced the Th2 cytokine production by OVA-specific splenocytes, while influenza virus did not. These data confirm our previously reported findings that only the two pneumoviruses, PVM and RSV, enhanced pulmonary Th2 cytokine mRNA expression, and lung pathology [8]. Intracellular cytokine staining pointed out that a higher percentage of OVA-specific $CD4^+$ T cells produced IL-4 when OVA-allergic mice were infected with RSV, whereas OVA-specific $CD8^+$ T cells were not affected by RSV. This finding indicates that RSV enhanced the allergic Th2 response by stimulating systemic OVA-specific $CD4^+$ T cells. The results presented in the current article are not in correspondence with the recently reported findings by Peebles et al [161]. They studied the production of IL-4, IL-5 and IFN-γ by pulmonary $CD4^+$ and $CD8^+$ lymphocytes in a similar allergic mice model, but could not detect increased IL-4- and IL-5-positive T cells in OVA/RSV-treated mice compared to OVA/mock-treated mice. This difference might be due to a discrepancy between pulmonary and splenic T cell responses, or *in vitro* stimulation of lymphocytes with an a-specific stimulus (PMA) by Peebles et al [161]. In the present study splenic T cells were specifically stimulated with either OVA or RSV, thus preventing the generation of a mixed immune response.

A possible mechanism explaining the RSV-induced enhancement of the allergic immune response could be the altered anti-viral immune response. The results presented in the current article demonstrate that a subset of RSV-specific $CD8^+$ T cells produce more IL-4 when mice were previously sensitised and challenged to OVA, i.e. when a pre-existing Th2 cytokine environment is present. In contrast, no increased percentages of influenza-specific IL-4 producing $CD8^+$ T cells were obtained from OVA/influenza-treated mice. The increased

percentage of RSV-specific IL-4 producing $CD8^+$ cells was not accompanied by an increase of IL-4 in culture supernatants of RSV-stimulated splenocytes. A possible reason for this discrepancy could be the relatively small amount of CD8 cells present (ca 20% of CD4 cells) and their low ability to produce IL-4 (30 to 100 fold lower than CD4 cells) [47].

Experimental *in vitro* studies have demonstrated that activation of $CD8^+$ T cells in the presence of IL-2 or IL-12 resulted in $CD8^+$ T effector cells that produce IL-2 and IFN-γ upon reactivation [47]. In contrast, the presence of IL-4 during effector cell activation promoted the development of $CD8^+$ T cells that produce IL-4 and IL-5 [47;60]. In our allergic mice model, OVA-sensitisation and –challenge induced the development of a Th2 cytokine response. It has been demonstrated *in vivo* that lymphocytic choriomeningitis virus (LCMV)-specific $CD8^+$ T cells produce IL-5, when mice were previously sensitised to OVA [45]. Coyle et al [45] observed a direct relation between virus-specific $CD8^+$ T lymphocytes producing IL-5 in the lungs of allergic mice, and increased eosinophilia and AHR. It is reasonable to assume that in the present study RSV enhances the allergic Th2 immune response via a comparable mechanism as described for LCMV [45]. Though we demonstrated the switch of RSV-specific $CD8^+$cells to produce IL-4 instead of IL-5. Besides the direct effect of RSV-induced IL-4 and IL-5 on the allergic phenotype, IL-4 produced by RSV-specific $CD8^+$ cells could in turn stimulate OVA-specific $CD4^+$ and $CD8^+$ T cells to enhance the Th2 cytokine production resulting in further aggravation of the allergic inflammation. In contrast to Coyle et al [45], no decrease in the number of RSV-specific IFN-γ producing $CD8^+$ T cells and IFN-γ concentration in splenocyte cultures from RSV-infected allergic mice were detected. It remains unknown why IFN-γ production was not inhibited by the increased Th2 cytokine levels in these mice. The necessity of a pre-existing Th2 cytokine environment for RSV to subsequently enhance respiratory allergy appeared from previous experiments in which RSV infection only enhanced allergic disease when the immune system was already Th2 primed by the allergen (OVA), and not when infection was given before allergic sensitisation [9;159].

The reason why the OVA-induced switch of virus-specific $CD8^+$ T cells to IL-4 production is only observed for RSV and not for influenza virus is not clear. A possible explanation could be the Th2 immune-modulating capacity of the RSV G attachment protein. Immunisation with purified G protein induces a $CD4^+$ Th2 immune response upon subsequent RSV infection [80;193]. The OVA-induced inflammation and Th2 cytokine milieu in mice might favour the immune response to G and further stimulate the Th2-inflammatory response [68]. In contrast to RSV, influenza virus lacks a Th2-stimulating G protein. Furthermore, we have demonstrated that PVM and RSV were mainly located in the epithelial cells lining the alveoli and bronchi [8], stimulating these cells to secrete chemokines and other mediators involved in allergic inflammation [214;221]. In contrast, influenza virus was predominantly observed in the alveolar macrophages [8]. In summary, we demonstrated that RSV and PVM infection, but not influenza virus infection, of OVA-allergic mice enhanced the Th2 cytokine production of OVA-specific T cells in the spleen. The current study provides evidence that the pre-existing Th2 cytokine milieu modulates the RSV-specific $CD8^+$ T cells to produce IL-4. RSV infection

in turn stimulates the OVA-specific $CD4^+$ T cells to enhance IL-4 production. These results provide a mechanism that may explain the link between RSV infection and enhancement of allergic asthma.

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Chapter 7 General Discussion

Respiratory syncytial virus and asthma: lessons from the mice model

Publication in preparation

7.1 Introduction

Respiratory syncytial virus (RSV) is one of the most common paediatric infectious diseases world-wide, with 70% of children infected within the first year of life and almost 100% by age 3 [40]. Clinical symptoms induced by RSV infection vary widely. Most children infected with RSV develop mild upper respiratory tract infection, but a minority of RSV infected children develop severe bronchiolitis that requires hospitalisation and can be life-threatening [40]. Consequences of severe RSV bronchiolitis is the onset of recurrent wheezing, which can be prolonged for a long time after the initial infection [40]. However, the direct relation between severe RSV infection and the development of recurrent wheezing during childhood is unclear [127;222].
The increased prevalence and severity of asthma over the last decades [87] have led to considerable interest in the question whether or not severe RSV infection in infancy contributes to wheezing and/or the development of asthma later in childhood. Asthma during childhood is associated in most cases with sensitisation to inhaled allergens and characterised by allergic airway inflammation. The onset of asthma has been associated with viral lower respiratory tract infections [25]. In addition, acute viral respiratory infections can trigger wheezing and exacerbations of asthma in children and adults [98;131]. Whether RSV bronchiolitis during infancy remains a risk factor for enhanced allergic disease and asthma later in life is not clear [194]. While over 70% of all children are infected with RSV during their first year of life only a small part develops allergic illness. It is demonstrated that it is the severity of RSV infection which is associated with increased risk for the development of recurrent wheezing and asthma. It is possible that severe RSV lower airway inflammation leads to airway remodelling and affects lung development, or somehow targets allergic inflammation to the lower airways. It has been suggested that if RSV can trigger the "allergic process", this will occur only in subjects who are predisposed either by their genetic background, or by underlying lung disorders present before there first contact with RSV, that have "primed" their immune system and lungs [125;165]. In addition, Sigurs et al. reported that family history for asthma might have been a predisposing factor for the development of severe RSV bronchiolitis and the subsequent development of asthma in children [184]. In order to investigate the underlying immunological mechanisms involved in the interplay of RSV infection and allergic airway inflammation, animal models have been used . In the present article, we will review the murine models that explore how RSV infection has an impact on allergic disease.

7.2 Influence of RSV infection on pre-existing respiratory allergy

Murine models of allergen-induced pulmonary inflammation share many features with human asthma, including airway hyperreactivity (AHR), antigen-specific cellular and antibody response, amplification of Th2 cytokine (IL-4, IL-5, IL-13) levels, and induction of antigen-specific IgE. Ovalbumin (OVA, a chicken egg protein serving as an allergic antigen) is the most widely used

antigen to induce airway allergic inflammation [83;113], although the more relevant dust mite or cockroach allergens are also used. The different models reported in literature vary widely in the strain of mice used, the method of sensitisation to and challenge with OVA, and the time between allergen exposure and analysis of the different parameters [reviewed in [119]]. In its simplest form, OVA is injected intraperitoneally with or without a Th2 skewing adjuvant, such as aluminium hydroxide. Sensitisation to the antigen is followed 10-20 days later by a period of OVA aerosol challenge to the airways. As from 1 day after challenge, analysis of airway inflammation is performed.

Several authors have investigated the role of RSV infection on OVA-induced allergy in mice. The main variable between the different studies performed is the timing of RSV infection before or during the sensitisation and challenge protocol. In general, two groups can be distinguished, 1) those who investigated the influence of prior RSV infection on the later development of allergic inflammation, and 2) those who examined the influence of RSV infection on an already established allergic inflammation. Since RSV infections occur throughout life, allergic sensitisation can already be developed before the first RSV infection, although the first asthmatic symptoms in children might develop at a later age.

In this review we focus on the influence of an RSV infection on an already existing allergic inflammatory response in mice. We [6-9] and others [15;126;162] reported that RSV infection in already allergen-sensitised mice aggravated the allergic inflammation, characterised by increased Th2 cytokine production in lungs and spleen, enhanced and prolonged AHR, and eosinophilic infiltration and mucus secretion into the airways.

Th2 cytokines and chemokines

It is generally recognised that $CD4^+$ Th2 lymphocytes and their cytokines play a central role in allergic inflammation. The presence of Th2 cells in the airways of symptomatic patients together with the specific capacities of Th2-derived cytokines underlines their significance in this disease [26]. We reported that RSV infection, given during OVA challenge in already OVA-sensitised mice, resulted in increased mRNA expression of the Th2 cytokines IL-4, IL-5 and IL-13 in lung tissue [7;8]. In addition, RSV prolonged the period of Th2 cytokine expression up to one week after the last OVA-challenge [7]. Besides the increased cytokine mRNA levels in the lung, RSV infection enhanced the *in vitro* production of these Th2 cytokines by OVA-specific splenocytes [8;10]. The RSV-induced enhanced levels of IL-4, IL-5 and IL-13 have physiological consequences for allergen-induced lung disease. IL-4 is a central cytokine in the Th2 immune response. It is secreted by Th2 cells, mast cells, basophils and eosinophils. A major activity of IL-4 is activation of Th2 lymphocytes and promoting B cells to produce IgE antibody [44;54]. High concentrations of IL-4 has been detected in bronchoalveolar lavage (BAL) fluid [211] from patients with atopic asthma. Also the IL-4 mRNA was expressed at higher levels in BAL cells [170] and bronchial biopsies [231] from these patients. In murine models of allergic sensitisation and airway challenge, IL-4 appeared to be crucial for the

development of allergic airway inflammation [24] and AHR [43;107]. IL-4 plays a role in mast cell degranulation due to the cross-binding of IL-4-induced allergen-specific IgE by the allergen. Despite the ability of IL-4 to stimulate IgE production, we did not observe a correlation between IL-4 expression and IgE in RSV-infected OVA–allergic mice. Although RSV infection increased the level of pulmonary and splenic IL-4 in OVA-allergic mice, no changes in both total and OVA-specific IgE serum concentrations were detected [7-9]. A similar observation was made by Matsuse et al. who infected dust mite (DerF)-sensitised and -challenged mice with RSV [126]. In addition, Schwarze et al demonstrated in a study investigating the influence of RSV infection on subsequent allergic sensitisation, that increased IL-4 levels not resulted in enhanced levels of IgE in serum [178].

IL-13, is, independent of IL-4, crucial for the induction of allergic asthma in animal models and also stimulates IgE production by B cells [38;59;70]. Like for IL-4, RSV-enhanced the IL-13 mRNA expression in lung tissue of OVA-sensitised and challenged mice [8;9], and increased the production of IL-13 by OVA-stimulated splenocytes [7]. Thus, like for IL-4, IL-13 levels do not correlate with IgE levels. Therefore, it seems likely that beside IL-4 and IL-13 an other signal can be able to or is needed for IgE production. Vercelli et al reported that after the first signal delivered by IL-4 or IL-13, a second signal for the activation of IgE production is delivered. This signal is provided upon binding of CD40 on B cells to the CD40 ligand expressed on T cells, which finally results in production of IgE [210]. Presumably, the expression of CD40 and CD40 ligand in our mice model of allergic inflammation is not affected by RSV infection, hence no increase in serum IgE is detected.

The role of IL-13 in disease enhancement after RSV infection is reported by De Swart et al [51] who infected FI-RSV vaccinated (like OVA sensitisation and challenge known to skew the immune response towards Th2) macaques with RSV. They demonstrated that RSV-specific T cells predominantly produce IL-13 and IL-5 and suggested that an IL-13-associated mechanism was responsible for increased AHR in these animals [51].

IL-5 is mainly produced by Th2 cells and mast cells, and has been demonstrated to play a pivotal role in the development of airway eosinophilia and AHR during allergic sensitisation [79;175;177]. In our experiments, RSV infection enhanced the pulmonary expression of IL-5 in OVA-allergic mice, and increased the production of IL-5 by OVA-stimulated splenocytes [6;8]. However, the increase in IL-5 was not always correlated reproducible with increased eosinophilia in the lungs [7;8].

In contrast to our observations, Peebles et al. did not find increased Th2 cytokine levels in lung tissue when OVA allergic mice were infected with RSV during allergen challenge [161;163]. However, these authors observed increased airway hyperreactivity (AHR). A possible explanation for the discrepant results could be the difference in time points after infection and challenge at which the cytokine levels were determined. As demonstrated, RSV prolongs the Th2 cytokine mRNA expression in lung tissue of allergic mice [7]. When analysed before the end of the OVA-aerosol challenge [161], no differences between RSV infected and non-infected allergic mice are established yet. In addition, Peebles et al. also reported no changes in

pulmonary β-chemokine expression (eotaxin, macrophage-inflammatory protein (MIP)-1α, MIP-1β, (RANTES)) [161]. The β-chemokines are thought to control the migration of T helper cells and eosinophils to the asthmatic airway [121]. We demonstrated that RSV infection in allergen sensitised and challenged mice enhanced the pulmonary eotaxin mRNA expression [8].

Histopathologic changes

Without exception, the different murine models provided evidence for RSV-enhanced allergen-induced mucus production by airway epithelial cells in [7-9;15;163;179]. For example, Blyth et al found that extensive hyperplasia of airway goblet cells was induced by RSV infection during the course of ovalbumin aerosol exposure [15]. In addition, Peebles et al showed that RSV infection during allergen exposure clearly prolonged the expression of the mucin protein Muc5ac in the lung, compared to the expression in allergic mice that were sham inoculated [160]. These data suggest that the mucus production is essential for the increased AHR. Despite the RSV-enhanced levels of pulmonary IL-5 in OVA-sensitised and challenged mice, no increase in eosinophilia was observed in most of our experiments [8;9], indicating that IL-5 and eosinophilia are not directly correlated. Like us, Blyth et al [15] and Peebles et al [161;163] could not demonstrate increased levels of eosinophils in the lung tissue and BAL of allergic mice infected with RSV, although increased AHR was reported. Since IL-5 has been identified as an eosinophil attractant [79;175], increased eosinophilic inflammation was expected. Moreover, increased eosinophilia is associated with aggravated AHR [175;176]. However, Hessel et al. observed in a murine model of allergic asthma that AHR (one of the key characteristics of asthma) can develop without the presence of eosinophils in the lung [83], indicating that other cells or mechanisms are responsible for AHR[84].

7.3 The role of RSV-induced Th1 cytokines in RSV-enhanced Th2 associated allergic inflammation.

Peripheral blood mononuclear cells (PBMC) from normal children and adults infected with RSV showed a predominant type 1 like T cell response, characterised by the production of IFN-γ and low levels of Th2 cytokines [4;21]. Also in healthy BALB/c mice, primary RSV infection induced a predominant Th1 cytokine response [17;90;202]. As mentioned earlier, RSV infection during the provocation of an allergic inflammation in mice resulted in a more pronounced Th2-like immune and inflammatory response. However, a simultaneous increase in the expression of the Th1 cytokines IFN-γ and IL-12 is induced [7-9]. Since IFN-γ and IL-12 are both involved in immune regulation of the Th1/Th2 balance by inhibiting the Th2 pathway [16;201], the allergy-enhancing capacity of RSV seems in contrast to the induction of a strong RSV-specific Th1 immune response. We therefore hypothesised that IL-12 and IFN-γ do not inhibit the RSV-enhanced allergy. To investigate the role of IL-12 and IFN-γ in RSV-enhanced allergic inflammation, the cytokine and inflammatory responses in IL-12 and IFN-γ receptor

(IFN-γR) deficient mice were studied [6]. OVA-sensitisation and challenge without RSV infection did not result in differences in Th2 responses between IFN-γR$^{-/-}$, IL-12$^{-/-}$ and wild type mice [6]. Thus IFN-γ and IL-12 do not play a major regulatory role in the OVA-allergic response itself. These observations were also made by Brusselle et al [23]. In addition, IL-12 deficiency did not influence the Th2 immune and inflammatory response in RSV infected OVA-sensitised/challenged mice [6]. In a previous study by Boelen et al [18], IL-12 deficiency also did not influence the cytokine and inflammatory response after primary RSV infection. One explanation for the unaffected IL-4 and IL-5 responses in IL-12$^{-/-}$ mice is that another cytokine took over the role of IL-12 to compensate its absence. A likely candidate is IL-18, because IL-18 is, like IL-12, able to induce IFN-γ production by NK cells [129].

Because IFN-γ is an inhibitor of the Th2 immune response [16], lack of the IFN-γR may result in an unrestrained Th2 response [198]. Boelen et al. [18] showed in a primary RSV infection model that the absence of IFN-γR resulted in a shift towards a Th2 immune response, demonstrating that IFN-γ inhibits the development of a Th2 response upon RSV infection. Since similar levels of IFN-γ were observed in allergic and non-allergic mice infected with RSV, thus an RSV-enhanced allergic Th2 response could occur despite the presence of IFN-γ, suggesting that IFN-γ does not inhibit the Th2 response. However, absence of IFN-γR signalling in RSV-infected OVA-allergic mice resulted in a prolonged IL-4 and IL-13 production, enhanced pulmonary eosinophilia and increased mucus secretion, compared with identically treated wild-type mice [6]. Hence, RSV-induced IFN-γ diminished IL-4 and IL-13 production and histopathological changes. Therefore it appears that the virus infection during respiratory allergy has a dual role. The RSV-induced IFN-γ response restricts the Th2 response, which however appears insufficient for complete inhibition of the RSV-enhanced respiratory allergy. Thus, RSV probably enhances the allergic inflammatory and Th2 cytokine responses by an IFN-γ independent pathway.

7.4 Conditions for RSV-enhanced allergic inflammation: timing of infection

One major difference between the various mouse models used to study the relationship between RSV infection and aggravated allergic reactions, is the timing of infection during allergen sensitisation and challenge. The time interval between infection and allergen exposure seems to be critical for the influence of viral [203;229], or bacterial [39] infection on the development of respiratory allergy in mice. Indeed, the timing of RSV infection during OVA sensitisation/challenge is critical for RSV-enhanced allergy [5;65]. In our studies, RSV infection only enhanced the pulmonary Th2 cytokine and inflammatory response when mice were inoculated after OVA-sensitisation, thus in already Th2-skewed mice [9]. The increased Th2 cytokine mRNA responses coincided with alveolitis and hypertrophy of mucus-producing epithelial cells. Since alveolitis is specifically induced by RSV infection [8;17;18], the alveolitis is likely due to the effect of RSV alone and not to (RSV-enhanced) allergy. In contrast, the

hypertrophy of mucus-producing cells is not specifically enhanced by RSV and thus seems to be related to increases in Th2 cytokine expression. Like for Th2 cytokine expression and hypertrophy of mucus-producing cells, AHR was increased in mice infected with RSV after allergic sensitisation [159]. In contrast, Peebles et al. found that RSV infection given before allergic sensitisation decreased allergen-induced AHR, production of IL-13 in lung-tissue, and lung eosinophilia [159]. Although we observed no effect on allergic responses if RSV infection was given prior to allergen sensitisation and challenge, others found enhanced AHR and Th2 cytokine production [120;151;178]. The different outcomes may be due to variations between the BALB/c mice strains used [160]. A similar situation occurs in a primary infection model for RSV. Normally, primary RSV infection in BALB/c mice leads to a predominant type 1 immune response with undetectable levels of either IL-4, IL-5 or IL-13 [8], absence of eosinophilia and no AHR [163]. However, both Schwarze [178] and Lukacs [120] reported that primary RSV infection induced a Th2 cytokine response (IL-5 [178] or IL-13 [120]), airway eosinophilia and AHR. RSV infection that leads to Th2 responses and given before the onset of allergen sensitisation, could therefore be responsible for the development of enhanced allergic inflammation.

A possible explanation for the time-dependent effect of RSV-infection on OVA-allergy may be the OVA-induced Th2 cytokine milieu, present at the time of RSV infection. Among antigen-presenting cells, dendritic cells (DC) have a central role in regulation of the cellular immune response and T cell polarisation [150]. The outcome of T cell polarisation by DC can be either towards Th1 or Th2 development, depending on the microenvironment and the character of pathogen-induced inflammatory reactions [114]. In the OVA-sensitisation and challenge model, a Th2 cytokine environment is induced by OVA-sensitisation in lung tissue of mice. Presentation of RSV by DCs to T cells in this environment might stimulate T cells to become Th2 cells, aggravating the Th2 response. In contrast, when RSV infection is given before OVA-sensitisation and challenge, no Th2 cytokine environment is present yet and only the antiviral Th1 like immune response is developed.

7.5 Influence of a secondary RSV infection on respiratory allergy

Epidemiological data show that almost all children become infected with RSV in their first or second year of life [103], and that re-infections with RSV occur throughout life. This means that an RSV infection shortly before or during allergen-challenge might have been preceded by prior RSV infection episodes, for example before allergen-sensitisation. The RSV infection history could modulate virus replication and the immune and inflammatory response to a second RSV infection, thus influencing its potential effect on respiratory allergy. In addition, when prior RSV infection was given before allergen sensitisation it could diminish the allergic inflammation [159]. We demonstrated that prior RSV immunisation did not change the pulmonary cytokine mRNA expression of allergic mice infected with RSV during OVA challenge. Like Th2 cytokine mRNA expression, peribronchiolar and perivascular inflammation

are not altered by prior RSV immunisation [9]. These results are in accordance with those of Peebles et al. who observed that RSV immunisation before allergen sensitisation has no effect on lung IL-4 and IL-5 protein levels in OVA/RSV-treated mice [162]. However, they found that RSV immunisation protected against RSV-enhanced AHR. In our study, prior RSV immunisation diminished hypertrophy of mucus producing cells in OVA-allergic mice infected with RSV [9]. One might speculate that the increased hypertrophy of mucus producing cells is responsible for the enhanced AHR. In accordance with previously published data [17;162], prior RSV immunisation dramatically diminished the viral load after secondary infection, indicating protection against viral replication [9]. The lack of correlation between viral load and enhancement of allergy may indicate that the anti-viral inflammatory response during either a primary or secondary infection, rather than virus-induced lesions, is responsible for allergy-enhancement. So, despite the absence of virus replication, previous infection with RSV could not abrogate the RSV-enhanced OVA-induced Th2 cytokine expression in lungs.

7.6 Is RSV unique in enhancing respiratory allergy?

Besides RSV, also other respiratory viruses are known to exacerbate allergic asthma in children and adults. For example, rhinovirus induces and aggravates asthmatic exacerbations in children and adults [168;189;189]. In addition, influenza A virus infection is associated with asthma exacerbations in humans [61;199]. Furthermore, experimental studies in mice demonstrated that influenza A virus infection enhanced the sensitisation for allergic responses later in life [151;197].

It is not clear whether the RSV-enhanced respiratory allergic responses observed in mice are a result of non-specific or virus-specific stimuli. Therefore, we studied the influence of infection with two other respiratory viruses, pneumonia virus of mice (PVM) and influenza A virus [8]. PVM is closely related to RSV and also belongs to the family of *Pneumovirinae*. Since RSV is not a murine pathogen, large doses are required to infect mice and infection results in limited clinical disease [27;40]. In contrast, low doses of PVM are required for efficient infection [8;57]. For this reason, PVM has been used as a model to study the pathology and immune responses of primary RSV infection in humans [19;41]. In addition, Influenza A virus is a member of the virus family of Orthomyxoviridae.

Like for RSV infection, PVM and influenza A virus infection of mice result in a dominant Th1 like cytokine response in lungs and alveolar, peribronchiolar and perivascular inflammation. OVA-sensitisation and challenge did not affect the virus-induced Th1-cytokine expression. Like for RSV infection, PVM infection of allergic mice increased the expression of pulmonary IL-4, IL-5 and IL-13 mRNA and enhanced perivasculitis and infiltration of eosinophils in the peribronchiolar space [8]. In contrast, influenza A virus infection did not enhance the pulmonary Th2 cytokine mRNA expression in OVA-allergic mice, and even down-regulated IL-4, IL-5, and IL-13 mRNA responses. In addition, RSV and PVM enhanced Th2 cytokine production by OVA-stimulated splenocytes while influenza A virus did not [10]. These results

appear in contrast to other published data, which demonstrate that influenza virus infection enhances the Th2 inflammatory response in allergen-sensitised mice [151;197]. However, these studies examined the influence of infection on later development of allergic sensitisation rather than the effects of infection on a pre-existing allergy.

The key question is why RSV and PVM enhance the OVA-induced Th2 cytokine response, while influenza A virus does not. The difference in Th2-enhancement by the three viruses is likely explained by difference in virus-structure and pathogenesis between the virus families involved. A characteristic outer membrane protein of orthomyxoviruses is the HA protein, which is involved in attachment of virus to host cells and hemagglutination [112]. For attachment to host cells PVM and RSV are equipped with the G-protein, which is exclusively present in pneumoviruses [40], including the newly discovered human metapneumovirus [205]. The G protein is extensively glycosylated, and lacks homology to the HA protein or to any other known paramyxovirus protein [68]. A possible mechanism for enhancement of the OVA-induced Th2 cytokine response could be the immune-modulating capacity of the pneumovirus G protein. Evidence for this capacity is demonstrated by studies in which immunisation with purified G protein or vaccinia virus vectors expressing G, stimulate a Th2 immune response [80;96;193]. The OVA-induced pulmonary inflammation and Th2 cytokine milieu in mice might favour the immune response to G and further stimulate the Th2-inflammatory response [68]. Influenza virus seems to lack a Th2-stimulating protein.

A second possible mechanism that may explain the difference between the pneumoviruses and influenza A virus in enhancement of the OVA-induced Th2 cytokine response, could be the site of virus replication in lung tissue. PVM and RSV are mainly located in the epithelial cells lining the alveoli and bronchi, while influenza A virus was predominantly observed in the alveolar macrophages [4]. In addition, PVM and RSV were detected during a longer period of time in lungs of allergic mice than non-allergic mice, while influenza A virus was not. RSV and PVM infection of epithelial cells could enhance the production of β-chemokines (a.o. MIP-1α, eotaxin, RANTES) [221] and expression of ICAM-1 [214] by epithelial cells, which are both involved in allergic inflammation. In our model, influenza virus is predominantly located in macrophages [8], which produce, upon activation, different mediators, among them the cytokines IL-12, IL-1, TNF-α and IFN-γ, promoting cell-mediated immunity [226]. The synergistic effect of these cytokines could result in a stronger Th1-mediated inhibition of the Th2 cytokine response.

In summary, the paramyxoviruses RSV and PVM are both able to enhance the allergy-induced Th2 immune response in BALB/c mice, while the orthomyxovirus influenza A is not. Since hMPV is a member of the same virus family as RSV and PVM, and shares similar physiologic features like the G protein [204], it would be possible that hMPV also enhance allergic inflammation.

7.7 Proposed mechanisms of RSV-enhanced respiratory allergy

Several possible mechanisms are proposed to be responsible for RSV-enhanced allergy. The influence of RSV and other respiratory viruses on the development and exacerbation of allergic inflammation could be due to the epithelial damage induced by virus replication [64;131;151]. The disrupted epithelial layer might facilitate the enhanced uptake and presentation of allergen to the immune system [64]. However, in studies with prior RSV immunisation, enhanced Th2 responses still existed after the second RSV inoculation (during challenge), while no substantial virus replication occurred [9]. So, epithelial damage due to viral replication is unlikely. However, enhanced secretion of β-chemokines by the infected epithelial cells could play a role.

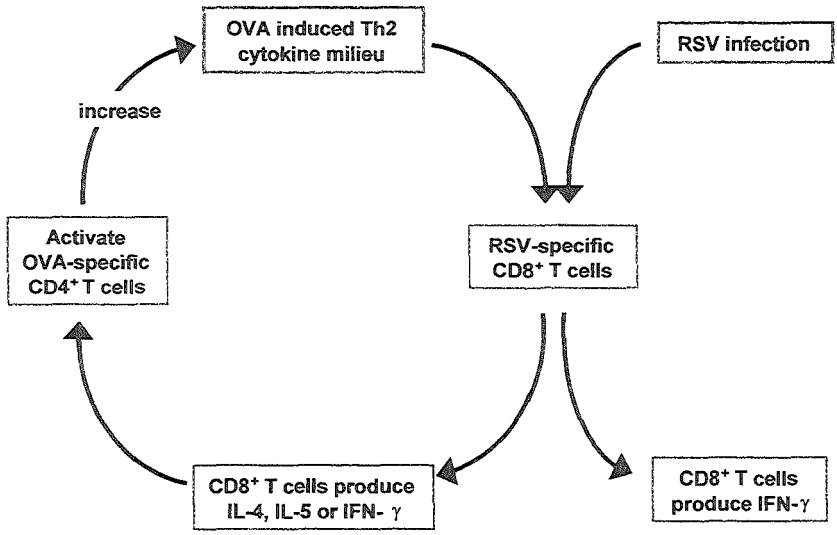

Figure 7.1. Schematic overview of the potential mechanism of RSV enhanced allergic Th2 immune response

The immune-modulating capacity of the pneumovirus G protein, which is demonstrated to stimulate a Th2 immune response [193], could also be responsible for the RSV-enhance allergy[80;96]. The OVA-induced pulmonary inflammation and Th2 cytokine milieu in mice might favour immune response to G and further stimulate the Th2-inflammatory response [68]. An other mechanism explaining the RSV-induced modulation of the allergic immune response could be an altered anti-viral immune response (fig. 7.1). Experimental *in vitro* studies have demonstrated before that activation of $CD8^+$ T cells in the presence of IL-2 or IL-12, results in $CD8^+$ T effector cells that produce. IL-2 and IFN-γ upon reactivation [47]. In contrast, the presence of IL-4 during effector cell generation promotes the development of IL-4 and IL-5

producing $CD8^+$ T cells [47;60]. In addition, it is demonstrated *in vivo* that lymphocytic choriomeningitis virus (LCMV)-specific $CD8^+$ T cells produce IL-5, when mice were previously sensitised to OVA [45]. Virus-specific $CD8^+$ T lymphocytes producing IL-5 in the lungs of allergic mice could be related to increased eosinophilia and AHR [45]. In accordance with these experiments, we demonstrated that a subset of RSV-specific $CD8^+$ T cells produce IL-4 when mice were previously sensitised and challenged to OVA [10]. It is reasonable to assume that in this allergy-model, RSV-specific $CD8^+$ T cells produce both IL-4 and IL-5. Besides the direct effect of RSV-induced IL-4 and IL-5 on the allergic phenotype, IL-4 produced by RSV-specific $CD8^+$ cells could in turn stimulate OVA-specific $CD4^+$ T cells to enhance the Th2 cytokine production resulting in aggravated allergic inflammation. The OVA-induced IL-4 production by RSV-specific $CD8^+$ T cells may provide the immunological mechanism for the observed influence of moment of RSV infection during OVA sensitisation and challenge (fig. 7.1). Since influenza A virus infection during OVA challenge did not enhance the allergic responses in mice, such IL-4 producing $CD8^+$ T-cells are not expected. Indeed, no increased percentages of influenza-specific $CD8^+$ T cells obtained from OVA/influenza-treated mice were positive for IL-4 [10].

7.8 Concluding remarks

Since decades, studies in humans have been performed to investigate the role of RSV infection on the development and exacerbation of allergic asthma. Because these studies were inconclusive, mice models were developed to study the research questions that remained unanswered.

Epidemiological studies have revealed a strong association between respiratory viral infections on severe asthma exacerbations in children [99;100] and adults [146], where RSV appeared one of the most important respiratory viruses involved. The results form human studies could be confirmed in mice. The mouse models gave the opportunity to investigate the immunological and pathological consequences of an RSV infection during allergic inflammation. RSV infection in the presence of allergic airway inflammation potentiate and prolong pulmonary Th2 cytokine responses [7], induce extensive mucus secretion into the airways [7;8;15;163], and increase airway hyperreactivity (AHR) [126;163]. This increased allergic airway inflammation and AHR was developed independently of changes in serum IgE levels and eosinophilic infiltration into the airways [15], both known to affect hyperreactivity of the airways [7;8;163]. From the mouse models we learned that activation of mucus production by mucus producing cells, rather than eosinophilic infiltration is correlated with increased AHR and thus asthmatic symptoms.

Almost all children have been infected once with RSV before the age of three. Therefore, the RSV infections that aggravate asthma in children [99;100] and adults [146] must be secondary infections. Whether the first infection (partly) protects against the exacerbated allergic responses induced by the second (or third) infection, cannot be investigated in humans. In allergic mice, prior RSV immunisation diminished the RSV-enhanced pulmonary pathology and

AHR, but failed to inhibit the increase in pulmonary Th2 cytokine mRNA expression [9;162]. So prior RSV immunisation only partly diminished the RSV-enhanced allergic inflammation. Whether the increased Th2 responses still have pathophysiologic consequences needs to be investigated.

RSV may not only affect already established allergic inflammation, but also promote the development and onset of allergic inflammation. Many epidemiological studies have demonstrated that severe RSV-induced bronchiolitis during early childhood is a risk factor for the development of allergic asthma later in life (table 7.1 [183]). However, most RSV infections induce mild symptoms. Whether these mild infections are a risk for development of allergic asthma is not easy to investigate in humans, since an uninfected control group is not available. Like in most humans, primary RSV infections in mice induce mild lung disease and a predominant Th1 immune response. When mice were infected with RSV, no enhancement of the allergic immune response after subsequent allergen sensitisation and challenge was detected [9;159]. Taken the human and mouse studies together, it seems that severe RSV disease upon infection increases the risk of development of allergic asthma later in life while mild infections are not.

Several factors are known that increase the risk for the development of severe RSV disease upon infection. Among them are premature birth and lung- or hearth disease. Mostly children below 3 months of age develop a severe course of RSV infection.

The immune system of both premature and very young children is not fully developed and is predominantly Th2 skewed [165], suggesting that a Th2 immune response influences the

Table 7.1. Studies investigating the link between respiratory syncytial virus bronchiolitis and reactive airway disease

Year	Number of children (RSV/control)	Hospitalised	Age at follow up (years)	Presence of wheezing/ asthma (RSV versus control)	*P* (odds ratio)	Reference
1982	130/111	Yes	10	42% versus 19%	0.001	[167]
1984	100/200	Yes	7	N/A	N/A	[136]
1984	59/177	No	8	44% versus 14%	<0.0001	[128]
1987	51/24	Yes	2	60% versus 4%	<0.01	[31]
1992	73/73	Yes	6	43% versus 15%	<0.001	[143]
1993	70/70	Yes	2	44% versus 13%	=0.001	[154]
1995	47/93	Yes	3	23% versus 1%	<0.001	[185]
1997	61/47	Yes	9-10	34% versus 13%	(3.59)	[148]
1999	N/A	No	6	N/A	(4.3)	[194]
	N/A	No	13	N/A	(1.4; N.S)	
1999	105/105 and 102	Yes	3	N/A	NS at age 3 years	[220]
2000	47/93	Yes	7.5	23% versus 2%	<0.001	[184]

N/A: no percentages or figures given.

severity of RSV infection. This is in accordance with results obtained from mice studies which demonstrate that a pre-existing Th2 milieu (induces by OVA-sensitisation) is necessary for RSV to enhance allergic inflammation [9;159]. Thus, what already was suggested for humans is confirmed by mice experiments.

Epidemiologic studies have revealed that genetic predisposition also plays a role in the development of severe RSV infection. Hoebee et al. demonstrated that severe RSV bronchiolitis was associated with polymorphisms in the genes encoding for IL-4 and IL-4 receptor α [86]. These gene-polymorphisms are positively associated with asthma [149;173]. Since both severe RSV disease and allergic asthma demonstrate comparable symptoms, maybe an identical genetic predisposition is involved. Indeed, Sigurs et al reported that family history for asthma correlates with the development of severe RSV bronchiolitis and the subsequent development of asthma in children [184].

Based on human and murine studies, we conclude that if RSV can trigger the "allergic process", this will occur in subjects with a Th2 immune environment due to their genetic background, an immature (Th2 skewed) immune response or other underlying disorders that have "primed" their immune system and lungs before the first contact with RSV [125] (fig. 7.2).

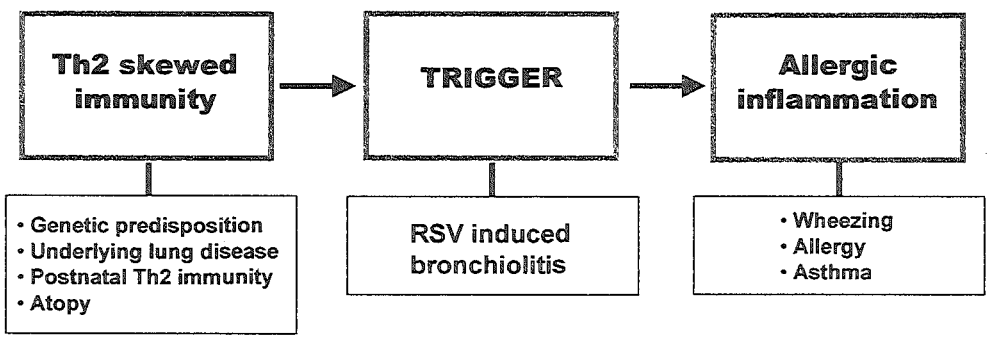

Figure 7.2. Mechanism by which RSV may influence the development of allergic inflammation.

Several mechanisms may be responsible for the RSV-enhanced allergic inflammation. First, increased permeability of the airway mucosa to allergens due to RSV-induced epithelial damage could be the cause. However, in studies with prior RSV immunisation, enhanced Th2 responses still existed after the second RSV inoculation (during challenge), while no substantial virus replication occurred [9]. So, epithelial damage due to viral replication is unlikely. Second, enhanced secretion of β-chemokines by the infected epithelial cells could play a role. Third, the OVA-induced Th2 cytokine environment may influence the presentation of RSV by DC's to T cells, stimulating differentiation of naive T-cells into Th2 cells rather than Th1 cells. In addition, the OVA-induced Th2 cytokine response may induce virus-specific $CD8^+$ T cells in the lung that produce IL-4 and IL-5, instead of IFN-γ, which subsequently enhances the

allergic inflammatory response [10]. This model is presented in fig. 7.1 and again stressed the finding that pre-existing Th2 immunity is responsible for the allergy enhancing effect of RSV.
Finally, the mouse model showed the unique capacity of the pneumoviruses RSV and PVM, to aggravate allergic inflammation in mice [8]. This may indicate that the recently discovered human metapneumovirus (hMPV), an other member of the same virus family, which contains similar viral characteristics as RSV and PVM [204;205], may influence allergic sensitisation and obstructive airway disease in humans.
In summary, both animal and human studies make it clear that the combination of RSV and allergy augments lung immunopathology and physiologic dysfunction. The extent as to which the animal models reflect human immunopathology remains to be assessed. Although already many features of the immunological mechanism responsible for the RSV-enhanced allergic inflammation are investigated (predominantly in mice), further investigation will be necessary to help us determine what interventions can be used to minimise the impact of RSV infection on allergy and asthma.

Abbreviations

AHR	airway hyperresponsiveness
APC	antigen presenting cell
APC	allophycocyanin
BAL	bronchoalveolar lavage
CD	cluster of differentiation
cDNA	copy desoxyribonuclease acid
DNA	desoxyribonuclic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell scanner
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
hMPV	human metapneumovirus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MCP	monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
mRNA	messenger ribonuclic acid
OD	optical density
OVA	ovalbumin
PCR	polymerase chain reaction
PE	phycoerythrin
PerCp	peridin chlorophyl protein
pfu	plaque-forming units
PVM	pneumoniavirus of Mice
RANTES	regulation upon activation normal T cell-expressed and secreted
RNA	ribonuclic acid
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction
Th	T helper
TNF	tumor necrosis factor

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Nederlandse samenvatting

Respiratoir Syncytieel Virus (RSV) is de meest voorkomende verwekker van luchtweginfecties bij jonge kinderen. Geschat wordt dat geduren de het eerste levensjaar ca. 70% van alle kinderen geïnfecteerd wordt met RSV en op driejarige leeftijd zijn bijna alle kinderen ten minste één keer geïnfecteerd geweest. De klinische symptomen volgend op infectie met RSV kunnen echter sterk verschillen. De meeste kinderen ontwikkelen een milde infectie van de bovenste luchtwegen, maar in een klein deel van de infecties kan zich een ernstige, soms levensbedreigende, bronchiolitis ontwikkelen. Een ernstig verlopende RSV-infectie in jonge kinderen kan leiden tot het ontwikkelen van piepend ademhalen dat tot lang na de eigenlijke infectie kan voortduren. De toegenomen astma-prevalentie gedurende de laatste decennia heeft geleid tot een verhoogde interesse in de vraag of ernstige RSV-infectie in zuigelingen bijdraagt aan de ontwikkeling van terugkerende periodes van piepen en astma gedurende de kinderleeftijd. Met het oog op de pathologische overeenkomsten tussen RSV-bronchiolitis en allergische astma wordt gespeculeerd dat gelijke immuun-pathogenetische mechanismen verantwoordelijk zijn voor de ontwikkeling van beide ziektes. Astma in kinderen is in de meeste gevallen geassocieerd met de sensitisatie tegen inhalatieallergenen en wordt gekarakteriseerd door allergische, type-2-geassocieerde, luchtwegontsteking. Vooral CD4-positieve T helper (Th)-type-2 lymfocyten en de cytokinen die zij produceren (o.a. interleukine (IL) –4, IL-5 en IL-13) spelen een centrale rol tijdens een allergische ontsteking. Hoewel een milde RSV-infectie gekarakteriseerd wordt door een dominante Th1-immuunrespons, wordt gesuggereerd dat een ernstige RSV-bronchiolitis wordt veroorzaakt door een allergische Th2 immuunrespons.

Al sinds tientallen jaren worden er studies in mensen uitgevoerd om de rol van RSV-infectie op de ontwikkeling en ernst van allergische astma te onderzoeken. Omdat humane studies onvoldoende antwoorden opleverden op de vraag of RSV inderdaad verantwoordelijk is voor de ontwikkeling en versterking van allergische astma, zijn er diermodellen ontwikkeld om deze vragen te kunnen beantwoorden. Het muismodel biedt ons de mogelijkheid om nauwkeurig het onderliggende immuunmechanisme en de interactie tussen RSV-infectie en allergische luchtwegontsteking te bestuderen. Het doel van dit proefschrift is om, gebruik makend van een muismodel, te onderzoeken of RSV infectie de respiratoire allergie beïnvloedt, en welke immuunmechanismen hier verantwoordelijk voor zijn.

Epidemiologische studies hebben aangetoond dat acute virale luchtweginfecties periodes van ernstige astmatische ontsteking in kinderen en volwassenen kunnen induceren. RSV is één van de belangrijkste oorzaken van luchtweginfecties die in deze studies is aangetoond. De resultaten van humane studies zijn bevestigd met experimentele studies in muizen. De muismodellen gaven de mogelijkheid om de immunologische en pathologische gevolgen van een RSV-infectie tijdens allergische ontsteking te onderzoeken. In **hoofdstuk 2** van dit proefschrift hebben we aangetoond dat RSV-infectie in de aanwezigheid van allergische luchtwegontsteking leidt tot een versterkte pulmonaire Th2-cytokinerespons, en een verhoogde excretie van mucus in de luchtwegen. Door andere onderzoekers is aangetoond dat toegenomen mucussecretie

overeenkomt met een versterkte hyperreactiviteit van de luchtwegen (AHR). Er was echter geen correlatie tussen de toegenomen allergische ontsteking en veranderingen in IgE-concentraties in serum en infiltratie van het aantal eosinofielen in longweefsel. De resultaten van deze studie hebben ons geleerd dat, in plaats van eosinofiele infiltratie in de luchtwegen, de activatie van Th2-cytokinen en mucus-secretie door mucus-producerende cellen gecorreleerd is met versterkte AHR en dus met astmatische symptomen.

RSV-infectie in gezonde kinderen en volwassenen induceert over het algemeen een Th1 immuun respons, welke gekarakteriseerd wordt door de productie van IFN-γ en IL-12. Ook in muizen resulteert een primaire RSV-infectie in een sterke Th1-immuun respons. In **hoofdstuk 2** hebben we beschreven dat RSV-infectie van allergische muizen niet alleen de allergische Th2 respons versterkt, maar ook een sterke Th1 cytokine (IL-12 en IFN-γ) respons induceert die niet beïnvloed is door de aanwezigheid van een allergische Th2 immuun respons. Omdat zowel IFN-γ als IL-12 betrokken zijn bij de regulatie van de Th1/Th2 balans door de ontwikkeling van de Th2-route te blokkeren, lijkt de allergie-versterkende capaciteit van RSV in contrast met de sterke RSV-geïnduceerde Th1 immuunrespons. In **hoofdstuk 3** beschrijven we een studie waarin we de functie van zowel IL-12 als IFN-γ in de RSV-versterkte allergische ontsteking bestuderen in muizen die deficient zijn in IL-12 of de IFN-γ receptor. De resultaten van deze studie laten zien dat IL-12 geen rol speelt in de ontwikkeling van allergische ontsteking, nog in de door RSV-versterkte allergie. IFN-γ blijkt echter wel een remmend effect te hebben op de door RSV infectie versterkte respiratoire allergie. Omdat de expressie van IFN-γ na RSV-infectie even hoog is in allergische en niet-allergische muizen, concluderen we echter dat, hoewel IFN-γ de allergische Th2 respons remt, het niet in staat is om de versterking van de allergie door RSV geheel te voorkomen.

Bijna alle kinderen zijn voor het derde levensjaar ten minste één keer geïnfecteerd geweest met RSV. We kunnen daarom concluderen dat de RSV-infecties die astma verergeren in kinderen en volwassenen secondaire infecties moeten zijn. Omdat we in mensen niet kunnen onderzoeken of voorafgaande RSV-infecties (gedeeltelijke) bescherming bieden tegen verergering van astma-symptomen na een volgende infectie, hebben we deze vraag onderzocht in muizen. In **hoofdstuk 4** tonen we aan dat RSV-immunisatie voorafgaand aan allergische sensitisatie de pulmonaire pathologie na de secondaire RSV infectie (tijdens allergeen challenge) doet verminderen. Er is echter geen vermindering van de pulmonaire Th2-cytokine mRNA expressie waargenomen. Daarom is geconcludeerd dat RSV-immunisatie maar gedeeltelijk de RSV-versterkte allergische ontsteking kan verminderen. Het is alleen nog onduidelijk of de versterkte Th2-immuunrespons in geïmmuniseerde muizen ook nog steeds een pathologisch effect kan veroorzaken.

Het kan mogelijk zijn dat RSV niet alleen een effect heeft op een al bestaande allergische ontsteking, maar ook op de ontwikkeling van allergische sensitisatie en ontsteking. Veel

epidemiologische studies hebben laten zien dat ernstige RSV-bronchiolitis in jonge kinderen een risicofactor vormt voor de ontwikkeling van allergische astma op latere leeftijd (tabel 7.1). De meeste RSV-infecties veroorzaken echter slechts milde ziektesymptomen. Omdat er geen niet-geïnfecteerde controlegroep beschikbaar is, is het vrijwel onmogelijk om te onderzoeken of milde RSV-infecties een risicofactor vormen voor de ontwikkeling van allergische astma in kinderen. Ook in muizen veroorzaakt een primaire RSV-infectie een milde ontsteking van de luchtwegen, gekarakteriseerd door een pre-dominante Th1-immuunrespons. In **hoofdstuk 4** hebben we aangetoond dat wanneer muizen voorafgaand aan allergische sensitisatie en challenge worden geïnfecteerd met RSV, er geen versterking van de allergische immuunresponsen is waar te nemen. Zowel humane als muisstudies suggereren dat alleen een ernstig verlopende RSV-infectie het risico op de ontwikkeling van allergische ontsteking versterkt, terwijl een milde RSV-infectie dit niet doet.

Verschillende factoren kunnen een rol spelen in de ontwikkeling van een ernstige RSV-infectie, onder andere prematuriteit en hart- en longaandoeningen bij jonge kinderen. Een ernstig verlopende RSV-bronchiolitis wordt meestal waargenomen bij kinderen jonger dan drie maanden. Het is aangetoond dat het immuunsysteem van zowel te vroeg geboren kinderen als zuigelingen nog niet volledig is volgroeid en vooral Th2-gepolariseerd is. Dit suggereert dat een Th2-immuunrespons de ernst van RSV-infectie kan beïnvloeden. Deze aanname is in overeenstemming met de resultaten uit muisexperimenten zoals deze beschreven zijn in **hoofdstuk 4.** Daar bewijzen we dat een al aanwezig Th2-cytokinemilieu, welke geïnduceerd is door OVA sensitisatie, essentieel is voor RSV om de allergische ontsteking te kunnen versterken.

Omdat zowel ernstige RSV-infectie als astma overeenkomstige symptomen laten zien, zou het kunnen zijn dat beide ziektes een gemeenschappelijke genetische oorsprong hebben. Een epidemiologische studie in kinderen heeft inderdaad aangetoond dat het voorkomen van astma in de familie samenhangt met de ontwikkeling van ernstige RSV-bronchiolitis en dus met de daaropvolgende ontwikkeling van astma in kinderen. Gebaseerd op humane en muisstudies concluderen we, dat als RSV het allergische proces kan initiëren, dit alleen zal gebeuren in personen met een immuunrespons die vooral Th2-gestuurd is. Deze Th2-gestuurde immuunrespons kan het gevolg zijn van de genetische predispositie, een onvolgroeide (Th2-gestuurde) immuunrespons of andere onderliggende lichamelijke aandoeningen die het immuunsysteem en de luchtwegen hebben geprimed voor het eerste contact met RSV (fig. 7.2).

Verschillende mechanismen kunnen verantwoordelijk zijn voor de door RSV-versterkte allergische ontsteking. Ten eerste de verhoogde permeabiliteit van de luchtweg-mucosa voor allergenen als gevolg van door RSV veroorzaakte epitheelschade. De studies in **hoofdstuk 4** hebben echter aangetoond dat na voorafgaande immunisatie met RSV, secondaire RSV-infectie (tijdens allergeen-challenge) nog steeds de Th2-cytokine-respons kan versterken, terwijl er geen

substantiële virusreplicatie in de epitheelcellen plaatsvond. Waarschijnlijk is de epitheelschade als gevolg van virusreplicatie dus geen mogelijke oorzaak voor de versterkte allergische ontsteking. Ten tweede, het door OVA-sensitisatie en -challenge geïnduceerde Th2-cytokine-milieu in de luchtwegen kan van invloed zijn op de presentatie van RSV-antigeen door dendritische cellen aan T cellen, en zo de differentiatie van naïeve T-cellen in Th2-cellen in plaats van in Th1-cellen stimuleren. Een ander mogelijk mechanisme is onderzocht in **hoofdstuk 6.** Daar tonen we aan dat de OVA-geïnduceerde Th2-cytokinerespons de RSV-specifieke $CD8^+$ T-cellen moduleert, waardoor deze in plaats van IFN-γ, IL-4 en IL-5 gaan produceren. De door de RSV-specifieke $CD8^+$ cellen geproduceerde Th2-cytokinen kunnen vervolgens de allergische ontstekingsrespons verergeren.

Tenslotte, naast RSV zijn er meerdere respiratoire virussen waarvan bekend is dat ze ook van invloed kunnen zijn op de ontwikkeling en verergering van allergische astma in mensen en muizen. In **hoofdstuk 5** hebben we onderzocht of het mechanisme waarmee RSV de allergische ontsteking beïnvloedt uniek is voor dit virus. Onze resultaten laten zien dat de pneumovirussen RSV en PVM (een muis-virus dat nauw verwant is aan RSV) de unieke eigenschap hebben om de allergische ontsteking in de muis te versterken. Gezien de grote overeenkomst tussen deze twee pneumovirussen en het recent ontdekte humane Metapneumovirus (hMPV, ook een lid van de zelfde virusfamilie) kan verwacht worden dat hMPV ook de allergische luchtwegontsteking zou kunnen beïnvloeden.

Samenvattend kunnen we zeggen dat zowel studies in muizen als mensen duidelijk hebben gemaakt dat de combinatie van RSV-infectie en allergische astma de (immuun)pathologie in de long doet toenemen. Ondanks het feit dat er al veel onderzoek is verricht naar de mechanismen die verantwoordelijk zijn voor de door RSV versterkte allergische ontsteking, is er nog meer onderzoek nodig om vast te kunnen stellen welke interventies gebruikt kunnen worden om de impact van RSV-infectie op allergie en astma in de toekomst te verkleinen.

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Curriculum vitae

Marion Barends werd geboren op 19 juni 1973 te Urk. In juni 1990 behaalde zij het HAVO diploma aan de Christelijke Scholengemeenschap "De Brug" in Lelystad. In hetzelfde jaar startte zij met de Hogere Laboratorium Opleiding aan de Hogeschool van Utrecht, richting Medische Microbiologie. Tijdens deze studie heeft zij een stage gelopen bij de toenmalige afdeling voor Virologie van het RIVM te Bilthoven, waar onderzoek werd verricht naar een getransformeerde muize-L-cellijn voor gebruik in de poliovirus diagnostiek. Na het behalen van het diploma in juni 1994 ging zij aansluitend Medische Biologie studeren aan de Vrije Universiteit te Amsterdam. Gedurende de doctoraalfase van deze studie heeft zij een onderzoeksstage gedaan bij het Eijkman-Winkler Instituut voor Microbiologie van het Academisch Ziekenhuis te Utrecht, in samenwerking met het Laboratorium voor Infectieziekteonderzoek van het RIVM te Bilthoven. Daar ontwikkelde zij een RFLP methode en onderzocht ze of deze methode geschikt was voor de surveillance van penicilline resistente *Streptococcus pneumoniae.* In oktober 1997 studeerde Marion Barends af waarna ze in augustus 1998 is gestart als Assistant In Opleiding bij het Laboratorium voor Infectieziektenonderzoek, in samenwerking met de afdelingen kindergeneeskunde en virologie van de Erasmus Universiteit Rotterdam. Tijdens dit promotie-onderzoek heft zij de invloed van RSV-infecties op de ernst en ontwikkeling van respiratoire allergie in een muismodel bestudeerd. Dit onderzoek vormt de basis voor het huidige proefschrift. Op dit moment is Marion Barends als Laboratory Scientist werkzaam bij de Shoklo Malaria Research Unit (SMRU) in Mae Sot, Thailand. De SMRU is een onderdeel van het Wellcome-Mahidol University-Oxford Tropical Medicine Research Programme.

List of Publications

1. **Barends, M, Boelen A, de Rond L, Kwakkel J, Dormans J, Bestebroer T, Neijens HJ, Kimman, TG.** Influence of respiratory syncytial virus infection on cytokine and inflammatory responses in allergic mice.
Clinical & Experimental Allergy 2002; 32:463-471.

2. **Boelen A, Kwakkel J, Barends M, de Rond L, Dormans J, Kimman TG.** Effect of lack of interleukin-4, interleukin-12, interleukin-18, or the interferon-γ receptor on virus replication, cytokine response, and lung pathology during respiratory syncytial virus infection in mice.
Journal of Medical Virology 2002, 66:552-560.

3. **Barends M, Boelen A, de Rond L, Dormans J, Kwakkel J, van Oosten M, Neijens HJ, Kimman TG.** Respiratory syncytial virus enhances respiratory allergy in mice despite the inhibitory effect of virus-induced interferon-γ.
Journal of Medical Virology 2003, 69:156-162.

4. **Barends M, de Rond CGH, Dormans JAMA, van Oosten M, Boelen A, Neijens HJ, Osterhaus ADME, Kimman TG.** Respiratory syncytial virus, Pneumoniavirus of mice, and influenza A virus differently affect respiratory allergy in mice.
Clinical & Experimental Allergy, accepted for publication.

5. **Barends M, van Oosten M, de Rond CGH, Dormans JAMA, Osterhaus ADME, Neijens HJ, Kimman TG.** Respiratory Syncytial Virus-enhanced allergic inflammation depends on timing of infection and is affected by prior RSV immunisation.
Journal of Infectious Diseases, accepted for publication

6. **Barends M, van Oosten M, de Rond CGH, Lambrecht BN, Kimman TG.** Mutual enhancement of RSV- and allergen-specific Th2, but not Th1 systemic cellular immune responses in a mouse model of respiratory allergy.
Submitted

7. **Barends M, van Oosten M, Osterhaus ADME, Neijens HJ, Kimman TG.** Respiratory syncytial virus and asthma: lessons from the mice model.
Review in preparation

Appendix

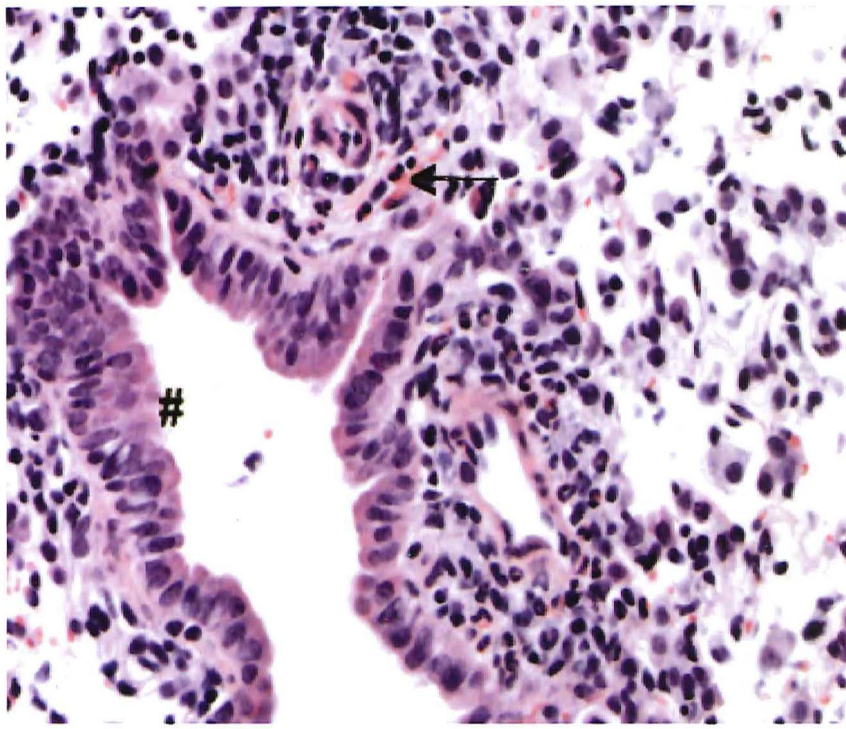

Figure 4.3. Lung of OVA-sensitised and challenged mice inoculated with RSV on day 35. Lung pathology is analysed at day 43 of the experimental protocol (i.e. day 3 after the last OVA-challenge). A marked hypertrophy of mucus producing cells in bronchiolar epithelium is observed (#), together with perivascular infiltration of eosinophils (arrow) and lymphocytes (1200x).

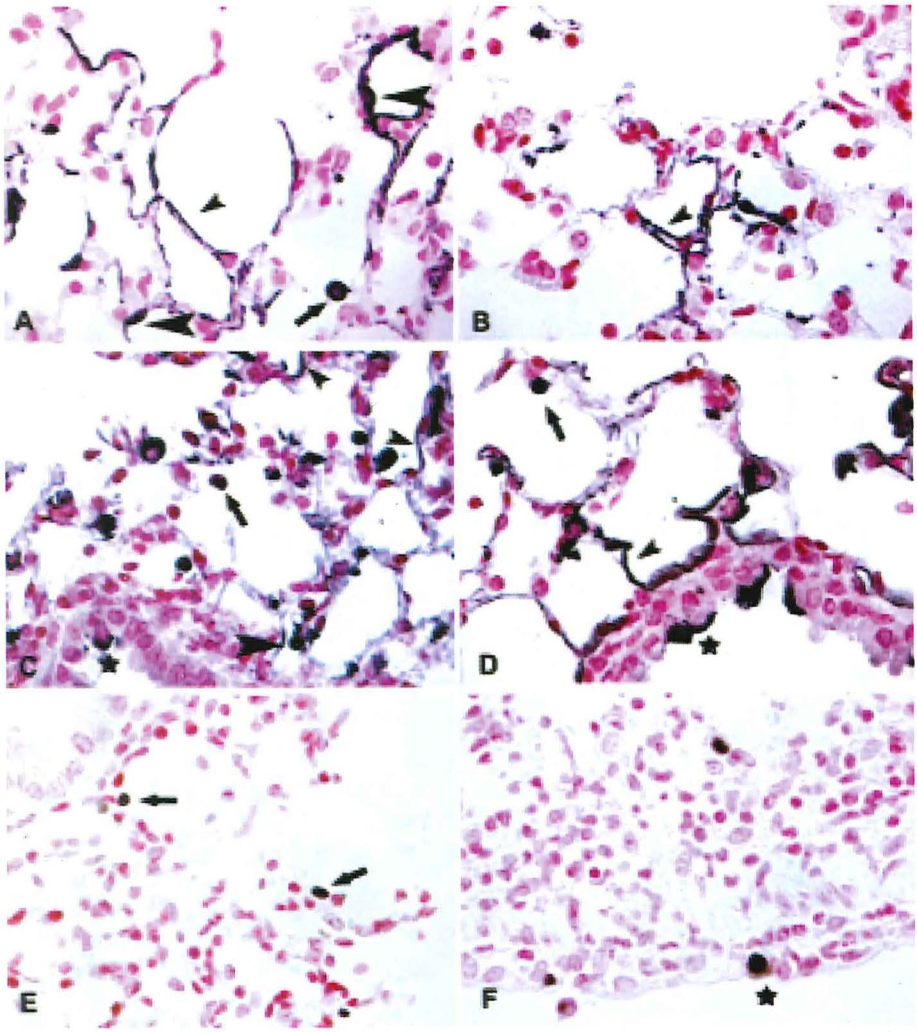

Figure 5.4. Sections of lungs from mice intranasally inoculated with RSV [A, B], PVM [C, D] and influenza [E, F] with [A, C, E] or without [B, D, F] ovalbumin treatment are shown. All pictures represent immunoperoxidase staining 6 days post virus-inoculation (1 day after the last OVA-challenge). Sections were counter-stained with nuclear fast red, and examined using light microscopy (660x). Immunostaining for RSV is observed in alveolar macrophages (arrow in A), alveolar type I (small arrowhead in A,B) and type II epithelial cells (large arrowhead in A), independently of the OVA-treatment. Immunoreactivity for PVM is seen in the cytoplasm of bronchiolar cells (non-ciliated, non-mucus-secreting epithelial cells, asterix in C,D), in alveolar type I (small arrowhead in C,D), and type II epithelial cells (large arrowhead in C), and alveolar macrophages (arrow in C,D), independent of OVA-treatment. Alveolar macrophages (arrow in E) and bronchiolar cells (asterisk in F) stained positive for influenza independently of ovalbumin treatment.