Mycobacteria and the *Nramp1* gene in asthma

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(met een samenvatting in het Nederlands)

Proefschrift

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Chapter **1**

General Introduction

1. Asthma

The word "asthma", meaning panting, was already employed by Greek physicians of antiquity such as Hippocrates. Aretaeus of Cappadocia in the second century AD gave the first accurate clinical description of asthma: "the symptoms of its approach are heaviness of the chest; sluggishness to one's accustomed work, and to every other exertion; difficulty of breathing in running of on a steep road", and acknowledged that the disease was potentially fatal. In 1910 it was suggested that asthmatics are individuals sensitive to a specific substance, asthma was since then considered an anaphylactic phenomenon (1). Anaphylaxis was a phenomenon discovered before by Richet and Portier in 1902; they showed that after first contact with a specific toxin, animals developed increased sensitivity to this substance, so called anaphylaxis (against protection). This phenomenon of hyperresponsiveness was later in 1906 called "atopy" or allergy by Clemens of Pirquet, who defined allergy as an "altered capacity of the body to react to a foreign substance" (2).

Since, research in allergy and asthma has developed rapidly during the last 50 years and asthma is now considered a complex syndrome with many clinical phenotypes. Asthma is now recognized and defined by characteristics such as a variable degree of airway obstruction, bronchial hyperresponsiveness and airway inflammation. Allergic mechanisms are of importance in about 80% of childhood asthma and in about 40-50% of adult asthma (2). On the other hand, a substantial group of asthmatic patients have no sign of atopy, characterized by no elevated serum levels of IgE (3) and negative skin tests. Most likely, other mechanisms than the classical IgE-mediated allergic cascade are involved in this asthma type.

Airway inflammation
Crucial in the development of airway inflammation in allergic asthma is the allergic cascade (Fig. 1). In the beginning of this cascade, inhaled allergens are encountered and captured by antigen presenting cells, which subsequently migrate to the draining lymph nodes. There they present the processed allergen to T and B cells. Interactions between those cells elicit responses that are characterized and influenced by cytokines and the presence or absence of costimulatory molecules. A subdivision of T cells, Th2 cells are associated with allergic diseases. Activation of these cells by antigen presenting cells leads to the production of cytokines such as interleukin (IL) -4, IL-5 and IL-13. For a switch by B cells to the production of IgE, IL-4 and IL-13 are considered crucial (4). Once synthesized, IgE antibodies circulate in the blood before binding to the high affinity IgE receptor (FcεRI) on the mast cell in tissue or on peripheral-blood basophils. Allergen binding to mast cell bound IgE causes the activation of membrane and cytosolic pathways which subsequently cause the release of preformed mediators such as histamine, the synthesis of prostaglandins and leukotrienes and the transcription of cytokines by mast cells (5). These mediators cause the so-called early phase asthmatic reaction, which is characterized by constriction of airway smooth muscle, vascular leakage and mucus production. This early phase reaction is
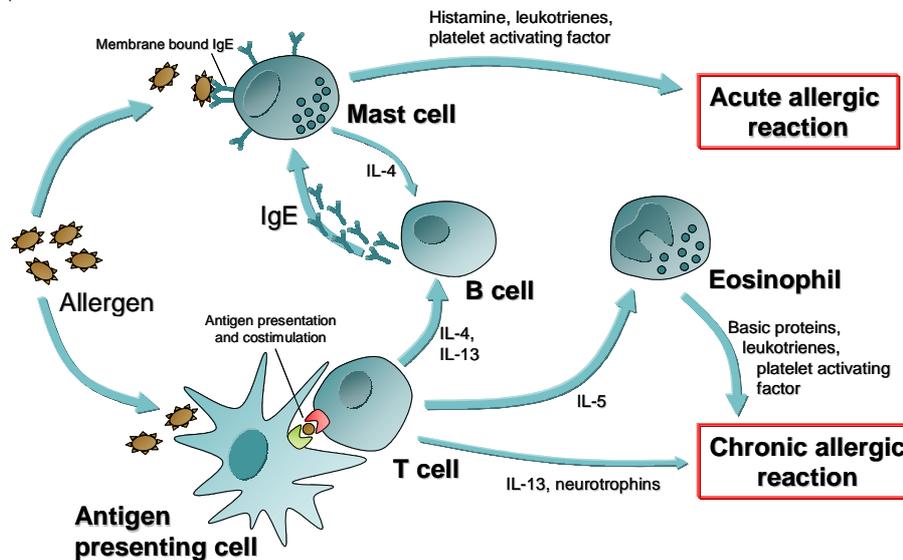
Figure 1. *The allergic cascade.*

four to six hours later followed by the late phase reaction. This phase is characterized by excessive inflammation of the airways resulting in airway narrowing induced by various mediators derived from inflammatory cells.

      Several cells are involved in inflammation of the airways. First, T cells, as mentioned before, play an important role as initiators but also as cells maintaining airway inflammation. The action of eosinophils and mast cells is regulated by products produced by these cells. Whether only Th2 type CD4+ T cells are involved in asthma is still matter of controversy. In human patients with asthma the levels of interferon-(IFN) -γ, a Th1 cytokine, are increased as well, and in mice antigen-specific Th1 cells may not protect or prevent Th2-mediated allergic disease, but rather may cause acute lung pathology (6). Probably both Th1 as Th2 cells play a role at different stages in the pathogenesis of asthma. Secondly, the main effector cell involved in airway inflammation in asthma is the eosinophil (7). The mature eosinophil has dense intracellular granules that are sources of inflammatory proteins such as leukotrienes, major basic protein and peroxidase (8). A number of cytokines regulate the function of eosinophils. Among these is IL-5 the most dominant, this cytokine stimulates the release of eosinophils into the circulation and prolongs their survival (9). Thirdly, macrophages play a dual role in allergic responses and inflammation in the airways. On one hand, macrophages are recruited to the airways of allergic subjects following allergen challenge (10). Moreover, macrophages are important effector cells in inflammation in the airways (11). On the other side, macrophages are important immunoregulators by inducing a state of reversible T cell non-responsiveness during local antigenic stimulation, which subsequently affects the allergic response (12). Finally, tissue resident structural cells, including endothelial cells, epithelial cells, fibroblasts and smooth muscle cells may also be an important source of inflammatory mediators in asthma as well (13).

Airway hyperresponsiveness

One of the most important manifestations of both allergic as non-allergic asthma is the development of airway hyperresponsiveness. Airway hyperresponsiveness is defined as an exaggerated bronchoconstrictor response to not only allergens but to non-specific stimuli as well, including cold air, moisture or chemicals such as methacholine (14). Interestingly, the degree of hyperresponsiveness of the airways is related with the severity of asthma (15). Until now, several interrelating mechanisms for the generation of airway hyperresponsiveness have been proposed. First, airway inflammation, mediated by eosinophils, evokes mediators which damage the epithelial airway tissue. This exposes sensory nerve endings, which will be more easily stimulated by exogenous triggers, and therefore contribute to the development of airway hyperresponsiveness. However, the role of eosinophils in inducing airway hyperresponsiveness remains controversial since it has been shown that airway hyperresponsiveness can occur in the absence of eosinophilic inflammation (16). In these cases T cell-mediated immune responses can be the causative mechanism of airway hyperresponsiveness, since T cells constitute a large proportion of the inflammatory cells in the airways. T cells may, trough secretion of mediators, directly influence development of airway hyperresponsiveness (17), but act most likely indirectly trough enhancement and initiation of the allergic inflammation by secretion of inflammatory chemokines and cytokines. In addition, tissue resident structural cells, including endothelial and epithelial cells, fibroblasts and smooth muscle cells both directly or indirectly initiate and maintain airway hyperresponsiveness (13).

Animals models for allergic asthma

Experimental support for the above mentioned mechanisms is largely derived from animal models for asthma. Although rat and guinea pig models are relevant and reflecting the human situation, the mouse model is most used, probably due to the superior availability of immunological tools, such as congenic, transgenic and knock out mouse strains, antibodies and recombinant protein mediators. Although the mouse model does not replicate human disease perfectly, much of what is known about the immunobiology of allergic pulmonary inflammation and airway hyperresponsiveness is applicable in humans. In order to achieve the replication of human disease, most mouse models applied were developed to exhibit the two most important features of asthma: Airway inflammation and airway hyperresponsiveness.

Although differences exist, in most models, mice are sensitized with antigens like ovalbumin or house dust mite with or without an adjuvant like aluminium hydroxide (alum). After sensitization, mice are challenged once or repeatedly with the antigen via inhalation or tracheal installation (18). After challenge, many parameters of allergy and allergic asthma can be measured, such as airway hyperresponsiveness *in vitro* or *in vivo*, antigen-specific IgE, IgG1 or IgG2a levels, the cellular composition of the bronchoalveolar lavage, morphological changes in the lungs and alterations in expression of mRNA and proteins levels.

2. The "hygiene hypothesis"

Allergic diseases, such as allergic asthma, are steadily increasing in developed countries (19). Whereas hay fever was a rare disorder restricted to the privileged class in the 19th century, at present almost a quarter of the population in some western countries demonstrates sensitization to one or more common environmental allergens. For instance, in countries like Great Britain and Australia, a quarter of the children under the age of 14 years have asthma and a fifth has eczema (20). Most likely, the cause of the rise in allergic diseases must be sought in environmental factors, of which several have been suggested, such as changes in allergen exposure due to housing isolation or breast feeding (21), changes in environmental pollution (22), and changes in the diet and the gut flora (23). However, the suggestion that a change in the level and the kind of early childhood infections would be a major factor influencing the development of allergic diseases has drawn most attention and discussion. A largely neglected study in 1976 by Gerrard and colleagues demonstrated lower IgE levels in rural living Metis indians in Canada when compared with the white community there. They stated: "atopic disease is the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria and helminths" (24). Hereafter, in 1989, Strachan denominated the suggested relationship between hygiene and allergic disease the "hygiene hypothesis" (25).

Basically, this hypothesis states that improved hygiene in industrialized societies, with improved public health measures and the use of vaccines and antibiotics, has reduced the incidence of infections that normally stimulate the immune system in some way that mitigates against asthma (26). The data that supports this theory is extensive but controversial. Several epidemiological studies showing that a large family size or early placement in day care settings (27), and therefore presumed high exposure to infectious agents, protects against the development of asthma. Other studies show that exposure to farm animals early in life reduces the likelihood of developing atopic sensitization or asthma, probably due to higher bacterial endotoxin levels (28). However, the inverse relationship between exposure to common childhood infections and development of allergic asthma is not confirmed in several other studies. (29-31). Moreover, the specific infectious pathogens or specific mechanism that are responsible for the observed effects have not been identified yet, therefore the relationship between infection and the development of asthma remains controversial and indefinite.

Th1, Th2 and regulatory T cells
The first proposed mechanism for the hygiene hypothesis was based on the T helper type 1 and 2 (Th1 and Th2) dichotomy in specific murine and human immune reactions (32). Th2 cells, by secreting mediators such as IL-4 and IL-5, play a central role in initiating and sustaining the allergic and asthmatic response by regulating the production of IgE and the growth, differentiation and recruitment of eosinophils (33). Th1 cells, secreting IFN-γ and involved in the immune response to a number of

intracellular pathogens, dampen the activity of Th2 cells (32). The immune system in newborns is weakly Th2-biased (33), infection in infants would lead to stimulation of this Th2-biased immune system to mature and more Th1-biased immune responses, characterized by the production of IFN-γ and IL-12. For this reason, a lack of infections would lead to less frequent activation of Th1 biased responses and, thereby, facilitate development of Th2-biased immune responses (34, 35).

In contrast with this initially widely accepted mechanism is the observation that a massive Th2-biased immune reaction as seen in helminth infections is associated with protection against the development of allergy and asthma (36, 37). Moreover, the incidence of Th1-mediated autoimmune diseases such as Type 1 diabetes and multiple sclerosis is increasing as well (38). It is therefore important to realize that the connecting link between allergy and asthma and autoimmune diseases is the fact that they all represent poorly regulated and exaggerated immune responses. Most of the regulation of the immune system is done by different regulatory cells, including the specialized regulatory T cells (39). These cells prevent the activity of effector T cells and other inflammatory cells, by secreting inhibitory cytokines such as IL-10 and TGF1-ß, but also by direct contact with effector T cells or antigen presenting cells (40). For that reason, the hygiene hypothesis probably is not explained by a mechanism which involves the Th1/Th2 balance, but may involve a mechanism employing a balance between regulated and disregulated immune responses (**Figure 2**) (26, 38).
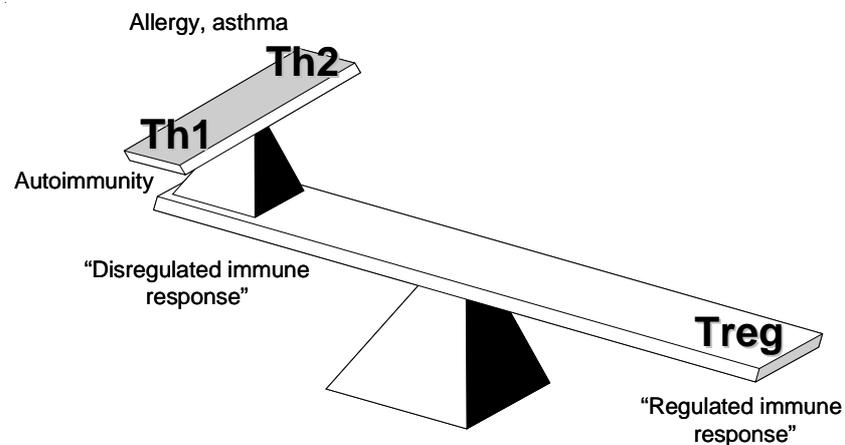
Figure 2. The balance between regulated and disregulated immune response. If regulatory activity is low, disregulated immune response will develop. The nature of this disregulated response depends on the balance between Th1 (autoimmune disease) or Th2 (allergy and asthma). (Adapted from GAW Rook and LR Brunet, Biologist 2002. 49(4): 145-149)

Mycobacteria

If the increase in diseases due to faulty immune regulation is due to decreased exposure to certain microorganisms, it may be possible to use certain bacterial species as protective or therapeutical vaccines against diseases such as allergic

asthma. In this respect, mycobacterial infections or vaccines are most studied. Most species of mycobacteria are non-pathogenic and very common in mud and untreated water. Mycobacteria do not belong to the regular commensal flora, and therefore, the exposure to these bacteria is regulated by lifestyle. In addition, their impact on the immune system is indicated by the existence of CD1-restricted T cells which seem to recognize mostly mycobacterial components (41). Furthermore, under certain circumstances mycobacterial infections have a strong capacity to elicit Th1 responses. The experimental data in animal models of allergic asthma supporting the candidature of mycobacteria in the hygiene hypothesis are quite convincing. Vaccination with mycobacteria suppressed the development of several allergic and asthmatic manifestations in the mouse (42-44) and in the rat (45). Treatment of mice with heat-killed *M. vaccae*, three weeks before the first ovalbumin immunization was able to reduce airway eosinophilia. This down regulatory effect of *M. vaccae* was probably mediated through the induction of specific regulatory T cells and mediated by IL-10 and TGF1-ß, and not by induction of Th1 responses (42). This was the first experimental proof that regulatory mechanisms (as elicited by mycobacteria) may be of importance in the hygiene hypothesis.

In contrast, the relationship between mycobacterial infection and the development of allergy and asthma in humans is highly controversial. The first epidemiological study in Japan in this respect showed that children that had been vaccinated with bacille Calmette Guérin (BCG) shortly after birth and responded with positive tuberculin reactions at 6 and 12 years of age, showed reduced incidences of allergic symptoms and asthma compared to vaccinated children with negative tuberculin reactions (46). A similar study in Guinea-Bissau confirmed these findings (47). In contrast, hereafter, several retrospective studies in a variety of countries failed to demonstrate a negative correlation between BCG vaccination during early childhood and the subsequent development of atopy or asthma (reviewed in 30). In contrast, studies using BCG (48) or heat-killed *M. vaccae* in established asthma (49) or atopic dermatitis (50) showed beneficial effects of mycobacterial treatment, although the effect of *M. vaccae* in asthma patients was not confirmed (51). Factors causing these discrepancies may be the age of the child and the frequency of vaccination with BCG or *M. vaccae*, the BCG strain used, and the varying natural exposure to mycobacteria (including *M. tuberculosis*) resulting in different types of skin-test-positivity and efficacy of BCG(52). In addition, a genetic relationship in the inverse correlation between (mycobacterial) infection and the development of allergy and asthma may be important as well, which will be discussed below.

3. Genes and asthma

Next to the above mentioned environmental factors, in the pathogenesis of asthma genetic factors undoubtedly play a role as well. A recent study suggested that 73% of asthma susceptibility is due to genetic factors (53). The complexity of the genetics of asthma and other atopy-associated phenotypes is reflected by the linkage of asthma

susceptibility to several chromosomal regions on chromosome 5, 6, 11, 14 and 12, which contain hundreds of candidate genes. Especially chromosome 5q31 has drawn a lot of attention because it contains a large number of candidate genes, including the genes for IL-12p40, IL-9, ß-adrenergic receptor and the IL-4 cytokine cluster which contains the genes for IL-4, IL-5 and IL-13 (54). Nonetheless, the significant increase in the prevalence of allergic diseases over the post decades cannot be explained by changes in gene frequencies alone. Therefore, it is likely that various existing gene factors interacting with changes in the environment, such as a decline in childhood infections, have caused a large percentage of the population to be susceptible for allergic disease.

Studies investigating interactions between genes and environmental factors in allergy and asthma are scarce and hampered by ethnic differences in the immune response to infections. Nevertheless, some interesting candidate genes that may provide a link between infection and allergic disease have appeared recently. For instance, a genetic variation on the regulation for CD14, the endotoxin receptor, is strongly correlated with IgE responses (55, 56) and IL-12B gene heterozygosis contributed to asthma severity (57) in humans. Other candidate genes in this respect are the genes for nitric oxide synthase 1, tumor necrosis factor-α (TNF-α) and IFN-γ, which are important mediators of resistance to infection (56). Since the ability to mount a response to mycobacterial antigens is highly heritable (58), a genetic contribution to the inverse relationship between mycobacterial infection and the development of allergy and asthma is very plausible as well.

Nramp1

The immune response to intracellular bacteria, including mycobacteria, is under control of the natural-resistance-associated macrophage protein 1 gene (*Nramp1*, formerly designated *Ity/Lsh/Bcg* and now designated *Slc11A1*) (59-61). This gene is located on chromosome 1 in mice or chromosome 2q35 in humans and encodes a 90-100 kDa phosphoglycoprotein (62) predicted to contain 12 transmembrane domains and a glycosylated extraplasmic loop. In mice, a naturally occurring Gly$\rightarrow$Asp mutation at amino acid 169 in a transmembrane region of the Nramp1 protein leads to a non-functional protein and makes mice susceptible to intracellular bacteria. *Nramp1* is exclusively expressed in primary macrophages and in granulocytic lineages (63). Therefore, the murine polymorphism of *Nramp1* is apparent as either low (*Nramp1*^{s} or *Nramp1*^{Asp}) or high (*Nramp1*^{r} or *Nramp1*^{Gly}) resistance of macrophages to the growth of intracellular organisms. The *Nramp1*^{s} polymorphism is associated with susceptibility to many microorganisms such as BCG, *Salmonella typhimurium, Leishmania donovani, Mycobacterium spp., Toxoplasma godii, Candida albicans* (59, 64). In humans, associations or linkage of NRAMP1 with susceptibility to tuberculosis and leprosy but also susceptibility to a range of autoimmune diseases have been demonstrated (65, 66).The effects of *Nramp1* on early-phase resistance to infections are related to the observation that *Nramp1*^{r} macrophages display faster and superior activation in response to intracellular bacteria, bacterial products, and IFN-γ than *Nramp1*^{s}

macrophages. This is indicated by the higher rate and degree of activation of the transcription factors NF-κB and STAT1 in *Nramp1*[r] macrophages (61, 66), resulting in among others a higher production of bactericidal nitric oxide (NO·) (65) and IFN-γ (67).

The precise biochemical function of the Nramp1 protein and how Nramp1 affects the macrophage activity is still matter of debate. It is now generally assumed that the Nramp1 protein is a divalent cation transporter in lysozomes and phagolysosomes of phagocytotic cells (68, 69). Nevertheless, the direction of the cation current has divided researchers into two schools. One view (**Figure 3A**) proposes that Nramp1 functions to deprive the intraphagosomal bacterium of the availability of cations such as iron and manganese and therefore limits the bacterial growth and antioxidant defence (70). The other view (**Figure 3B**), which has gained a weight of evidence, proposes that Nramp1 is an antiporter that can flux divalent cations in either direction against a proton gradient (64) dependent on the pH on either side of the membrane. As a result, Nramp1 delivers divalent cations from the cytosol into the acidic phagolysosome. Here, the Fenton reaction can use the cation iron to generate toxic hydroxyl radicals ($H_2O_2 + Fe^{2+} \rightarrow H_2O + OH\bullet$). In this way, the *Nramp1* gene is crucial in metabolism and clearance of iron acquired by phagocytosis, since iron in this manner can be released from macrophages via a lysosomal secretory way (71, 72). Likewise, it was found that *Nramp1*[s] macrophages are less capable of releasing iron than *Nramp1*[r] macrophages (71-74). As a result, high cytoplasmic iron levels in *Nramp1*[s] macrophages may cause mRNA stability for a range of activation markers (75-77), contributing to the low capacity of *Nramp1*[s] macrophages to become activated.
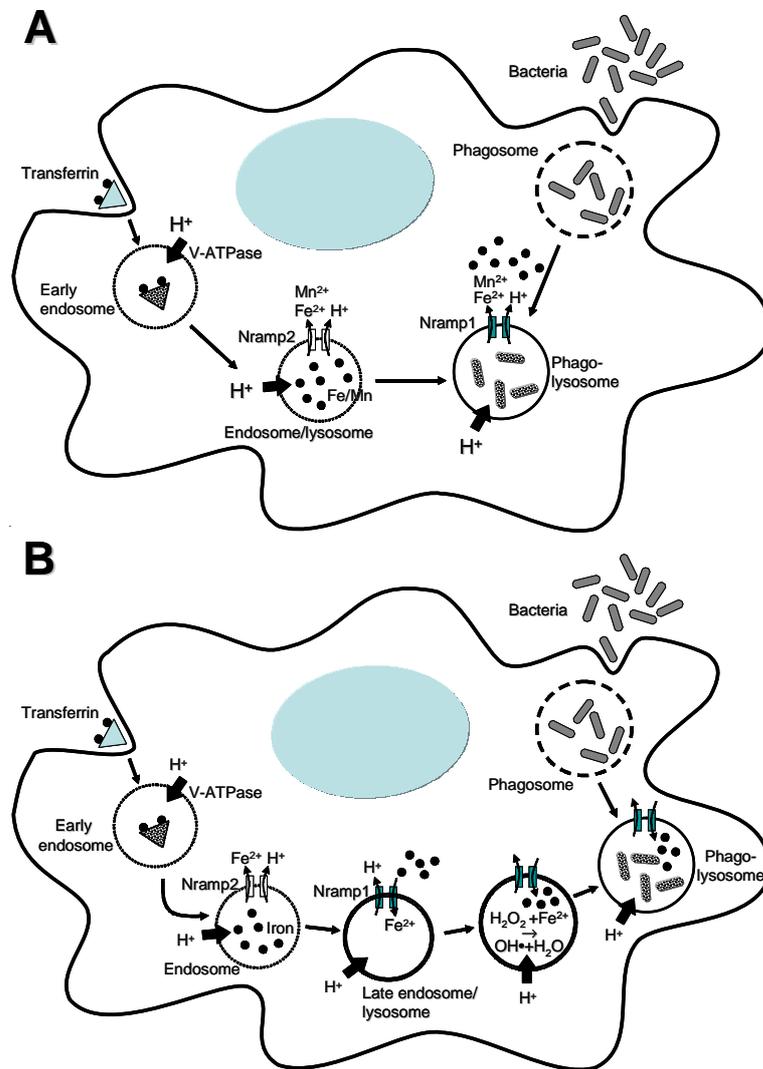
Figure 3. *Current proposed models of Nramp1 protein function in the macrophage.*
Figure 3A, *Model 1: Diferric transferrin is transported to an early endosome and Fe^{2+} is released after acidification of the vacuole due to recruitment of vesicular ATPase (V-ATPase). After phagocytosis, Nramp1, similar to Nramp2 (a family member of Nramp1), delivers divalent cations, like Fe^{2+} and Mn^{2+}, from the phagolysosome into the cytosol, and thereby removes essential nutrients for the microbe.*
Figure 3B, *Model 2: Diferric transferrin is transported to an early endosome and Fe^{2+} is released after acidification of the vacuole due to recruitment of vesicular ATPase (V-ATPase). Nramp2 symport activity delivers divalent cations, like Fe^{2+}, across the early endosomal membrane into the cytosol. Nramp1 antiport activity transports Fe^{2+} into acidic late endosomes, lysosomes and phagolysosomes where toxic radicals are formed via the Fenton and/or Haber-Weiss reaction.*

4. Aim and outline of this thesis

In brief, vaccination with mycobacteria may suppress the development of several allergic and asthmatic manifestations in the mouse. Since the immune response to intracellular bacteria, including mycobacteria, is under control of the *Nramp1* gene, we hypothesized that *Nramp1* affects the efficacy of a mycobacterial vaccine in the treatment of allergic asthma. Therefore, in this thesis, we addressed the following questions:

1. *Is heat-killed M. vaccae able to prevent or treat allergic and asthmatic manifestations in a mouse model of allergic asthma?*
For this purpose, mice were vaccinated with heat-killed *M. vaccae* before allergen sensitization (**Chapter 2**), or before allergen challenge (**Chapter 3**) to reflect prophylactic and therapeutic treatment. After challenge, allergic and asthmatic manifestations such as levels of IgE, airway hyperreactivity and airway eosinophilia were measured. Furthermore, the effect of *M. vaccae* in a milder model of allergic asthma was studied in chapter 3. In **chapter 4** we investigated whether *M. vaccae* must be administered during allergen exposure, either during sensitization or challenge, to be effective in lowering the allergic and asthmatic response.

2. *Does the Nramp1 gene, which controls the resistance to intracellular bacteria, influence sensitivity to induction of allergic asthma-like disease in mice?*
Therefore, in **chapter 5**, we compared wild type and *Nramp1* congenic mice as to their ability to develop allergic asthma-like disease. After final allergen challenge we measured various manifestations of allergy and asthma and Th2 type immune responses in the lung.

3. *Does the Nramp1 gene affect the efficacy of heat-killed M. vaccae in suppressing the allergic and asthmatic response?*
For that reason, in **chapter 6**, wild type and *Nramp1* congenic mice were treated with heat-killed *M. vaccae* during sensitization in an allergic asthma model. In order to investigate whether *Nramp1* affected the T cell-mediated response to *M. vaccae* as well, we investigated whether wild type and *Nramp1* congenic mice differed in the delayed type hypersensitivity reaction to *M. vaccae*. In addition, to provide a clue to the effects of *Nramp1*, we studied the effect of *M. vaccae* on *Nramp1* resistant or susceptible macrophages *in vitro*.

The final conclusions are summarized and discussed in **chapter 7**

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Chapter 2

Prophylactic treatment with heat-killed *M. vaccae* (SRL172) in a mouse model for allergic asthma

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Abstract

Mycobacteria and their components have attracted a lot of attention concerning the "Hygiene hypothesis", which suggests that a decline of infections during early childhood would favor the development of allergic asthma. In this study, we investigated whether prophylactic treatment with heat-killed *Mycobacterium vaccae* is able to inhibit the development of allergic and asthmatic manifestations in a mouse model. For this purpose, mice were treated with *M. vaccae* on day -21 (protocol 1) or days -14 and -7 (protocol 2). Hereafter, all mice were sensitized with ovalbumin/alum on days 0 and 14 and challenged by inhalation of ovalbumin or saline aerosols on days 28, 32 or 35 (protocol 1) or on days 35, 38 and 41 (protocol 2). We demonstrated that *M. vaccae* induced a strong Th1 response, as measured by delayed-type hypersensitity reactions to this agent. Prophylactic treatment of mice with *M. vaccae*, however, did not affect airway hyperresponsiveness and eosinophilia, IgE and IL-5 production 24 hours after ovalbumin challenge. These findings suggest that heat-killed *M. vaccae*, when administered prophylactically by the subcutaneous route, despite its capacity to induce a strong Th1 response, is unable to prevent the induction of allergic disease in a mouse model.

Introduction

Allergic diseases, such as allergic asthma, are steadily increasing in developed countries (1). Environmental changes have been targeted as the main cause of this trend, giving rise to the "hygiene hypothesis". This hypothesis suggested that a decline in infections during childhood would favor the development of allergic asthma (2, 3). A proposed mechanism for the hygiene hypothesis is based on the T helper type 1 and 2 (Th1 and Th2) dichotomy in specific murine and human immune reactions (4). Th2 cells, by secreting interleukins such as interleukin (IL) -4 and IL-5, play a central role in initiating and sustaining the allergic and asthmatic response by regulating the production of IgE and the growth, differentiation and recruitment of eosinophils (5). In contrast, Th1 cells, secreting interferon (IFN)-γ and involved in the immune response to a number of intracellular pathogens, have been proposed to dampen the activity of Th2 cells (4). For this reason, a lack of infections would lead to less frequent activation of Th1 cell responses and, thereby, facilitate development of Th2 cell type responses (6, 7). In contrast, a massive Th2 shift as seen in helminth infections is associated with protection against the development of allergy and asthma (8). Additionally, the incidence of Th1-mediated autoimmune diseases is increasing as well (9). These data suggest that the cause of the increase in allergic diseases cannot be merely be ascribed to a Th1/Th2 imbalance, but instead must have another immunological denominator.

Interestingly, mycobacterial infections make good candidates in the hygiene hypothesis (9, 10). Mycobacteria do not belong to the regular commensal flora, and therefore, the exposure is regulated by lifestyle. In addition, their impact on the immune system is indicated by the existence of CD1-restricted T cells which seem to recognize nothing else than mycobacterial components (11). The experimental data in animal models supporting the candidature of mycobacteria seems convincing. Several studies showed that vaccination with mycobacteria may suppress the development of allergic asthma in mouse models (12-14). Additionally, one species, *M. vaccae*, has proven to be effective in the treatment of atopic dermatitis in humans (15). However, the effect of *M. vaccae* in patients with established asthma remains controversial (16, 17).

Since mycobacterial infections are likely to occur before the onset of allergic disease, we hypothesized that treatment with mycobacteria before sensitization may be effective in lowering allergic asthma. Therefore, we investigated in a mouse model whether prophylactic treatment with heat-killed *M. vaccae* (SRL172) is able to prevent the induction of allergic asthma.

Materials and methods

Animals
Specified pathogen free male BALB/cByJIco mice were obtained from Charles River (Maastricht, The Netherlands). They were provided with food and water *ad libitum* and used when 5-6 weeks of age. All experiments were approved by the animal

ethics committee of the National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

Reagents

Vials of heat-killed *M. vaccae* (SRL172) were kindly provided by SR Pharma Ltd. (London, UK). Ovalbumin (grade V) and acetyl-ß-methylcholinechloride (methacholine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum hydroxide (AlumImject) was purchased from Pierce (Rockford, IL, USA).

Measurement of Delayed-Type Hypersensitivity (DTH)

For measurement of Delayed-Type Hypersensitivity (DTH) to heat-killed *M. vaccae*, mice were injected subcutaneously in the neck with 10^8 CFU (100 µg) heat-killed *M. vaccae* in 100 µl saline or saline alone on day 0 and 7. On day 21, mice were anaesthetized by an intramuscular injection of a mixture of xylazin and ketamin and ear thickness of both ears was measured using a spring loaded caliper (No. 293-561, Mitutoyo, Veenendaal, The Netherlands). Accordingly, all mice were challenged with an intradermal injection with 15 µg heat-killed *M. vaccae* in 20 µl saline in the left ear and saline alone in the right ear. Ear thickness of both ears was measured after anesthesia as described above, 24, 48, 96 and 216 hours after ear challenge.

Sensitization, treatment and challenge

All mice were sensitized by two i.p injections with 10 µg ovalbumin adsorbed onto 2.25 mg aluminum hydroxide in 100 µl saline on days 0 and 14. Prophylactic treated mice received a subcutaneous injection of 10^8 CFU (100 µg) heat-killed *M. vaccae* in 100 µl saline on day -21 (Protocol 1, see table 1) or 10^7 CFU (10 µg) on days -7 and -14 (Protocol 2, see table 1) before ovalbumin sensitization. Mice were challenged on days 28, 32 and 35 (Protocol 1) or on days 35, 38, 41 (Protocol 2) by inhalation of either ovalbumin or saline aerosols in a plexiglass exposure chamber for 20 minutes. The aerosols were generated by nebulizing an ovalbumin solution (10 mg/ml) in saline or saline alone using a Pari LC Star nebulizer (Pari Respiratory Equipment, Richmond, VA, USA).

Protocol	Treatment with *M. vaccae*	Sensitization	Challenge aerosol
1	Day -21: 10^8 CFU	Day 0 & 14	Day 28, 32 & 35
2	Day -14 & -7: 10^7 CFU	Day 0 & 14	Day 35, 38 & 41

Table 1. Experimental design as described in materials and methods.

Determination of airway responsiveness

Airway responsiveness to inhaled nebulized methacholine was determined 24 hours after the final challenge, in conscious, unrestrained mice using whole body plethysmography (BUXCO, EMKA, Paris, France). The airway response was expressed as enhanced pause (Penh), as described previously (18).

Bronchoalveolar lavage

After measurement of cholinergic airway responses, animals were sacrificed and bronchoalveolar lavage was performed, total number of cells was determined and cells were differentiated as described before (18). The supernatant of the first milliliter lavage fluid was separated and frozen at -70°C until further analysis.

Determination of serum levels of ovalbumin-specific immunoglobulins and determination of IL-5 in lung lavage fluid

Blood was withdrawn by heart puncture 24 hours after the last allergen challenge to prepare serum for determination of ovalbumin-specific IgE, IgG2a and IgG1 levels in serum by ELISA's as described previously (19). A commercially available ELISA kit (Pharmingen, San Diego, CA, USA) was used to assess the levels of IL-5 in the lung lavage fluid, according to the instructions of the manufacturers.

Statistical Analysis

The airway response curves to methacholine and the DTH responses were statistically analyzed by a general linear model of repeated measurements followed by post-hoc comparison between groups. Cell counts were statistically analyzed using the Mann-Whitney U test. All other analyses were performed using Student's t test. A probability value $p < 0.05$ was considered significant.

Results

DTH responses to heat-killed M. vaccae

To investigate whether heat-killed *M. vaccae* was able to elicit T cell-mediated responses, we measured DTH responses to this agent. This DTH response was characterized by measuring ear swelling after immunization and ear challenge with *M. vaccae* and defined as the difference in thickness between the left and right ear. Challenge with *M. vaccae* induced a small background swelling in non-sensitized mice (< 45 μm). However, a significantly stronger ear swelling is observed in sensitized mice compared to non-sensitized mice (Fig. 1) that peaked at 24 hours and virtually resolved at 96 hours.

Figure 1. *Ability of M. vaccae to induce a DTH reaction. Data are depicted as mean swelling of the left ear compared to the control right ear ± SEM in M. vaccae-sensitized and non-sensitized mice (n=4), measured 24, 48, 96 and 216 hours after challenge. * p < 0.01 compared to the non-sensitized group.*
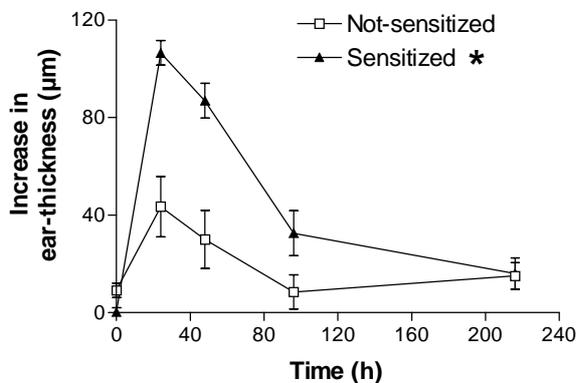
Airway response to methacholine

To investigate the effect of *M. vaccae* treatment on the development of airway hyperresponsiveness, we measured airway responses to increasing concentrations of methacholine 24 hours after final challenge. The dose response curves to metha-choline of ovalbumin-challenged mice were significantly higher than those of saline-challenged animals (Fig. 2). However, treatment with heat-killed *M. vaccae*, in nei-ther protocol, reduced the ovalbumin-induced airway hyperreactivity. *M. vaccae* treat-ment alone did not influence the airway reactivity (data not shown).
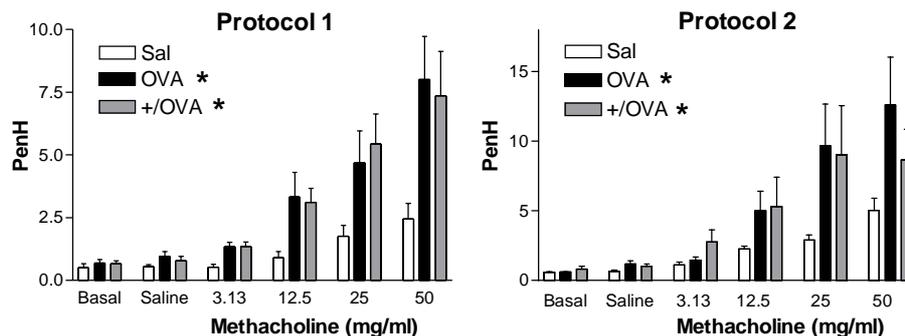

Figure 2. *Airway response to increasing concentrations of nebulized methacholine in ovalbumin-sensitized mice, expressed as Penh-values. Measurements were performed 24 hours after the final saline (Sal) or ovalbumin challenge of control (-/OVA) or with M. vaccae treated (+/OVA) mice. See table 1 for protocol 1 and 2. Data are represented as mean ± SEM, n=8. * p < 0.05 compared to the aline-challenged control group.*

Bronchoalveolar lavage cell counts

Furthermore, we studied the effect of allergen exposure and *M. vaccae* treatment on the cellular composition in the airways. Cell counts in the lung lavage fluid ob-tained 24 hours after final challenge showed that ovalbumin challenge induced a significant increase in the total number of cells in the lavage fluid compared to saline challenge (Fig. 3). This increase in total number of cells was mainly caused by an increase in the number of eosinophils, since no significant differences were ob-served in the number of other cell types between different groups. *M. vaccae* treat-ment did not lower the eosinophil numbers after ovalbumin challenge.
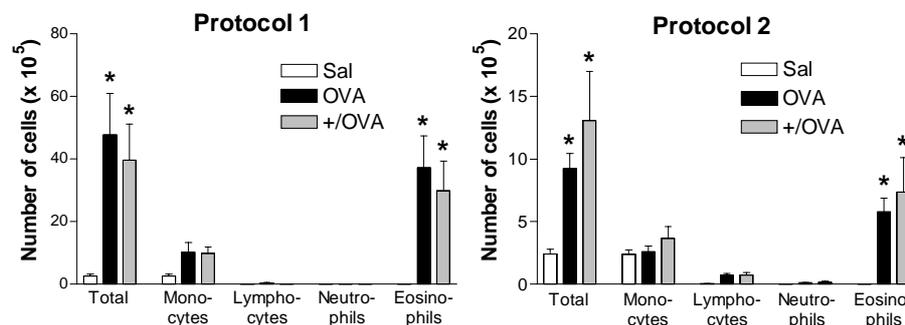

Figure 3. *Number of cells in the lung lavage fluid obtained from ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin challenge of control (-/OVA) or with M. vaccae treated (+/OVA) mice. See table 1 for protocol 1 and 2. Data are represented as mean cell number ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group.*

Serum levels of immunoglobulins

Additionally, we analyzed serum prepared from blood collected 24 hours after the final challenge to study possible effects of *M. vaccae* treatment on immunoglobulin levels. Ovalbumin challenge of sensitized mice induced an increase in the levels of ovalbumin-specific IgE compared to the saline-challenged animals (Fig. 4). In addition, ovalbumin challenge markedly increased serum levels of ovalbumin-specific IgG1 and IgG2a (Fig. 5). However, *M. vaccae* treatment did not reduce the levels of ovalbumin-specific IgE, IgG1 or IgG2a after ovalbumin challenge.

Figure 4. *Levels of ovalbumin-specific IgE measured by ELISA in serum of ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin challenge of control (-/OVA) or with M. vaccae treated (+/OVA) mice. See table 1 for protocol 1 and 2. Vertical bars indicate Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group.*
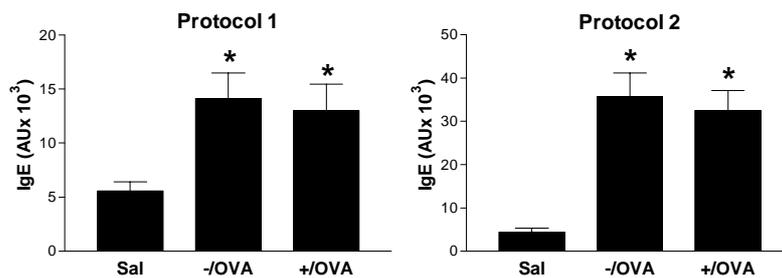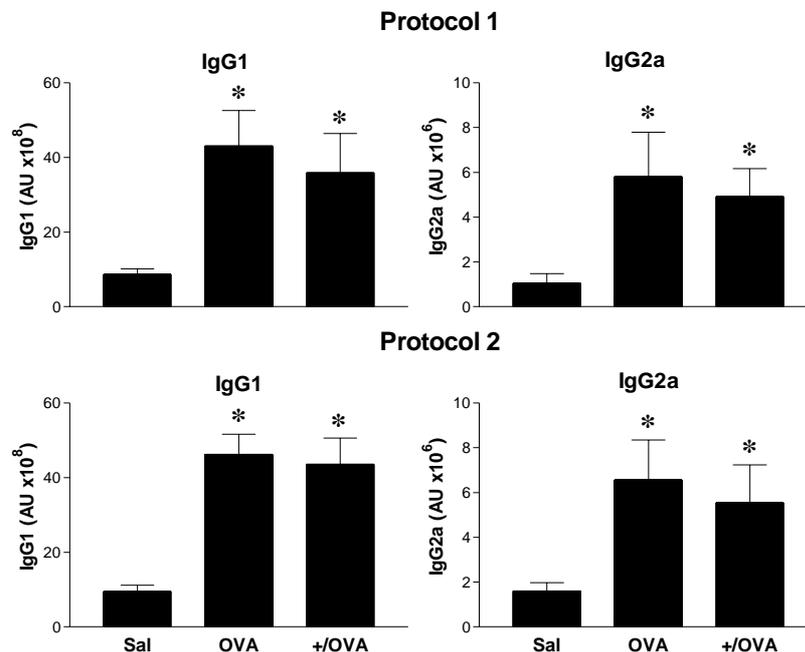

Figure 5. *Ovalbumin-specific IgG1 and IgG2a levels measured by ELISA in serum of ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin challenge of control (-/OVA) or with M. vaccae treated (+/OVA) mice. See table 1 for protocol 1 and 2. Vertical bars indicate Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group.*

IL-5 in lung lavage fluid

Finally, we measured the levels of IL-5 in the lung lavage fluid. The levels of IL-5 in the lung lavage fluid of ovalbumin-challenged mice were significantly increased as compared with saline-challenged mice (Fig. 6). We did not find a reduction in IL-5 levels in ovalbumin-challenged, *M. vaccae* treated mice.
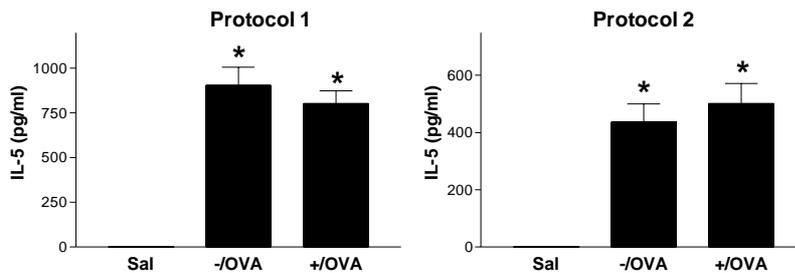

Figure 6. *Levels of IL-5 in lung lavage fluid obtained from ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin challenge of control (-/OVA) or with M. vaccae treated (+/OVA) mice. See table 1 for protocol 1 and 2. Data are represented as mean levels of IL-5 (pg/ml) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group.*

Discussion

Infection or vaccination with mycobacteria may prevent the development of allergic asthma. Therefore, we hypothesized that prophylactic treatment with mycobacteria may be effective in preventing the induction of allergic asthma. In contrast to this hypothesis, prophylactic treatment with *M. vaccae* 1 to 3 weeks before ovalbumin sensitization did not lead to an alteration in airway hyperreactivity, airway eosinophilia or levels of ovalbumin-specific IgE, IgG1 or IgG2a after ovalbumin challenge in our model. However, we demonstrated that heat-killed *M. vaccae* was able to elicit a strong DTH response, showing that *M. vaccae* generated strong T cell immunity in our experiments.

The strong capacity of *M. vaccae* to induce a DTH reaction in the mouse has been demonstrated by other investigators (20), although we are the first to report a DTH reaction to *M. vaccae* in the ear. Previous studies demonstrated that administration of both 10^7 and 10^8 CFU heat-killed *M. vaccae* is capable of eliciting a strong Th1 response, while 10^9 CFU elicited mixed Th1 and Th2 responses in spleen cell cultures (20, 21). For that reason, we conclude that the time and dose of *M. vaccae* used in our studies was sufficient to elicit a relatively pure and strong Th1 response, despite the fact that, *M. vaccae*, both viable and heat-killed, is rapidly cleared after immunization (22).

Despite the strong induction of a Th1 response, treatment with *M. vaccae* did not prevent the induction of allergic and asthmatic parameters in our mouse model. This seems to be in contrast to a number of other studies in which treatment with bacille Calmette-Guérin (BCG) or *M. vaccae* in comparable murine models was able to reduce allergic and asthmatic manifestations (12, 13, 23, 24). One of these studies showed that treatment of mice with heat-killed *M. vaccae*, three weeks

before the first ovalbumin immunization was able to reduce airway eosinophilia. This down regulatory effect was probably mediated through the induction of specific regulatory T cells and mediated by IL-10 and TGF1-ß, and not by induction of Th1 responses (12). These studies, however, applied an intratracheal allergen challenge, while in our studies an aerosol challenge was used. Therfore, a difference in the challenge protocol and hereby a difference in the severity of allergic and asthmatic manifestations may explain the lack of effect of *M. vaccae* treatment in our model.

Some other explanations for the lack of effect of heat-killed *M. vaccae* in lowering asthmatic symptoms in our model can be given. First, the subcutaneous route of administration of *M. vaccae* we used in this study might be not efficient for inducing the appropriate downregulation of the allergic and asthmatic response. It was demonstrated that BCG was most capable in reducing airway eosinophilia when given intranasally and less effective when given intraperitoneally or subcutaneously (23). Though, the intranasal route of administration of mycobacteria is not preferable because of serious side effects. For instance, intranasal application of heat-killed BCG, inhibited the development of allergen induced Th2 responses in the lung, but this inhibition was accompanied by severe inflammation in the airways of mice, that consisted of macrophages, neutrophils and lymphocytes. In our opinion, the subcutaneous route of administration of heat-killed *M. vaccae* has proven to be the most safe and thus far without side effects in mice and humans (15).

Finally, the timing of treatment might be important for *M. vaccae* treatment to be effective in reducing asthmatic responses. In this study, we administered *M. vaccae* prophylactically. However, therapeutic administration after or during establishment of allergic manifestations might prove to be more efficient. Future experiments will investigate the most effective moment for *M. vaccae* administration. In conclusion, we demonstrated that heat-killed *M. vaccae*, when administered prophylactically by the subcutaneous route, despite its capacity to induce a strong Th1 response, was unable to prevent the induction of allergic disease in a mouse model.

Acknowledgement

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Chapter 3

Therapeutic treatment with heat-killed *M. vaccae* (SRL172) in a mild and severe mouse model for allergic asthma

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Abstract

The hypothesis that lack of early childhood bacterial infections would favor the development of allergic disease, suggests that bacteria can be used as a potential treatment for allergic asthma. Therefore, in this study, we investigated the therapeutic potential of heat-killed *Mycobacterium vaccae* in two mouse models for allergic asthma. For this purpose, mice were sensitized with ovalbumin/alum (severe model) or ovalbumin alone (mild model) and challenged on days 77, 80 and 83 by inhalation of either ovalbumin or saline aerosols. Treatment of mice with *M. vaccae* on days 56 and 63, however, did not reduce airway hyperresponsiveness and eosinophilia, IgE and IL-5 production 24 hours after ovalbumin challenge in both mouse models. We therefore conclude that treatment with *M. vaccae* of sensitized mice before allergen exposure is not able to reduce the allergic and asthmatic response in a mild and severe model of allergic asthma.

Introduction

The prevalence of allergic asthma and other atopic disorders have increased dramatically to almost epidemiological proportions in the last decades, especially in industrialized countries (1). In contrast to this increase, few novel drug therapies for allergic disease have been developed and proven effective in clinical trials. Existing treatment consists of inhaled bronchodilators and anti-inflammatory drugs, which are effective in most asthmatic patients, but may have serious side effects, particularly in children and patients with severe asthma, who require high dose treatment. Moreover, these therapies require long-term daily administration, and do not affect the underlying (immune) responses of allergic asthma. Without a doubt, there is a need for better treatment targeted at the underlying mechanisms of asthma and giving a long-term, antigen-specific protective effect.

The "hygiene hypothesis" states that the relative lack of infections early in life could inhibit the development of allergic disease in genetically predisposed individuals (2, 3). In line with this hypothesis, certain vaccinations could prevent or even treat development of allergic disease. Support for this idea comes amongst others from rodent models in which bacteria and bacterial products inhibited the asthmatic response. For instance, Bacillus Calmette-Guérin (BCG) immunization before or during sensitization reduced the Th2 responses, eosinophilia and airway hyperresponsiveness after allergen challenge in rodents (4-6). Furthermore, bacterial products, such as heat-killed BCG (7), *Mycobacterium vaccae* (8), *Listeria monocytogenes* (9), bacterial unmethylated CpG motifs (10) and purified protein derivative (PPD) from *Mycobacterium tuberculosis* (11) suppressed the allergic and asthmatic responses in mice as well. The majority of these studies, however, administered bacteria or bacterial products prophylactically, either entirely before or during allergen sensitization, while few studies looked at the therapeutic potential of these agents.

Therefore, in this study, we investigated whether heat-killed *Mycobacterium vaccae*, which previously has been demonstrated to be effective in preventing allergic asthma, is able to lower the asthmatic response in a mouse model therapeutically, after allergen sensitization. To reflect the spectrum of human disease, we used two mouse models for allergic asthma, one reflecting more severe asthma, and one reflecting mild asthma.

Materials and methods

Animals
Specified pathogen free male BALB/cByJIco mice were obtained from Charles River (Maastricht, The Netherlands) and maintained under SPF conditions in macrolon cages. They were provided with food and water *ad libitum* and used when 5-6 weeks of age. All experiments were conducted in accordance with the Animal Care Committee of the Utrecht University.

Reagents

Vials of heat-killed *M. vaccae* (SRL172, 10 mg or 10^{10} CFU/ml) were kindly provided by SR Pharma Ltd. (London, UK). Diff-Quick staining solutions were purchased from Dade A.G. (Düdingen, Switzerland). Ovalbumin (grade V), acetyl-ß-methylcholine chloride (methacholine), bovine serum albumin (BSA), *o*-phenylenediamine-dichloride substrate (OPD), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum hydroxide (AlumImject) was obtained from Pierce (Rockford, IL, USA). Digoxigenin (DIG), anti-DIG-Fab fragments coupled to horse radish peroxidase and protease inhibitor were purchased from Roche Diagnostics (Basel, Switzerland) and anti-mouse IgE, biotinylated anti-mouse IgG1, biotinylated anti-mouse IgG2a was obtained from Pharmingen (San Diego, CA, USA). Peroxidase-conjugated streptavidin (Poly HRP) was purchased from the CLB (Amsterdam, The Netherlands). ELISA buffer contained 0.5% BSA, 2 nM EDTA, 136.9 nM NaCl, 50 nM Tris and 0.05% Tween-20.

Sensitization, treatment and challenge

Two mouse models of allergic asthma were used in this study (Table 1). Protocol 1 ("severe asthma") consisted of sensitization by two i.p. injections with 10 μg ovalbumin adsorbed onto 2.25 mg aluminum hydroxide in 100 μl saline on days 0 and 14. Protocol 2 ("mild asthma") consisted of 7 i.p. injections with ovalbumin (10 μg/0.5 μl saline) on alternate days between day 0 and 14. *M. vaccae* treated mice received a subcutaneous injection of 10^7 or 10^8 CFU (0.01 or 0.1 mg, respectively) heat-killed *M. vaccae* in 100 μl saline on days 56 and 63. The *M. vaccae* concentrations used are analogous with earlier experiments with this agent (12-15). Mice were challenged in both protocols on days 77, 80 and 83 by inhalation of either ovalbumin or saline aerosols in a plexiglass exposure chamber for 20 minutes. The aerosols were generated by nebulizing an ovalbumin solution (10 mg/ml) in saline or saline alone using a Pari LC Star nebulizer (Pari Respiratory Equipment, Richmond,VA, USA).

Protocol	Sensitization	Treatment with *M. vaccae*	Challenge aerosol
1. "Severe asthma"	Day 0 & 14: Ovalbumin/Alum	Day 56 & 63: 10^7 or 10^8 CFU	Day 77, 80 and 83
2. "Mild asthma"	Between day 0 and 14: 7 x ovalbumin	Day 56 & 63: 10^7 or 10^8 CFU	Day 77, 80 and 83

Table 1. Experimental design as described in materials and methods.

Determination of airway responsiveness

Airway responsiveness to inhaled nebulized methacholine was determined 24 hours after the final challenge, in conscious, unrestrained mice using whole body plethysmography (Buxco corp., Sharon, CT, USA). The airway response was expressed as enhanced pause (Penh), as described previously (16). After assessment of baseline

Penh values for 3 minutes, mice were subsequently subjected to aerosols of saline and increasing concentrations of methacholine (3.13, 12.5, 25, and 50 mg/ml saline) for 3 minutes. Aerosols were generated by a Pari LC Star nebulizer and each aerosol was followed by 3 minutes of recording to assess the average Penh value.

Bronchoalveolar lavage

After measurement of cholinergic airway responses, animals were sacrificed and bronchoalveolar lavage was performed. For this purpose, lungs of mice were lavaged once with 1 ml PBS at 37°C containing 5% BSA and protease inhibitor and 4 times with 1 ml saline at 37°C. Lung lavage cells of each mouse were collected after centrifugation, pooled and resuspended in 150 µl saline. The supernatant of the first lavage was separated and frozen at -70°C until further analysis. For differential cell counts, cytospin preparations were made and stained with Diff-Quick. Cells were differentiated into monocytes, eosinophils, lymphocytes and neutrophils by standard morphology.

Determination of serum levels of ovalbumin-specific immunoglobulins

Blood was withdrawn by heart puncture 24 hours after the last allergen challenge to prepare serum for determination of antibody levels in serum by ELISA's using microtiter plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% Tween-20 for washing between incubations. To determine ovalbumin-specific IgE levels, wells were coated overnight at 4°C with 1 µg/ml of anti-mouse IgE in PBS, followed by blocking for 1 hour and incubation of the wells with diluted serum samples and duplicate dilution series of an ovalbumin-specific IgE reference serum, prepared as described previously (17) for 2 hours. Thereafter, wells were incubated for 1 hour with 1 µg/ml of DIG-conjugated ovalbumin followed by incubation with anti-DIG-Fab fragments coupled to horse radish peroxidase, according to manufacturer's instructions. To assess ovalbumin-specific IgG1 or IgG2a levels, wells were coated with 10 µg/ml ovalbumin in PBS. After blocking, diluted serum samples or duplicate dilution series of a reference standard serum obtained from multiply ovalbumin-boosted mice were added. Hereafter, wells were incubated with 1 µg/ml of biotinylated anti-mouse IgG1, or 1 µg/ml of biotinylated anti-mouse IgG2a for 2 hours, followed by 1:10,000 diluted Poly-HRP for 1 hour. For color development, 0.4 mg/ml of OPD and 4 mM H_2O_2 in PBS was used and the reaction was stopped by adding 4 M H_2SO_4. The optical density was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

Determination of IL-5 in lung lavage fluid

A commercially available ELISA kit (Pharmingen) was used to assess levels of IL-5 in the lung lavage fluid, according to the instructions of the manufacturers. The detection limit for this ELISA was 32 pg/ml.

Statistical Analysis

All data are expressed as mean ± SEM. The airway response curves to methacholine and the DTH responses were statistically analyzed by a general linear model of repeated measurements followed by post-hoc comparison between groups. Data were log10 transformed before analysis to equalize variances in all groups. Cell counts were statistically analyzed using the Mann-Whitney U test. All other analyses were performed using Student's *t* test. A probability value $p < 0.05$ was considered significant.

Results

Airway response to methacholine

To study the effect of *M. vaccae* treatment on the development of airway hyperresponsiveness, we measured airway responses to increasing concentrations of methacholine, 24 hours after final challenge. The dose response curves to methacholine of ovalbumin-challenged mice were significantly higher than those of saline-challenged animals in both protocols (Fig. 1). This airway hyperresponsiveness was significantly higher in protocol 1 ("severe asthma") than protocol 2 ("mild asthma"). Treatment with heat-killed *M. vaccae*, however, failed to reduce the ovalbumin-induced airway hyperreactivity in either protocol.
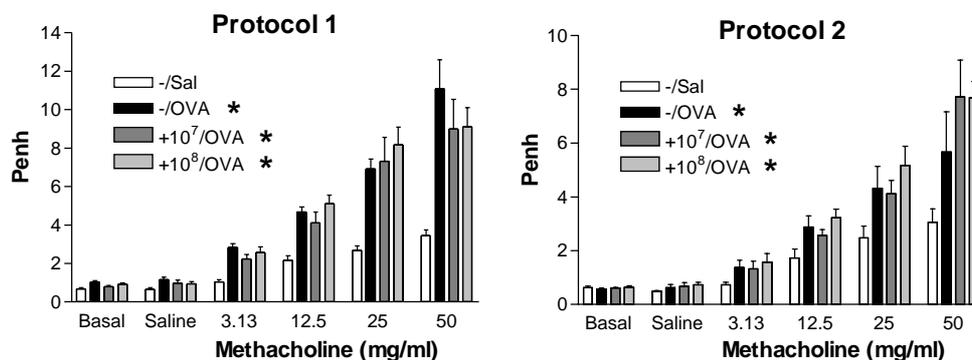

Figure 1. Airway response to increasing concentrations of nebulized methacholine in ovalbumin-sensitized mice, expressed as Penh-values. Measurements were performed 24 hours after the final saline (Sal) or ovalbumin challenge of control (-/OVA) or with 10^7 CFU (+10^7/OVA) or 10^8 CFU (+10^8/OVA) M. vaccae treated mice. See table 1 for protocol 1 and 2. Data are represented as mean ± SEM, n=8. * $p < 0.05$ compared to the saline-challenged control group.

Bronchoalveolar lavage cell counts

Furthermore, we studied the effect of allergen exposure and *M. vaccae* treatment on the cellular composition in the airways. Cell counts in the lung lavage fluid obtained 24 hours after final challenge showed that ovalbumin challenges induced a significant increase in the total number of cells in the lavage fluid compared to saline challenge (Fig. 2). This increase in total number of cells was mainly caused by an increase in the number of eosinophils, since no major increase was observed in

the number of other cell types between different groups. The eosinophilic response in the airways was 10 times higher in protocol 1 ("severe asthma") compared with protocol 2 ("mild asthma"). *M. vaccae* treatment did not lower the eosinophil numbers after ovalbumin challenge in both protocols.
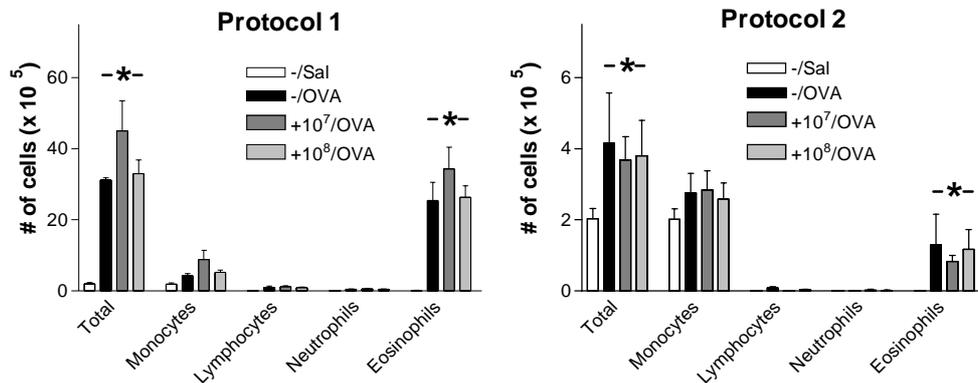

Figure 2. *Number of cells in the lung lavage fluid obtained from ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin challenge of control (-/OVA) or with 10^7 CFU (+10^7/OVA) or 10^8 CFU (+10^8/OVA) M. vaccae treated mice. See table 1 for protocol 1 and 2. Data are represented as mean cell number ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group.*

Serum levels of immunoglobulins

Additionally, we analyzed serum prepared from blood collected 24 hours after the final challenge to study possible effects of *M. vaccae* treatment on immunoglobulin levels. Ovalbumin challenge of sensitized mice induced an increase in the levels of ovalbumin-specific IgE compared to the saline-challenged animals (Fig. 3). In addition, ovalbumin challenge markedly increased serum levels of ovalbumin-specific IgG1 and IgG2a (Fig. 4). The increase in IgE and IgG1 was significantly higher in protocol 1 ("severe asthma") compared with protocol 2 ("mild asthma"). In contrast, the levels of IgG2a did not differ between protocol 1 or 2. *M. vaccae* treatment did not affect the levels of ovalbumin-specific IgE, IgG1 or IgG2a after ovalbumin challenge.

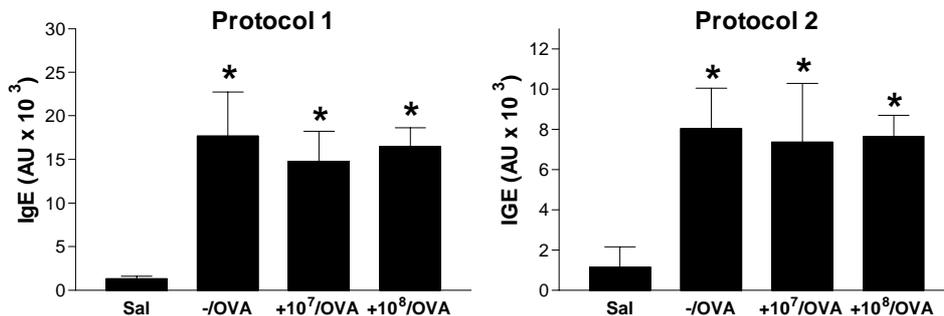**Figure 3.** *Levels of ovalbumin-specific IgE measured by ELISA in serum of ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin challenge of control (-/OVA) or with 10^7 CFU (+10^7/OVA) or 10^8 CFU (+10^8/OVA) M. vaccae treated mice. See table 1 for protocol 1 and 2. Vertical bars indicate Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group.*

Protocol 1

Protocol 2

*Figure 4. Ovalbumin-specific IgG1 and IgG2a levels measured by ELISA in serum of ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin challenge of control (-/OVA) or with 10^7 CFU (+10^7/OVA) or 10^8 CFU (+10^8/OVA) M. vaccae treated mice. See table 1 for protocol 1 and 2. Vertical bars indicate Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group.*

IL-5 in lung lavage fluid

Finally, we measured the levels of IL-5 in the lung lavage fluid. The levels of IL-5 in the lung lavage fluid of ovalbumin-challenged mice were significantly increased as compared with saline-challenged mice (Fig. 5). This increase was higher in protocol 1 ("severe asthma") compared with protocol 2 ("mild asthma"). *M. vaccae* treatment did not affect the levels of IL-5.

Figure 5. *Levels of IL-5 in lung lavage fluid obtained from ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin challenge of control (-/OVA) or with 10^7 CFU (+10^7/OVA) or 10^8 CFU (+10^8/OVA) M. vaccae treated mice. See table 1 for protocol 1 and 2. Data are represented as mean levels of IL-5 (pg/ml) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group.*
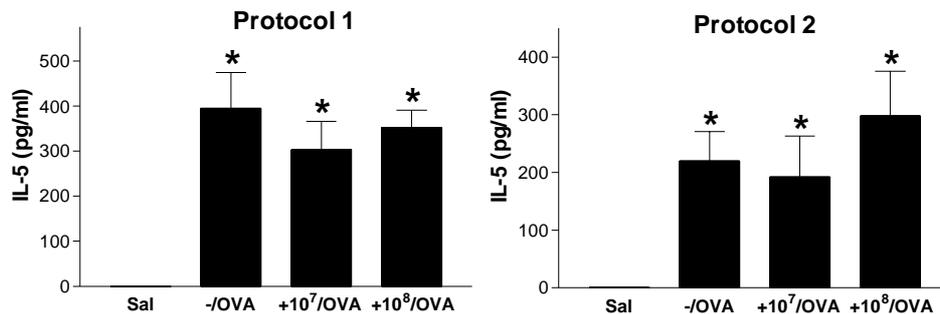
Discussion

This study examined the therapeutic potential of *M. vaccae* to inhibit the allergic response in two mouse models of allergic asthma. Since the ovalbumin/alum adjuvant model could push the pathology too far to be recoverable, we used both an adjuvant and a non-adjuvant, mild model for allergic asthma in our studies as well. Results clearly show that allergic and asthmatic manifestations measured are drastically higher in the severe model compared with the mild model. However, therapeutic treatment with *M. vaccae* starting after sensitization, 3 weeks before ovalbumin challenge did not lead to an alteration in airway hyperreactivity, airway eosinophilia or levels of ovalbumin specific IgE, IgG1 or IgG2a or IL-5 after ovalbumin challenge in either model for allergic asthma. This implicates that the negative results with *M. vaccae* in our experiments are not a consequence of the model used. This study append earlier experiments (chapter2) in which we demonstrated that *M. vaccae* administration 1 to 3 weeks before allergen sensitization did not able to inhibit allergic and asthmatic responses as well. In addition, we showed before that challenge with heat-killed *M. vaccae* is able to elicit strong DTH responses after *M. vaccae* vaccination, demonstrating that time and dose of *M. vaccae* was sufficient to elicit a relatively pure and strong Th1 response, despite the fact that, *M. vaccae*, both viable and heat-killed, is rapidly cleared after immunization (14).

However, despite the strong induction of a Th1 response, treatment with *M. vaccae* did not prevent the induction of allergic and asthmatic parameters in our mouse model.

Some explanations for the lack of effect of *M. vaccae* can be given. First, while most studies administer mycobacteria before, during or shortly after sensitization (4, 5, 7, 18), in this study *M. vaccae* was given therapeutically a long period after sensitization. In a murine non-adjuvant ovalbumin model for allergic disease it was found that after sensitization, Th2 memory was present even after more than 400 days (19). Therefore, it is be possible that long after sensitization and allergen exposure the allergic and asthmatic response is irreversible. Secondly, the route of *M. vaccae* administration might be of importance. Other studies demonstrated that treatment with mycobacteria in allergic asthma models were most effective when mycobacteria were administered locally by the intranasal route. For instance, it was demonstrated that intranasal administration of BCG was superior over intraperitoneal or subcutaneous routes in lowering the asthmatic response in mice (4, 20). On the other hand, intranasal application of both live and heat-killed BCG is accompanied by serious inflammation in the airways of mice which consists of macrophages, lymphocytes and neutrophils. Therefore, subcutaneous administration of mycobacteria, as used in our study has proven to be the safest and thus far only accepted route of administration.

Finally, crucial in treatment of allergic asthma with *M. vaccae* may be the time of inoculation. In this study, *M. vaccae* was administered in the absence of allergen exposure. As suggested by other investigators (6, 21) mycobacteria may be

only efficient when administered at the time of allergens exposure. Future experiments will investigate whether allergen exposure is necessary for mycobacterial treatment to be effective.

We conclude that heat-killed *M. vaccae*, administered a long time after allergen sensitization is unable to ameliorate allergic and asthmatic manifestations in a mild and more severe mouse model for allergic asthma. Future research will focus on the most efficient moment for *M. vaccae* administration.

Acknowledgement

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Chapter 4

M. *vaccae* (SRL172) administration during allergen sensitization or challenge suppresses asthmatic features

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Abstract

Based on the "hygiene hypothesis", bacterial infections could prevent or even treat allergic disease. Therefore, we examined the effect of heat-killed *Mycobacterium vaccae* in a mouse model of allergic asthma. Mice were sensitized with ovalbumin/ alum on days 0 and 14. Hereafter, mice were challenged on days 35, 39, and 42 by inhalation of either ovalbumin or saline aerosols. Mice received an injection with 10^6, 10^7 or 10^8 CFU heat-killed *M. vaccae* on days 0 and 14 or 10^7 CFU on days 35 and 39. On day 43, the airway responsiveness of the mice to increasing concentrations of methacholine was assessed, blood was withdrawn to measure serum parameters and lung lavage was performed to detect cytokines and inflammatory cell number. Treatment of ovalbumin-sensitized mice with 10^7 CFU *M. vaccae* either during sensitization or challenge suppresses airway hyperresponsiveness, airway eosinophilia and IL-5 production after ovalbumin-challenge. The increase in ovalbumin-specific serum IgE and IL-4 by respiratory challenges with ovalbumin were only diminished after *M. vaccae* treatment (10^7 CFU) during sensitization. In conclusion, heat-killed *M. vaccae* prevents allergic and asthmatic manifestations in a mouse model and more importantly, *M. vaccae* suppresses features of asthma during challenge, which opens possibilities for new therapeutic interventions.

Introduction

Atopic asthma is a disease characterized by airway hyperresponsiveness to a variety of specific and non-specific stimuli, by chronic pulmonary inflammation with eosinophilia and by IgE mediated immune responses (1). The underlying mechanisms of the development of atopic asthma have become clearer over the past decade. Nevertheless, the prevalence of asthma has steadily increased by 75% during the last decade in the USA (2). The specific cause for this rise is not known, but one explanation which has gained much attention is the "hygiene hypothesis". It suggests that the increase in allergic diseases is caused by a cleaner environment and fewer childhood infections (3, 4). Mechanistic explanations for the hygiene hypothesis usually comprised a disbalance in Th1 and Th2. Th1 responses are induced during most bacterial and viral infections, and may be able to downregulate the allergic Th2 responses. Accordingly, a reduction in the overall microbial burden will result in weakening Th1 responses and therefore unrestrained Th2 responses, resulting in an increase in allergy (5, 6). This possible mechanism is contradicted by the observations that Th2-mediated helminth infections are negatively associated with allergy and that the prevalence of Th1-mediated autoimmune diseases are increasing as well (7, 8). Therefore, other mechanisms must be taken into account as well to explain the hygiene hypothesis.

Good candidates in providing a mechanism for the hygiene hypothesis may be the group of mycobacterial infections. Changes in exposure to these bacteria are related to changes in the environment and to a western lifestyle, since mycobacteria are present in soil and untreated water, but not in concrete environments (8). In addition, the existence of CD1-restricted T cells, that recognize nothing else than mycobacterial components, indicate the impact mycobacteria have on the immune system (9). In animal models, inoculation with bacillus Calmette-Guérin (BCG) or *Mycobacterium vaccae* reduced allergic sensitization, eosinophilic and airway hyperresponsivess responses to allergen (10-13). In contrast, several retrospective human studies failed to demonstrate a correlation between BCG vaccination during early childhood and the subsequent development of atopy or asthma (reviewed in (14). It seems that differences exist between prophylactic or therapeutic treatment of allergic disorders with mycobacteria in humans, since some investigators demonstrated a positive therapeutic effect of Bacille Calmette-Guérin (BCG) or *M. vaccae* on established asthma (15, 16) or atopic dermatitis (17). In particular, *M. vaccae*, which shares several immunodominant epitopes with other mycobacteria, and which induces a strong cell-mediated immune response, may be used as a potential therapeutic asthma vaccine.

The purpose of this study was to investigate in a mouse model whether heat-killed *M. vaccae* (SRL172) is not only able to suppress the induction of allergic asthma but can also downregulate established allergic asthma. In addition, we investigated whether the treatment with *M. vaccae* is dependent on allergen exposure in our model.

Materials and methods

Animals
Specified pathogen free male BALB/cByJlco mice were obtained from Charles River (Maastricht, The Netherlands) and maintained under SPF conditions in macrolon cages. They were provided with food and water *ad libitum* and used when 5-6 weeks of age. All experiments were conducted in accordance with the Animal Care Committee of the Utrecht University.

Reagents
Vials of heat-killed *M. vaccae* (SRL172, 10 mg or 10^{10} CFU/ml) were kindly provided by SR Pharma Ltd. (London, UK). Diff-Quick staining solutions were purchased from Dade A.G. (Düdingen, Switzerland). Ovalbumin (grade V), acetyl-ß-methylcholine chloride (methacholine), bovine serum albumin (BSA), *o*-phenylenediamine-dichloride substrate (OPD), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum hydroxide (AlumImject) was obtained from Pierce (Rockford, IL, USA). Digoxigenin (DIG), anti-DIG-Fab fragments coupled to horse radish peroxidase and protease inhibitor were purchased from Roche Diagnostics Corp. (Basel, Switzerland) and anti-mouse IgE, biotinylated anti-mouse IgG1, biotinylated anti-mouse IgG2a was obtained from Pharmingen (San Diego, CA, USA). Peroxidase-conjugated streptavidin (Poly HRP) was purchased from the CLB (Amsterdam, The Netherlands). ELISA buffer contained 0.5% BSA, 2 nM EDTA, 136.9 nM NaCl, 50 nM Tris and 0.05% Tween-20.

Sensitization, treatment and challenge
All mice were sensitized by two intraperitoneal injections with 10 µg ovalbumin adsorbed onto 2.25 mg aluminum hydroxide in 100 µl saline (ovalbumin/alum) on days 0 and 14. Hereafter, mice were challenged on days 35, 39, 42 by inhalation of either ovalbumin or saline aerosols in a Plexiglas exposure chamber for 30 minutes. The aerosols were generated by nebulizing an ovalbumin solution (10 mg/ml) in saline or saline alone using a Pari LC Star nebulizer (Pari Respiratory Equipment, Richmond, VA, USA; particle size 2.5-3.1 µm) driven by compressed air at a flow rate of 6 l/min. *M. vaccae* treated mice received a subcutaneous injection in the neck with 10^6, 10^7 or 10^8 CFU heat-killed *M. vaccae* in 100 µl saline on days 0 and 14, immediately before the i.p. ovalbumin/alum injection. A separate group of mice received a subcutaneous injection in the neck with 10^7 CFU heat-killed *M. vaccae* in 100 µl saline on days 35 and 39, immediately before receiving an ovalbumin aerosol.

Determination of airway responsiveness
Airway responsiveness to inhaled nebulized methacholine was determined 24 hours after the final challenge, in conscious, unrestrained mice using whole body plethysmography (Buxco corp., Sharon, CT, USA). The airway response was expressed as enhanced pause (Penh), as described previously (18, 19). Briefly, animals were placed

in a whole body chamber to record differences in pressure between this chamber and a reference chamber for calculation of Penh values. After assessment of baseline Penh values for 3 minutes, mice were subsequently subjected to aerosols of saline and increasing concentrations of methacholine (3.13, 12.5, 25, and 50 mg/ml saline) for 3 minutes. Aerosols were generated by a Pari LC Star nebulizer and each aerosol was followed by 3 minutes of recording to assess the average Penh value from 10 or 5 valid breaths.

Bronchoalveolar lavage

After measurement of cholinergic airway responses, animals were sacrificed and bronchoalveolar lavage was performed. For this purpose, lungs of mice were lavaged once with 1 ml PBS at 37°C containing 5% BSA and protease inhibitor and 4 times with 1 ml saline at 37°C. Lung lavage cells of each mouse were collected after centrifugation, pooled and resuspended in 150 µl saline. Total numbers of cells were determined using a Bürker-Türk chamber (Omnilabo, Breda, The Netherlands). For differential cell counts, cytospin preparations were made and stained with Diff-Quick. Cells were differentiated into monocytes, eosinophils, lymphocytes and neutrophils by standard morphology. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated. The supernatant of the first lavage was separated and frozen at -70°C until further analysis.

Determination of serum levels of ovalbumin-specific immunoglobulins

Blood was withdrawn by heart puncture 24 hours after the last allergen challenge to prepare serum for determination of antibody levels in serum by ELISA's using microtiter plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% Tween-20 for washing between incubations. To determine ovalbumin-specific IgE levels, wells were coated overnight at 4°C with 1 µg/ml of anti-mouse IgE in PBS, followed by blocking for 1 hour and incubation of the wells with diluted serum samples and duplicate dilution series of an ovalbumin-specific IgE reference serum, prepared as described previously (20) for 2 hours. Thereafter, wells were incubated for 1 hour with 1 µg/ml of DIG-conjugated ovalbumin followed by incubation with anti-DIG-Fab fragments coupled to horse radish peroxidase, according to manufacturer's instructions.

To assess ovalbumin-specific IgG1 or IgG2a levels, wells were coated with 10 µg/ml ovalbumin in PBS. After blocking, diluted serum samples or duplicate dilution series of a reference standard serum obtained from multiply ovalbumin-boosted mice were added. Hereafter, wells were incubated with 1 µg/ml of biotinylated anti-mouse IgG1, or 1 µg/ml of biotinylated anti-mouse IgG2a for 2 hours, followed by 1:10,000 diluted Poly-HRP for 1 hour. For color development, 0.4 mg/ml of OPD and 4 mM H_2O_2 in PBS was used and the reaction was stopped by adding 4 M H_2SO_4. The optical density was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

Determination of IL-4, IL-5 and IL-10 in lung lavage fluid

Commercially available ELISA kits (Pharmingen) were used to assess levels IL-4, IL-5 and IL-10 in the lung lavage fluid, according to the instructions of the manufacturers. The detection limit for IL-4, IL-5 and IL-10 were 8, 32 and 60 pg/ml, respectively.

Statistical Analysis

All data are expressed as mean ± SEM. The airway response curves to methacholine were statistically analyzed by a general linear model of repeated measurements followed by post-hoc comparison between groups. Data were log10 transformed before analysis to equalize variances in all groups. Cell counts were statistically analyzed using the Mann-Whitney U test. All other analyses were performed using Student's *t* test. A probability value $p < 0.05$ was considered significant.

Results

Airway response to methacholine

To investigate the effect of *M. vaccae* treatment on the development of airway hyperresponsiveness, airway responses of ovalbumin-sensitized mice to increasing concentrations of methacholine, 24 hours after final ovalbumin or saline challenge, were measured and expressed as Penh (Fig. 1). The dose response curves to metha-
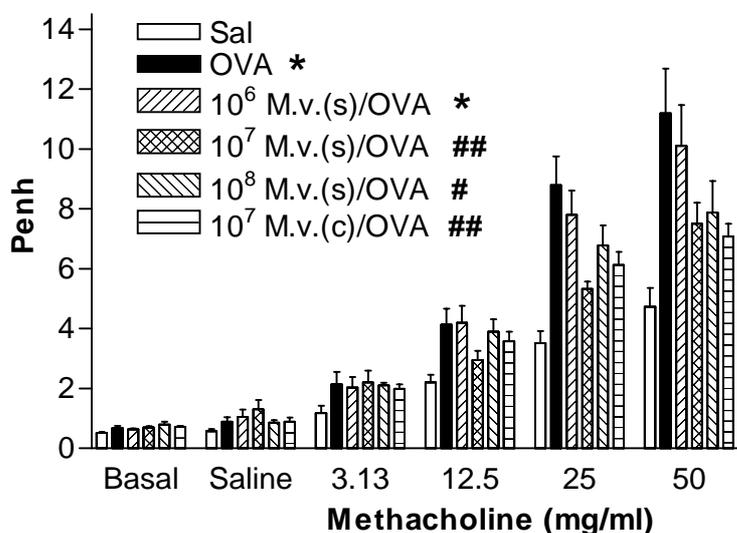
Figure 1. *Airway response to increasing concentrations of nebulized methacholine in ovalbumin-sensitized mice, expressed as Penh-values. Measurements were performed 24 hours after the final saline (Sal) or ovalbumin (OVA) challenge. Mice were treated with 10^6 (10^6 M.v.(s)/OVA), 10^7 (10^7 M.v.(s)/OVA), or 10^8 (10^8 M.v.(s)/OVA) CFU M. vaccae during ovalbumin sensitization or with 10^7 (10^7 M.v.(c)/OVA) CFU M. vaccae during ovalbumin challenge. Data are represented as mean ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group. # p < 0.05 or ## p < 0.01 compared to the ovalbumin-challenged control group.*

choline of ovalbumin-challenged mice were significantly higher than those of the saline-challenged animals, demonstrating ovalbumin-induced hyperresponsiveness. Treatment with 10^7 and 10^8 CFU, in contrast to 10^6 CFU, heat-killed *M. vaccae* during ovalbumin sensitization lowered the airway hyperresponsiveness significantly. Interestingly, when 10^7 CFU *M. vaccae* was administered during ovalbumin challenge, it was effective as well in lowering the airway hyperresponsiveness. *M. vaccae* treatment alone did not influence the airway reactivity (data not shown).

Bronchoalveolar lavage cell counts

Furthermore, we studied the effect of allergen exposure and *M. vaccae* treatment on the cellular composition in the airways. Cell counts in the lung lavage fluid obtained 24 hours after final challenge showed that ovalbumin challenge induced a significant increase in the total number of cells in the lavage fluid compared to saline challenge (Fig. 2). This increase in total number was mainly caused by an increase in the number of eosinophils, although the number of other cell types (monocytes, lymphocytes and neutrophils) was increased to a certain extent as well. Treatment with 10^7 CFU *M. vaccae* during ovalbumin sensitization significantly reduced the number of eosinophils in the lung lavage fluid, while 10^6 or 10^8 CFU *M. vaccae* did not significantly reduce the number of eosinophils. In addition, treatment with 10^7 CFU *M. vaccae* during challenge was effective in lowering the eosinophilia in the lung as well.

Figure 2. Number of cells in the lung lavage fluid obtained from ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin (OVA) challenge. Mice were treated with 10^6 (10^6 M.v.(s)/OVA), 10^7 (10^7 M.v.(s)/OVA), or 10^8 (10^8 M.v.(s)/OVA) CFU M. vaccae during ovalbumin sensitization or with 10^7 (10^7 M.v.(c)/OVA) CFU M. vaccae during ovalbumin challenge. Data are represented as mean cell number ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group. # p < 0.05 or ## p < 0.01 compared to the ovalbumin-challenged control group.

Serum levels of immunoglobulins

Additionally, we analyzed the serum prepared from blood collected 24 h after the final challenge, to study possible effects of *M. vaccae* treatment on allergen-specific antibody levels. Ovalbumin challenge of sensitized mice induced an increase in the levels of ovalbumin-specific IgE (Fig. 3), IgG1 and IgG2a (Fig. 4) compared to saline-challenged mice. Treatment with 10^7 CFU *M. vaccae* during sensitization significantly reduced serum levels of ovalbumin-specific IgE and IgG1, in contrast to *M. vaccae* treatment during challenge, which did not affect levels of these immunoglobulins. *M. vaccae* treatment did not change the serum levels of ovalbumin-specific IgG2a.
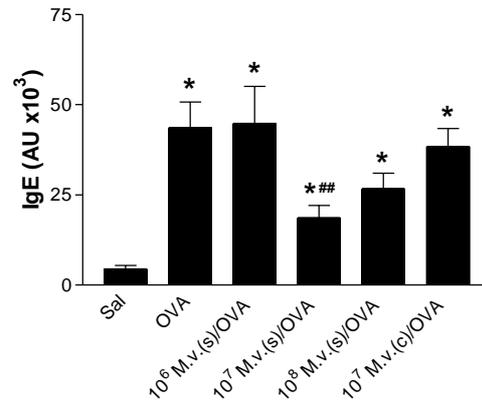
*Figure 3. Levels of ovalbumin-specific IgE, measured by ELISA in serum of ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin (OVA) challenge. Mice were treated with 10^6 (10^6 M.v.(s)/OVA), 10^7 (10^7 M.v.(s)/OVA), or 10^8 (10^8 M.v.(s)/OVA) CFU M. vaccae during ovalbumin sensitization or with 10^7 (10^7 M.v.(c)/OVA) CFU M. vaccae during ovalbumin challenge. Bars indicate Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group. ## p < 0.01 compared to the ovalbumin-challenged control group.*
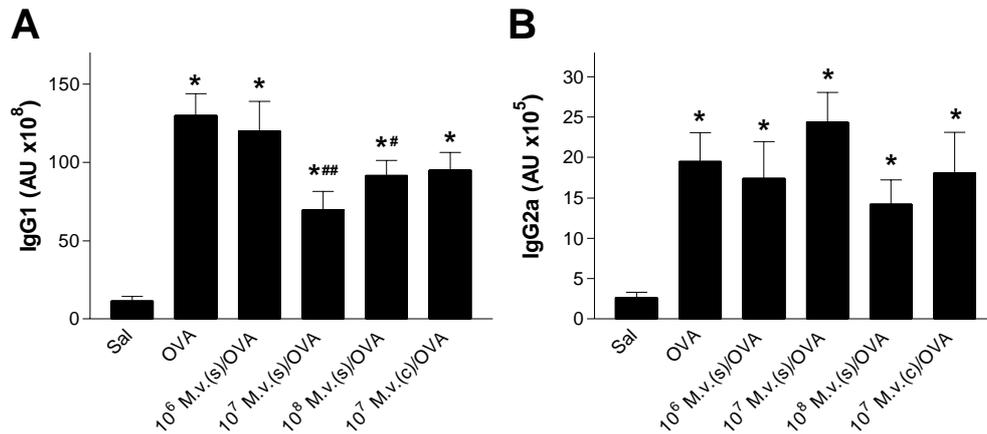
*Figure 4. Ovalbumin-specific IgG1 (A) and IgG2a (B) levels as measured by ELISA in serum of ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin (OVA) challenge. Mice were treated with 10^6 (10^6 M.v.(s)/OVA), 10^7 (10^7 M.v.(s)/OVA), or 10^8 (10^8 M.v.(s)/OVA) CFU M. vaccae during ovalbumin sensitization or with 10^7 (10^7 M.v.(c)/OVA) CFU M. vaccae during ovalbumin challenge. Vertical bars indicate Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group. # p < 0.05 or ## p < 0.01 compared to the ovalbumin-challenged control group.*

Cytokines in lung lavage fluid

Finally, cytokine levels were measured in the lavage fluid, to study the cytokine profile in the lung 24 h after challenge. Lung lavage fluid of ovalbumin-sensitized mice contained no detectable levels of IL-4 and IL-5 and low levels of IL-10 after saline challenge (Fig. 5). Ovalbumin challenge caused a significant increase in the levels of these cytokines in both strains as compared to the saline-challenged controls. Interestingly, the increase in IL-4 was significantly lower in mice treated during sensitization with 10^7 CFU *M. vaccae*. In addition, the level of IL-5 was reduced in mice treated during sensitization with 10^7 CFU *M. vaccae* but also in mice treated with *M. vaccae* during challenge. No effect of *M. vaccae* treatment was observed on the levels of IL-10 in the lavage fluid. IFN-γ was not detectable in the lavage fluid, irrespective of ovalbumin challenge or *M. vaccae* treatment (data not shown).

A

B

C
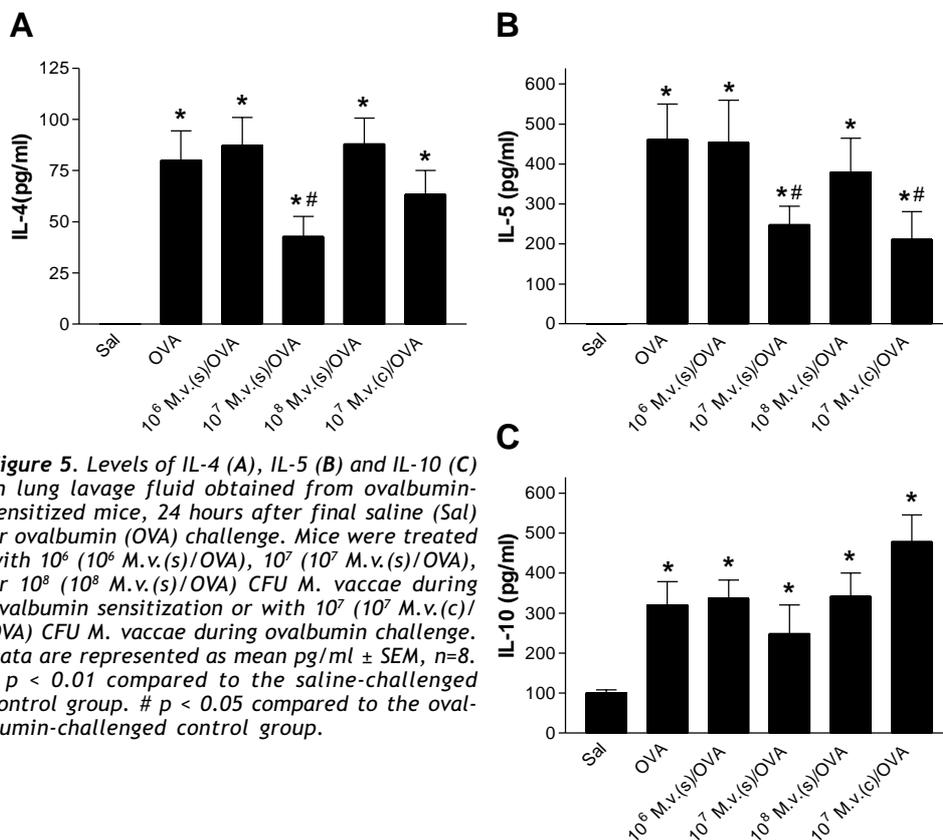
*Figure 5. Levels of IL-4 (A), IL-5 (B) and IL-10 (C) in lung lavage fluid obtained from ovalbumin-sensitized mice, 24 hours after final saline (Sal) or ovalbumin (OVA) challenge. Mice were treated with 10^6 (10^6 M.v.(s)/OVA), 10^7 (10^7 M.v.(s)/OVA), or 10^8 (10^8 M.v.(s)/OVA) CFU M. vaccae during ovalbumin sensitization or with 10^7 (10^7 M.v.(c)/OVA) CFU M. vaccae during ovalbumin challenge. Data are represented as mean pg/ml ± SEM, n=8. * p < 0.01 compared to the saline-challenged control group. # p < 0.05 compared to the ovalbumin-challenged control group.*

Discussion

This study demonstrates that heat-killed *M. vaccae* reduces important manifestations of atopic asthma in a mouse model, in which we used two approaches of *M. vaccae* treatment, during or after the onset of allergic disease. We demonstrated that *M. vaccae* treatment during sensitization suppressed the airway

hyperresponsiveness, eosinophilia, serum levels of IgE, and the Th2 cytokines IL-4 and IL-5 in the lung lavage fluid after ovalbumin challenge. This effect was dose-dependent, with 10^7 CFU being the optimal dose for *M. vaccae* treatment, while 10^8 CFU appeared less and 10^6 CFU not effective in lowering the allergic and asthmatic response. Moreover, therapeutic treatment with 10^7 CFU *M. vaccae* during challenge was effective in reducing the airway hyperresponsiveness and airway eosinophilia after challenge, interestingly, accompanied by a lower level of IL-5 in the lavage fluid.

The results presented in this study complete earlier experiments in which 10^7 or 10^8 CFU *M. vaccae* administered 1-3 weeks before ovalbumin sensitization or challenge did not decrease parameters of atopic asthma in the same mouse model (J.J.Smit, submitted). These data and the present study suggest that timing of *M. vaccae* administration is crucial: *M. vaccae* should be present during allergen exposure to be effective. These results seem in contrast with results by other investigators who demonstrated that treatment with 10^8 CFU heat-killed *M. vaccae* three weeks before ovalbumin sensitization or ovalbumin challenge reduced airway eosinophilia and airway hyperresponsiveness to methacholine (11, 21). Differences in study design may account for this observed difference, such as the dose and frequency of *M. vaccae* and the time and intensity of allergen sensitization and challenge.

Next to the observed effects of *M. vaccae* on development of airway hyperresponsiveness and eosinophilia, we demonstrated that *M. vaccae* downregulates allergen-specific IgE responses and the Th2 cytokines IL-4 and IL-5 in the lung as well. Although the first study describing beneficial effects of heat-killed *M. vaccae* demonstrated that 10^7 CFU *M. vaccae* downregulated the levels of IgE and the production of IL-5 by splenic cells (10), following studies, using 10^8 CFU *M. vaccae*, failed to do so (11, 21). It seems that a lower dose of *M. vaccae* (10^7 CFU) is more effective than a higher dose (10^8 CFU,) in lowering Th2 type responses. Interestingly, a low dose of *M. vaccae* (10^7 CFU) has been described to prime a Th1 response in the absence of a Th2 component, while a higher dose (10^8 and 10^9 CFU) induce a mixed Th1/Th2 response in the spleen of mice (22). The different cytokine secretion related to the *M. vaccae* immunizing dose may explain the observed effects on Th2 responses in our model.

We observed a decrease in the IL-4 and IL-5 response after *M. vaccae* treatment during sensitization, but interestingly, a decrease in IL-5 after treatment during challenge as well. This lowered Th2 response in both treatment regimes may provide a possible mechanism for the observed effects of *M. vaccae* on allergen induced eosinophilia and airway hyperresponsiveness, both downstream manifestations of asthma. Many subsequent studies in mice and man have confirmed the pivotal role of allergen-specific Th2 cells in the pathogenesis and maintenance of allergic asthma, their cytokines orchestrating the inflammatory immune response resulting in airway symptoms (reviewed in (23). Regardless of the downregulation of Th2 cytokines in the lavage fluid, we did not observe an upregulation of Th1 responses, as characterized by the levels of IFN-γ in the lung lavage fluid or the Th1-

related IgG2a antibodies in serum in our experiments. The beneficial effects of mycobacteria, such as BCG and *M. vaccae*, in treating allergic disease might be caused by their capacity to strongly induce Th1 type responses. Since both Th1 as Th2 cells cross-regulate each other's development and activity (24), the upregulation of Th1 responses by mycobacteria might have dampened the Th2 response. The lack of a Th1 response in our experiments suggests that mechanisms other than simple changes in the balance between Th1 and Th2 are responsible for the observed effects.

Another mechanism for the observed effects of *M. vaccae* comes from recent studies by Zuany-Amorim which showed that treatment with *M. vaccae* gives rise to induction of CD4^{+}CD45RB^{lo} T regulatory cells, which conferred protection against airway inflammation, probably mediated through IL-10 and TGF1-ß (21). Studies in mouse models confirmed a role for IL-10 in suppressing airway inflammation and cytokine production, although paradoxical effects on airway hyperresponsiveness have been found (25, 26). For that reason, we measured IL-10 in the lung lavage fluid after final challenge. We did not observe a significant change in the levels of IL-10 in the lung lavage fluid after *M. vaccae* treatment, either during sensitization or challenge. Therefore, the role of IL-10 in the observed effects of *M. vaccae* remains unclear in our model, although we cannot exclude a role of IL-10 in direct cell-cell interactions or temporal responses after *M. vaccae* treatment, since we measured cytokines in lavage fluid 24 hours after the final challenge.

Several other animal studies showed that mycobacteria are effective in lowering allergic and asthmatic responses (11-13, 27). The use of a heat-killed vaccine as used in our study is preferable above a viable preparation, in particular when converting to possible human use. Heat-killed *M. vaccae* has undergone extensive toxicological and safety assessment in humans, while BCG may exert side effects and poses a risk of bacterial disease. In addition, BCG immunization provides variable protection rates against tuberculosis (28), illustrating the incapacity of this agent to elicit a T cell-mediated response in a substantial proportion of the human population. In addition, the subcutaneous route of administration that we used in our experiments is strongly preferable above administration of mycobacteria directly at the site of allergen exposure, which is often used in animal experiments. Intranasal treatment with viable or heat-killed BCG most effectively lowered asthmatic responses when compared with systemic administration, but this was always accompanied by significant inflammation in the lung (12, 29).

In conclusion, as shown in our study, *M. vaccae* downregulates allergen-induced, Th2-mediated allergic and asthmatic responses during the development of allergic disease. Importantly, *M. vaccae* is able to lower these responses after the onset of allergic disease as well. Furthermore, *M. vaccae* must be administered during allergen exposure to be effective in our model. Therefore, the contradictive results in clinical trails using *M. vaccae* (30-32) may be due to the duration and level of exposure to allergens during the treatment period in these studies. New insights in the mechanism of action of *M. vaccae* need to be gained and possible genetic factors influencing *M. vaccae* treatment remain to be investigated.

Acknowledgement

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Influence of the macrophage bacterial resistance gene, *Nramp1* (*Slc11a1*), on the induction of allergic asthma in the mouse

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Abstract

Based on the hygiene hypothesis that lack of early childhood bacterial infections would favor development of allergic disease, we hypothesize that genes controlling antibacterial resistance may be important as well. We, therefore, studied whether *Nramp1* alleles, that determine resistance (*Nramp1^r*) or susceptibility (*Nramp1^s*) to intracellular bacteria at the macrophage level, affect sensitivity to induction of allergic asthma. *Nramp1^s* and congenic *Nramp1^r* mice were sensitized with ovalbumin/alum on days 0 and 14 and challenged with ovalbumin or saline aerosols on days 42, 45 and 48. On day 49, airway responsiveness was assessed, blood was withdrawn, and lung lavage was performed. We demonstrated that ovalbumin sensitization and challenge of *Nramp1^s* and *Nramp1^r* mice caused comparable airway hyperreactivity and airway eosinophilia and a similar increase in serum levels of ovalbumin-specific IgG1 and IgG2a. Ovalbumin challenge, however, induced significantly lower serum levels of total and ovalbumin-specific IgE and significantly lower mast cell degranulation in *Nramp1^r* mice as compared with *Nramp1^s* mice. In addition, ovalbumin challenge of *Nramp1^r* mice led to significantly less release of Th2 cytokines into the airways. Results show that *Nramp1* can affect the development of allergy but not the development of airway hyperresponsiveness in the mouse.

Introduction

Allergic asthma is a chronic inflammatory disorder, characterized by a variable degree of reversible airflow obstruction, bronchial hyperresponsiveness, and airway inflammation (1). The inflammation is considered crucial to development of the disorder and is mediated by a Th2 type immune response that results in allergen-specific IgE production. Re-encounter of the allergen then triggers mast cell degranulation, recruitment of eosinophils, and release of mediators by various cells (2). Allergy, as characterized by a Th2-mediated hypersensitivity reaction to common environmental proteins, is considered as a major risk factor for the development of allergic asthma in humans. However, non-specific airway hyperresponsiveness, a characteristic feature of asthma may develop independently of components of allergy such as IL-4, IL-5 and IgE, (3, 4). The prevalence of allergic asthma has risen steadily the last decades to 20-30% of the population of developed countries (5). The reason for this rise is not known, but reportedly may include changes in housing, in exposure to indoor and outdoor pollutants, and changes in the diet (6). Another explanation which has gained much attention is the "hygiene hypothesis". It states that lack of infections during early childhood would favor the development of IgE-mediated allergic diseases (7, 8). The lack of infections would lead to less frequent activation of Th1 cell responses and, therefore, facilitate development of Th2 cell type responses (5, 9). However, other reports contest the association between infections and allergic diseases, asthma included (10) or the explanation that reduced Th1-type responses lead to enhanced Th2-type responses (11, 12).

The hygiene hypothesis, being based on epidemiological studies, is supported by various experimental animal studies (13-15). Supporting evidence for the hygiene hypothesis in humans was provided by a retrospective study in Japan. Children that had been vaccinated against tuberculosis with bacille Calmette-Guérin (BCG) shortly after birth and responded with positive tuberculin reactions at 6 and 12 years of age, showed reduced incidences of asthma and other manifestations of IgE-mediated allergic disease, compared to vaccinated children with negative tuberculin reactions (16). This association between BCG vaccination and development of IgE-mediated allergic disorders remains controversial. Recent Swedish studies showed that vaccination of allergy prone children with BCG before 6 months of age did not change the development of allergic disease at 5 and 8 years of age (17, 18). The conflicting results of the Japanese and Swedish studies may be caused by the differences in experimental setup, but may also implicate that genetic factors, including those related to the capacity to respond to (mycobacterial) infections, are involved in the inverse relationship between allergy and infection.

In mice, the capacity of macrophages to respond to intracellular microorganisms, such as BCG (19), *Salmonella typhimurium* (20) and *Leishmania* sp. (21) is under control of the polymorphic natural-resistance-associated macrophage protein 1 gene (*Nramp1*, formerly designated *Ity/Lsh/Bcg* and now designated *Slc11a1*) (22-24). In humans, associations or linkage of *NRAMP1* with susceptibility to tuberculo-

sis, leprosy and a range of autoimmune diseases have been demonstrated as well (25, 26).

The murine polymorphism of *Nramp1* is apparent as either low (*Nramp1^s*) or high (*Nramp1^r*) resistance of macrophages to the growth of intracellular organisms. The increased capacity of *Nramp1^r* mice to limit the growth of these organisms as compared to *Nramp1^s* mice has been related to the observation that *Nramp1^r* macrophages display faster and superior activation in response to intracellular bacteria, bacterial products, and IFN-γ than *Nramp1^s* macrophages. This is indicated by the higher rate and degree of activation of the transcription factors NF-κB and STAT1 in *Nramp1^r* macrophages (22, 26), resulting in among others a higher production of bactericidal nitric oxide (25) and IFN-γ (27).

Whereas it is obvious that the rate and degree of macrophage activation play an important role in the non-specific resistance to microorganisms, the degree of macrophage activation has also been associated with the height of specific immune responses. Notably, experiments with high and low antibody responder Biozzi mice revealed a negative correlation between macrophage activity and the antibody response (reviewed in (28). Since *Nramp1* is one of the genes known to determine macrophage responsiveness to different stimuli, we hypothesize that *Nramp1* may influence the induction of allergic asthma as well. We, therefore, compared *Nramp1* congenic BALB/c mice as to their ability to develop allergic asthma-like disease, using ovalbumin as the allergen.

Materials and methods

Animals

Breeding pairs of wild type BALB/cAnPt (*Nramp1^s*) and congenic C.D2-*Vil6* (*Nramp1^r*) mice were kindly provided by Dr. M. Potter (National Cancer Institute, Bethesda, MD, USA). The C.D2-*Vil6* mouse was originally derived from crossing a BALB/cAnPt and a DBA/2NPt (*Nramp1^r*) mouse which was crossed back to BALB/cAnPt for 23 times (29). Specified pathogen free (SPF) mice were obtained by performing Caesarian sections and further breeding and maintenance under SPF conditions in macrolon cages in the animal facilities of the National Institute for Public Health and the Environment (Bilthoven, The Netherlands). Mice were provided with food and water *ad libitum* and used when 5-6 weeks of age. The *Nramp1* status of the mice was regularly checked using PCR technique as described before (30). The experiments were approved by the animal ethics committee of the National Institute of Public Health and the Environment.

Reagents

Diff-Quick staining solutions were purchased from Dade A.G. (Düdingen, Switzerland). Ovalbumin (grade V), acetyl-ß-methylcholine chloride (methacholine), BSA, *o*-phenylenediamine-dichloride substrate (OPD), LPS and PMA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum hydroxide (AlumImject) was obtained from Pierce (Rockford, IL, USA). Digoxigenin (DIG), anti-DIG-Fab fragments

coupled to horse radish peroxidase and protease inhibitor were purchased from Roche Diagnostics Corp. (Basel, Switzerland) and anti-mouse IgE, biotinylated anti-mouse IgG1, biotinylated anti-mouse IgG2a and anti-CD40 was purchased from Pharmingen (San Diego, CA, USA). Mouse IgE reference serum was purchased from ICN Pharmaceuticals (Cosa Mesa, CA, USA). Peroxidase-conjugated streptavidin (Poly HRP) was purchased from the CLB (Amsterdam, The Netherlands).

ELISA buffer contained 0.5% BSA, 2 nM EDTA, 136.9 nM NaCl, 50 nM Tris and 0.05% Tween-20. K-medium consisted of RPMI 1640 medium supplemented with 10% FCS, 4 mM L-glutamine, 5x 10^{-5} M 2-ME, 1 mM sodium pyruvate, 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 0.1 mM nonessential amino acids, all obtained from Life Technologies (Breda, The Netherlands). Bone marrow-derived mast cells culture medium consisted of K-medium containing 5% FCS, 20% pokeweed mitogen-stimulated spleen-conditioned medium (Life Technologies) and 1 ng/ml of rIL-3 (Peprotech, London, England). IFN-γ and IL-9 were purchased from Genentech (San Fransisco, CA, USA). Oligo dT 12-18 and dNTPs were purchased from Pharmacia (North Peapack, NJ, USA) and first strand buffer, DTT, M-MLV Reverse Transcriptase, PCR buffer and Taq DNA polymerase were obtained from Life Technologies.

Sensitization and challenge

All mice were sensitized by two i.p injections of 10 µg ovalbumin adsorbed onto 2.25 mg aluminum hydroxide in 100 µl saline on days 0 and 14. On days 42, 45 and 48, mice were challenged by inhalation of either ovalbumin or saline aerosols in a Plexiglas exposure chamber for 20 minutes. The aerosols were generated by nebulizing an ovalbumin solution (10 mg/ml) in saline or saline alone using a Pari LC Star nebulizer (Pari Respiratory Equipment, Richmond, VA, USA)

Determination of airway responsiveness

Airway responsiveness to inhaled nebulized methacholine was determined 24 hours after the final challenge, in conscious, unrestrained mice using whole body plethysmography (Buxco corp., Sharon, CT, USA). The airway response was expressed as enhanced pause (Penh) as described previously (31, 32). Briefly, animals were placed in a whole body chamber to record differences in pressure between this chamber and a reference chamber for calculation of Penh values. After assessment of baseline Penh values for 3 minutes, mice were subsequently subjected to aerosols of saline and increasing concentrations of methacholine (3.13, 12.5, 25, and 50 mg/ml saline) for 3 minutes. Aerosols were generated by a Pari LC Star nebulizer and each aerosol was followed by 3 minutes of recording to assess the average Penh value from 10 or 5 valid breaths.

Bronchoalveolar lavage

After measurement of cholinergic airway responses, animals were sacrificed and bronchoalveolar lavage was performed. For this purpose, lungs of mice were lavaged once with 1 ml PBS at 37°C containing 5% BSA and protease inhibitor and 4 times

with 1 ml saline at 37°C. Lung lavage cells of each mouse were collected after cen-trifugation, pooled and resuspended in 150 µl saline. Total numbers of cells were determined using a Bürker-Türk chamber (Omnilabo, Breda, The Netherlands). For differential cell counts, cytospin preparations were made and stained with Diff-Quick. Cells were differentiated into mononuclear phagocytes, eosinophils, lymphocytes and neutrophils by standard morphology. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated. The super-natant of the first lavage was separated and frozen at -70°C until further analysis.

Determination of total and ovalbumin-specific immunoglobulins
Blood was withdrawn by heart puncture 24 hours after the last allergen challenge to prepare serum for determination of antibody levels in serum by ELISA's using microtiter plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% Tween-20 for washing between incubations. To determine IgE levels, wells were coated overnight at 4°C with 1 µg/ml of anti-mouse IgE in PBS, followed by blocking for 1 hour. For measur-ing ovalbumin-specific IgE, diluted serum samples and duplicate dilution series of an ovalbumin-specific IgE reference serum, prepared as described previously (33) were added and wells were incubated for 2 hours. Hereafter, wells were incubated for 1 hour with 1 µg/ml of DIG-conjugated ovalbumin followed by incubation with anti-DIG-Fab fragments coupled to horse radish peroxidase, according to manufacturer's instructions. For determination of the total IgE levels, the wells were incubated with diluted serum samples and duplicate dilution series of an IgE reference serum for 2 hours. Hereafter, wells were incubated with 1 µg/ml of biotinylated anti-mouse IgE for 1.5 hours, followed by 1:10,000 diluted peroxi-dase-conjugated streptavidin.

To assess ovalbumin-specific IgG1 or IgG2a levels, wells were coated with 10 µg/ml ovalbumin in PBS. After blocking, diluted serum samples or duplicate dilu-tion series of a reference standard serum obtained from multiply ovalbumin-boosted mice were added. Hereafter, wells were incubated with 1 µg/ml of biotinylated anti-mouse IgG1, or 1 µg/ml of biotinylated anti-mouse IgG2a for 2 hours, followed by 1:10,000 diluted Poly-HRP for 1 hour.

For color development, 0.4 mg/ml of OPD and 4 mM H_2O_2 in PBS was used and the reaction was stopped by adding 4 M H_2SO_4. OD was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

Determination of IL-4, IL-5, IL-10, IL-13 and MMCP-1 in lung lavage fluid
Levels of IL-4, IL-5, IL-10 and IL-13 in the lung lavage fluid were analyzed by sand-wich ELISA using antibody pairs and standards purchased from Pharmingen, ac-cording to manufacturer's instructions. A commercially available ELISA kit was used to assess levels of mucosal mast cell protease-1 (MMCP-1, Moredun Scientific Ltd. Midlothian, UK).

Cell culture and stimuli

Primary cells and cell lines were cultured in K-medium and stimulated with different agents in 24-well plates or culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands), unless specified otherwise:

1. Unstimulated C10 mouse epithelial cells (CMRL 1066, Maastricht University, The Netherlands). 2. RAW 264.7 mouse (BALB/c) macrophages (ATCC, Manassas, VA, USA) stimulated with IFN-γ (50 U/ml) and LPS (10 µg/ml) for 3 hours. 3. J774 A.1 mouse macrophages (ATCC) stimulated with IFN-γ (50 U/ml) and LPS (10 µg/ml) for 3 hours. 4. A20 mouse B cells (ATCC) cultured in K-medium with 5% FCS and stimulated with anti-CD40 (1 µg/ml) for 3 hours. 5. Unstimulated bone marrow-derived mast cells (BMMC's) which were obtained from femurs of *Nramp1*[s] BALB/c mice and cultured in BMMC medium, as described before (34). 6. CFTL-12 mouse mast cells (J. Pierce, NIH, Bethesda, MD, USA), cultured in K-medium with 5% FCS and rIL-3 (1 ng/ml) and stimulated with PMA (10 ng/ml) for 3 hours. 7. 3DO mouse T cells (ATCC) stimulated with IL-9 (1 ng/µl) for 10 days. 8. BMMC's cultured in BMMC medium, stimulated with PMA (10 ng/ml) for 3 hours.

Nramp1 PCR analysis

Total RNA was extracted from the cells with TRIzol reagent according to manufacturer's instructions (Life Technologies). Hereafter, reverse transcription was performed by incubating 1 µg total RNA for 1 hour at 42 °C in the presence of 500 µg/ml of Oligo dT 12-18, 10 mM dNTPs, 5x first strand buffer, 0.1 M DTT and 200 U/ml M-MLV Reverse Transcriptase. Amplification of the resulting DNA was performed in 30 µl PCR buffer with 5 U/µl Taq DNA polymerase and *Nramp1* specific PCR primers (sense, 5'-GCACCTCAGCCAGCGCTTTG-3'; antisense, 5'-TGCCCACAGCCCGGACAGGT -3') for 30 seconds at 94 °C, 30 seconds at 65 °C, and 1 minutes at 72 °C for 35 cycles. The PCR products were separated on 1.5% agarose gels (ICN), stained with 0.5 µg/ml ethidium bromide and visualized under UV light using a Gel-Doc system (Bio-Rad).

Statistical Analysis

All data are expressed as mean ± SEM. The airway response curves to methacholine were statistically analyzed by a general linear model of repeated measurements followed by post-hoc comparison between groups. Data were log10 transformed before analysis to equalize variances in all groups. Cell counts were statistically analyzed using the Mann-Whitney U test. All other analyses were performed using Student's *t* test. A probability value $p < 0.05$ was considered significant.

Results

Airway response to methacholine

Airway responses of ovalbumin-sensitized mice to increasing concentrations of methacholine 24 hours after final ovalbumin or saline challenge were measured

and expressed as Penh (Fig. 1). Saline-challenged *Nramp1*[s] and *Nramp1*[r] control mice showed similar (*p* > 0.10) shallow increases in Penh in response to increasing doses of aerosolized methacholine. The dose response curves to methacholine of ovalbumin-challenged mice were significantly (*p* < 0.05) higher than those of the saline-challenged animals. However, no difference in hyperresponsiveness to methacholine was observed between ovalbumin-challenged *Nramp1*[s] and *Nramp1*[r] mice.
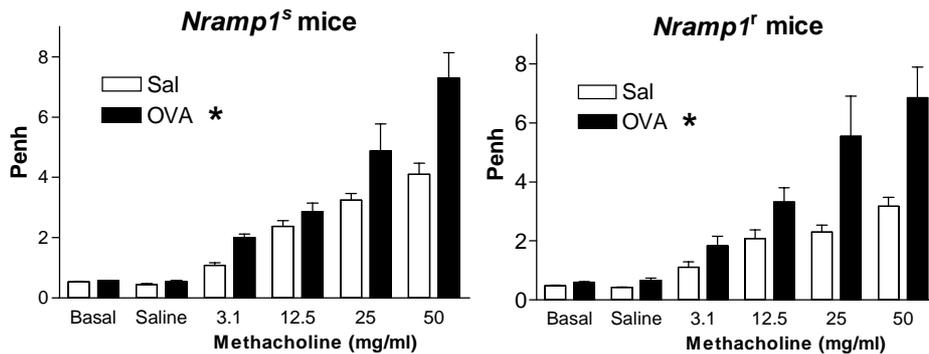

*Figure 1. Airway response to increasing concentrations of nebulized methacholine of ovalbumin-sensitized Nramp1[s] or Nramp1[r] mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Unrestrained plethysmography measurements were performed for 3 minutes after each exposure to methacholine and expressed as Penh-values. Data are represented as mean ± SEM, n=8. * p < 0.05 compared to the saline-challenged control groups.*

Bronchoalveolar lavage cell counts

Cell counts in the lung lavage fluid obtained 24 hours after final challenge showed that ovalbumin challenges induced a significant (*p* < 0.01 in both strains) increase in the total number of cells in the lavage fluid compared to saline challenge (Fig. 2). This increase in total number was mainly caused by an increase in the number of eosinophils. No significant differences in eosinophil numbers were observed between ovalbumin-challenged *Nramp1*[s] and *Nramp1*[r] mice. In addition, no significant differences were observed in number of lymphocytes and neutrophils between the different groups.

*Figure 2. Number of cells in lung lavage fluid obtained from ovalbumin-sensitized Nramp1[s] or Nramp1[r] mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Cells were isolated by lung lavage and differentiated in monocytes, lymphocytes, neutrophils and eosinophils. Data are represented as mean cell number ± SEM, n=8. * p < 0.01 compared to the saline-challenged control groups.*

Serum levels of immunoglobulins

The serum antibody levels before challenge (data not shown) and after saline-challenge were similar in both strains (Fig. 3). Ovalbumin challenge of sensitized mice induced a comparable increase in the levels of ovalbumin-specific IgG1 and IgG2a in both strains compared to the saline-challenged animals (Fig. 3A, B). In addition, ovalbumin challenge of *Nramp1^s* mice markedly increased serum levels of total and ovalbumin-specific IgE (Fig. 3C, D) compared to the saline-challenged animals. Interestingly, the *Nramp1^r* mice showed a considerably lower IgE response upon ovalbumin challenge.
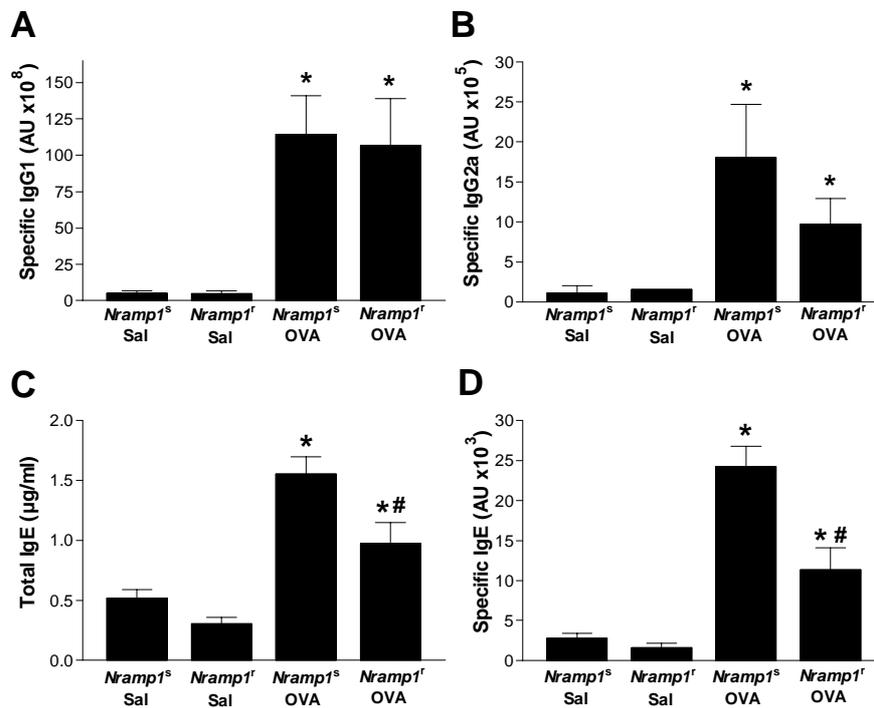
Figure 3. *Ovalbumin-specific IgG1 (A), IgG2a (B), and total (C) and ovalbumin-specific (D) IgE levels measured by ELISA in serum of ovalbumin-sensitized Nramp1^s or Nramp1^r) mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Vertical bars indicate mean Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control groups.*

Cytokines in lung lavage fluid

Lung lavage fluid of ovalbumin-sensitized mice contained no detectable levels of IL-4, IL-5 and IL-13 and low levels of IL-10 after saline challenge (Fig. 4). Ovalbumin challenge caused a significant increase in the levels of these cytokines in both strains as compared to the saline-challenged controls. Lung lavage fluid of *Nramp1^r* mice, however, contained half to one third the amount of cytokines as found in lavage fluid of *Nramp1^s* mice. IFN-γ was not detectable in the lavage fluid, irrespective of treatment and mouse strain (detection limit ELISA: 10 pg/ml, data not shown).

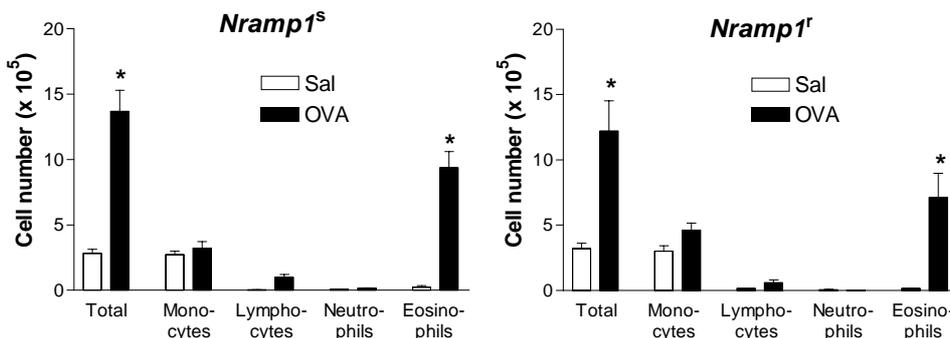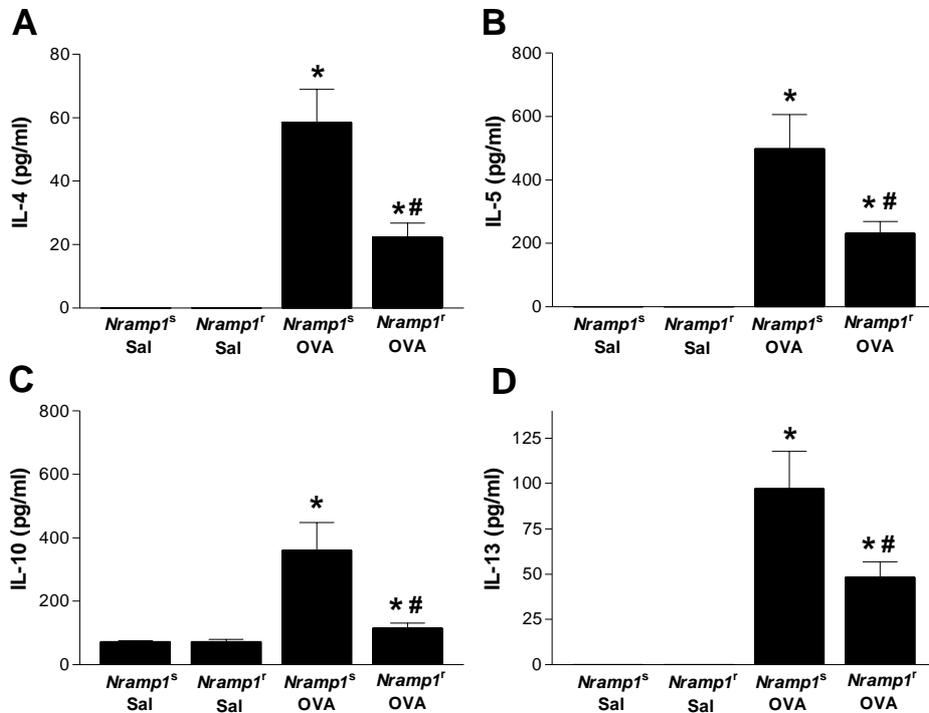**Figure 4.** *Levels of IL-4 (A), IL-5 (B), IL-10 (C) and IL-13 (D) measured by ELISA in lung lave fluid obtained from ovalbumin-sensitized Nramp1^s or Nramp1^r mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Data are represented as mean pg/ml ± SEM, n=8. * p < 0.01 compared to the saline-challenged control groups. # p <0.05 compared to the ovalbumin-challenged Nramp1^s group.*

In vivo mast cell degranulation: MMCP-1 in lung lavage fluid

Ovalbumin challenge significantly increased levels of MMCP-1, a measure of the *in vivo* mast cell degranulation, in the lung lavage fluid, as compared to challenge with saline (Fig. 5). This increase was markedly less pronounced in the *Nramp1^r* mice.

Figure 5. *Levels of MMCP-1 measured by ELISA in lung lavage fluid obtained from ovalbumin-sensitized Nramp1^s or Nramp1^r mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Data are represented as mean ng/ml ± SEM, n=8. * p < 0.01 compared to the saline-challenged control groups. # p <0.05 compared to the ovalbumin-challenged Nramp1^s group.*
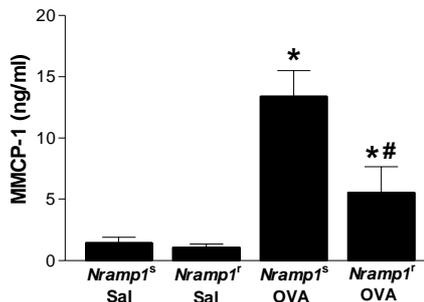
***Nramp1* gene expression** Total RNA of different cells and cell lines was analyzed by RT-PCR to assess *Nramp1* expression *in vitro* (Fig. 6). RAW 264.7 and J774 A.1 macrophages stimulated with IFN-γ and LPS showed marked *Nramp1* RNA expres-

sion. Unstimulated and PMA-stimulated bone marrow-derived mast cells (BMMC), PMA-stimulated CFTL-12 mast cells, unstimulated epithelial cells, anti-CD40-stimulated B cells and IL-9-stimulated T cells did not express *Nramp1* RNA.

Figure 6. RT-PCR analysis of Nramp1 mRNA in different cell cultures. Cells were cultured in the absence or presence of stimuli (see below) and total RNA was collected and analyzed by RT-PCR using primers specific for Nramp1. The resulting products were separated by agar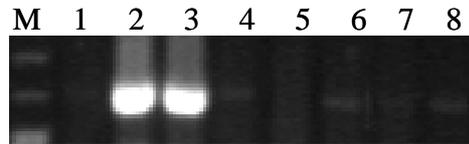ose gel electrophoresis, stained with ethidium bromide and visualized using the Geldoc system. Lanes: M. Marker (700, 600, 500 bp); 1. Unstimulated C10 epithelial cells; 2. RAW 264.7 macrophages stimulated with IFN-γ and LPS; 3. J774 A.1 macrophages stimulated with IFN-γ and LPS; 4. A20 B cells stimulated with CD40; 5. Unstimulated bone marrow-derived mast cells (BMMC); 6. CFTL-12 mast cells stimulated with PMA; 7. 3DO T cells stimulated with IL-9; 8. BMMC's stimulated with PMA.

Discussion

In the present study, we investigated whether the *Nramp1* gene influences sensitivity to induction of allergic asthma-like disease in mice. We demonstrated that ovalbumin sensitization and challenge of *Nramp1*s and *Nramp1*r mice caused comparable cholinergic airway hyperreactivity and airway eosinophilia and a similar increase in serum levels of ovalbumin-specific IgG1 and IgG2a. Ovalbumin challenge, however, induced markedly lower serum levels of total and ovalbumin-specific IgE in *Nramp1*r mice and markedly less mast cell degranulation in their airways as compared with *Nramp1*s mice. In addition, ovalbumin challenge of *Nramp1*r mice led to significantly less release of IL-4, IL-5, IL-10 and IL-13 into the airways than similar treatment of *Nramp1*s mice.

The lower IgE response in the *Nramp1*r mice in the absence of differences in the specific IgG1 and IgG2a responses is in line with experiments by Soo and co-workers (27). Using *Nramp1* congenic B10 mice, they demonstrated that *Nramp1*r mice mounted a lower IgE response than *Nramp1*s mice after infection with an attenuated strain of *Salmonella typhimurium*, but similar responses of total immunoglobulins (IgG1, IgG2a and IgM). Thus, the antibody response to a soluble protein antigen, like ovalbumin, and to viable salmonella bacteria is apparently similarly influenced by *Nramp1*, although the role of macrophages in both responses is likely to be different.

How *Nramp1* determines the height of the specific IgE response is not known, but macrophage activity as controlled by *Nramp1* may play a role. Airway macrophages of ovalbumin-sensitized BALB/c mice reportedly are activated upon respiratory ovalbumin challenge (35, 36), while the degree of macrophage activity is known to control the height of antibody responses (28). Notably, macrophages of

Biozzi high antibody responder mice were found to display less catabolic activity, antigen degradation, and bactericidal activity than macrophages of Biozzi low antibody responder mice (28). Likewise, *Nramp1*ʳ macrophages on B10 or BALB/c background were shown to display faster and/or more activation in reaction to various stimuli than *Nramp1*ˢ macrophages (37-39). Hence, the lower IgE production by the *Nramp1*ʳ strain as compared with the *Nramp1*ˢ strain may be related to more prominent pulmonary macrophage activation, including more NO· production, upon ovalbumin challenge of the *Nramp1*ˢ strain.

Elevated NO· production may be relevant, because NO· was found to be the mediator by which alveolar macrophages downregulate the presentation of protein antigens by pulmonary dendritic cells to primed rat T cells in vitro (40). Moreover, NO· was shown to be a potent inhibitor of T cell proliferation, irrespective of the cytokine profile (41). So, higher NO· production by sensitized *Nramp1*ʳ mice after ovalbumin challenge is likely to result in inhibition of T cell restimulation in these mice. This notion is compatible with our observation that the *Nramp1*ʳ mice had lower IL-4, IL-5, IL-10, and IL-13 levels in the lung lavage fluid after respiratory ovalbumin challenge than the *Nramp1*ˢ mice. The decreased IL-4 and IL-13 production in its turn conceivably would have led to lower production of IgE (42), as actually observed. However, why ovalbumin-specific serum levels of IgG1 were similar to those of *Nramp1*ˢ mice is unknown, since IgG1 production, like IgE production by ovalbumin/alum sensitized BALB/c mice, was found to be highly dependent on IL-4 (43).

Another issue is that the significantly lower IL-5 level in the airways of the *Nramp1*ʳ mice as compared with the *Nramp1*ˢ mice was not paralleled by reduced numbers of eosinophils. This may be explained by the significantly lower IL-10 level in the airways of *Nramp1*ʳ mice, since IL-10 is a potent inhibitor of allergen challenge-induced eosinophilic inflammation in the airways of BALB/c mice (44). Besides having anti-inflammatory activity, IL-10 was reported to increase cholinergic responsiveness of allergen-sensitized and -challenged BALB/c mice (44). We, however, observed the same degree of cholinergic responsiveness in both strains, although *Nramp1*ʳ mice produced significantly less IL-10 in the airways than the *Nramp1*ˢ mice. In addition, although IL-13 may be closely related to airway hyperresponsiveness (45), the significantly lower IL-13 levels in the airways of the *Nramp1*ʳ mice as compared with the *Nramp1*ˢ mice were not paralleled by a decrease in airway hyperresponsiveness. Data stress the complexity of development of nonspecific airway hyperresponsiveness and confirm that there are various mechanisms leading to this phenomenon (46).

Besides reduced IgE production, *Nramp1*ʳ mice displayed reduced mast cell degranulation. The reduced serum IgE levels, conceivably, may have led to reduced mast cell-bound IgE in the lungs and hence to less allergen-induced IgE cross-linking and degranulation. Moreover, circulating IgE was found to increase FceRI expression by mast cells in mice and so the sensitivity to and intensity of allergen-induced mast

cell degranulation in a concentration dependent way (47, 48). Furthermore, IL-4 and IL-10 were shown to be able to promote the proliferation of mast cells and allergen-induced degranulation of these cells (49-51). So, the reduced levels of IL-4 and IL-10 in the airways of *Nramp1*[r] mice may have led to the reduced mast cell degranulation as well. Decreased mast cell degranulation in *Nramp1*[r] mice may also be explained by the presumed increased NO· production by *Nramp1*[r] macrophages in response to ovalbumin challenge as stated above, since NO· was found to cause a concentration dependent inhibition of mast cell degranulation *in vitro* as well as *in vivo* (52, 53). Finally, since mast cells belong to the myeloid lineage and *Nramp1* is almost exclusively expressed by myeloid cells (54), it is possible that *Nramp1* is expressed in mast cells and, therefore, able to affect mast cell degranulation directly. PCR analysis, however, showed that *Nramp1* RNA is not expressed in stimulated cultures of mast cells. So, a direct effect of *Nramp1* on mast cell degranulation is unlikely.

We conclude that *Nramp1* can affect the development of allergy, but not the development of airway hyperresponsivess, an untoward symptom of asthma, in the mouse. Allergy is considered a risk factor for the development of asthma in humans. In our mouse model, components of allergy in *Nramp1*[r] were lower, including levels of IgE and IL-4 and IL-13. Eosinophilic inflammation, which has been related to airway hyperresponsiveness (55), however, was not affected by *Nramp1*, which might explain the lack of effect of *Nramp1* on development of airway hyperresponsiveness. Effects of *Nramp1* on macrophage function probably mediate its selective effects on Th2-mediated immunity. Since *Nramp1* controls the non-specific defense of macrophages against intracellular microorganisms, it is likely to affect the effects of intracellular bacteria on development of allergic diseases. This is subject of current studies.

Acknowledgement

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Chapter 6

The *Nramp1* (*Slc11a1*) gene controls the efficacy of *M. vaccae* treatment of allergic asthma in the mouse

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Abstract

Genes controlling antibacterial resistance may be important in the "Hygiene Hypothesis", which states that lack of bacterial infections during childhood would favor development of allergic disease. We, therefore, studied in a mouse model whether *Nramp1* (*Slc11a1*) alleles, that determine susceptibility (*Nramp1*^{s}) or resistance (*Nramp1*^{r}) to intracellular bacteria, affect the efficacy of heat-killed *Mycobacterium vaccae* in the treatment of allergic asthma. *Nramp1*^{s} and *Nramp1*^{r} mice were ovalbumin-sensitized and received an injection with 10^7 CFU heat-killed *M. vaccae* during sensitization. Treatment of ovalbumin-sensitized *Nramp1*^{s} mice with *M. vaccae* suppressed airway hyperresponsiveness, airway eosinophilia, antigen-specific IgE, and IL-4 and IL-5 production after ovalbumin aerosol challenge. In contrast, *M. vaccae* hardly affected these parameters in *Nramp1*^{r} mice. In addition, *Nramp1* clearly affected delayed-type hypersensitivity, and therefore T cell-mediated, responses to heat-killed *M. vaccae*. The influence of *Nramp1* on the efficacy of *M. vaccae* probably originates at the macrophage level since *Nramp1*^{s} macrophages are much less activated after *M. vaccae* stimulation *in vitro* when compared with *Nramp1*^{r} macrophages. In conclusion, heat-killed *M. vaccae* prevents allergic and asthmatic manifestations in a mouse model. However, the efficacy of *M. vaccae* is strongly affected by *Nramp1* alleles, which may have implications for further human application of *M. vaccae*.

Introduction

The prevalence of allergic asthma among children has risen drastically during the last decades, especially in western societies. An attractive explanation for this rise is offered by the "hygiene hypothesis" (1-3) which states that a lower level of infections during childhood could cause the increased prevalence of allergic diseases. One mechanism for this inverse correlation between allergic disease and infection is provided by the balance of Th1 and Th2 immune mechanisms. Since Th1 and Th2 responses are to a certain degree antagonistic, it is possible that certain infections that predominantly evoke Th1 responses might limit allergic Th2 responses (4, 5). In contrast, a massive Th2 shift as seen in helminth infections is associated with protection against the development of allergy and asthma (6). Additionally, the incidence of Th1-mediated autoimmune diseases is increasing as well (7). This suggests that both allergic and autoimmune diseases are a result of disordered immunoregulation instead of a misbalance in Th1 or Th2. Moreover, a common immunological and environmental variable probably causes the recent increase in these disorders (8, 9).

One such environmental variable might be mycobacterial exposure. Several animal studies showed that vaccination with mycobacteria prevented or treated allergic and asthmatic manifestations (10-13). Further support came initially from epidemiological studies which demonstrated that children that had been vaccinated with bacille Calmette-Guérin (BCG) shortly after birth and responded with positive tuberculin reactions at 6 and 12 years of age, exhibited reduced incidences of allergic symptoms and asthma compared to vaccinated children with negative tuberculin reactions (14). In contrast, several retrospective human studies hereafter failed to demonstrate a negative correlation between BCG vaccination during early childhood and the subsequent development of atopy or asthma (reviewed in (15). Factors causing this observed discrepancy may be the age at and frequency of vaccination with BCG, the BCG strain used, and the varying natural exposure to mycobacteria (including *M. tuberculosis*). More importantly, since the ability to mount a response to mycobacterial antigens is highly heritable (16), a genetic contribution to the inverse relationship between mycobacterial infection and the development of allergy and asthma is very plausible.

Interestingly, the immune response to intracellular bacteria, including mycobacteria, is under control of the *natural-resistance-associated macrophage protein 1* gene (*Nramp1*, recently designated *Slc11a1*) (17-19). In humans, association or linkage of *NRAMP1* with susceptibility to infectious diseases, but also atopy and autoimmune disease, has been demonstrated (20, 21). The murine polymorphism of *Nramp1* is apparent as either low (*Nramp1*[s]) or high (*Nramp1*[r]) resistance of macrophages to the growth of intracellular organisms. This has been related to the observation that *Nramp1*[r] macrophages display faster and superior activation in response to intracellular bacteria, bacterial products, and IFN-γ than *Nramp1*[s] macrophages (22, 23).

We previously demonstrated that heat-killed *M. vaccae* is able to prevent and suppress features of allergy and asthma in a mouse model (J.J.Smit, submitted). Since *Nramp1* controls the immune response to intracellular microorganisms such as *M. vaccae*, it is likely to affect the efficacy of *M. vaccae* in treatment of allergic disease. Therefore, in this study, we investigated the influence of *Nramp1* on the prevention of allergic asthma by heat-killed *M. vaccae* (SRL172) in a mouse model.

Materials and methods

Animals
Breeding pairs of wild type BALB/cAnPt (*Nramp1*s) and congenic C.D2-*Vil6* (*Nramp1*r) mice were kindly provided by Dr. M. Potter (National Cancer Institute, Bethesda, MD, USA). The C.D2-*Vil6* mouse was originally derived from crossing a BALB/cAnPt and a DBA/2NPt (*Nramp1*r) mouse which was crossed back to BALB/cAnPt for 23 times (24). Animals were maintained under SPF conditions in the animal facilities of the National Institute for Public Health and the Environment (Bilthoven, The Netherlands), provided with food and water *ad libitum* and used when 5-6 weeks of age. The *Nramp1* status of the mice was regularly checked using PCR technique as described before (25). The experiments were approved by the animal ethics committee of the National Institute of Public Health and the Environment.

Reagents
Vials of heat-killed *Mycobacterium vaccae* (SRL172, 10 mg or 10^{10} CFU/ml) were kindly provided by SR Pharma Ltd. (London, UK). Diff-Quick staining solutions were purchased from Dade A.G. (Düdingen, Switzerland). Ovalbumin (grade V), acetyl-ß-methylcholine chloride (methacholine), BSA, *o*-phenylenediamine-dichloride substrate (OPD), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum hydroxide (AlumImject) was obtained from Pierce (Rockford, IL, USA). Digoxigenin (DIG), anti-DIG-Fab fragments coupled to horse radish peroxidase and protease inhibitor were purchased from Roche Diagnostics Corp. (Basel, Switzerland). Anti-mouse IgE, biotinylated anti-mouse IgG1 and biotinylated anti-mouse IgG2a were obtained from Pharmingen (San Diego, CA, USA). Peroxidase-conjugated streptavidin (Poly HRP) was purchased from the CLB (Amsterdam, The Netherlands). ELISA buffer contained 0.5% BSA, 2 nM EDTA, 136.9 nM NaCl, 50 nM Tris and 0.05% Tween-20. DMEM medium was supplemented with 10% FCS, 4 mM L-glutamine, $5x 10^{-5}$ M 2-ME, 1 mM sodium pyruvate, 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 0.1 mM nonessential amino acids, all obtained from Life Technologies (Breda, The Netherlands.

Sensitization and challenge
All mice were sensitized by two i.p injections of 10 µg ovalbumin adsorbed onto 2.25 mg aluminum hydroxide in 100 µl saline on days 0 and 14. On days 35, 39 and 42, mice were challenged by inhalation of either ovalbumin or saline aerosols in a Plexiglas

exposure chamber for 20 min. The aerosols were generated by nebulizing an ovalbumin solution (10 mg/ml) in saline or saline alone using a Pari LC Star nebulizer (Pari Respiratory Equipment, Richmond, VA, USA) driven by compressed air at a flow rate of 6 l/min. *M. vaccae* treated mice received a subcutaneous injection with 10^7 CFU (0.01 mg) heat-killed *M. vaccae* in 100 µl saline on days 0 and 14, immediately before the i.p. ovalbumin/Alum injection.

Determination of airway responsiveness
Airway responsiveness to inhaled nebulized methacholine was determined 24 h after the final challenge, in conscious, unrestrained mice using whole body plethysmography (Buxco corp., Sharon, CT, USA). The airway response was expressed as enhanced pause (Penh) as described previously (26, 27). Briefly, animals were placed in a whole body chamber to record differences in pressure between this chamber and a reference chamber for calculation of Penh values. After assessment of baseline Penh values for 3 minutes, mice were subsequently subjected to aerosols of saline and increasing concentrations of methacholine (3.13, 12.5, 25, and 50 mg/ml saline) for 3 minutes. Aerosols were generated by a Pari LC Star nebulizer and each aerosol was followed by 3 minutes of recording to assess the average Penh value from 10 or 5 valid breaths.

Bronchoalveolar lavage
After measurement of cholinergic airway responses, animals were sacrificed and bronchoalveolar lavage was performed. For this purpose, lungs of mice were lavaged once with 1 ml PBS at 37°C containing 5% BSA and protease inhibitor and 4 times with 1 ml saline at 37°C. Lung lavage cells of each mouse were collected after centrifugation, pooled and resuspended in 150 µl saline. Total numbers of cells were determined using a Bürker-Türk chamber (Omnilabo, Breda, The Netherlands). For differential cell counts, cytospin preparations were made and stained with Diff-Quick. Cells were differentiated into monocytes, eosinophils, lymphocytes and neutrophils by standard morphology. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated. The supernatant of the first lavage was separated and frozen at -70°C until further analysis.

Determination of serum levels of ovalbumin-specific immunoglobulins
Blood was withdrawn by heart puncture 24 hours after the last allergen challenge to prepare serum for determination of antibody levels in serum by ELISA's using microtiter plates (Greiner Bio-One, Alphen a/d Rijn), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% Tween-20 for washing between incubations. To determine ovalbumin-specific IgE levels, wells were coated overnight at 4°C with 1 µg/ml of anti-mouse IgE in PBS, followed by blocking for 1 hour and incubation of the wells with diluted serum samples and duplicate dilution series of an ovalbumin-specific IgE reference serum, prepared as described previously (28) for 2 hours. Hereafter, wells were incubated for 1 hour with 1 µg/ml of DIG-conjugated ovalbumin

followed by incubation with anti-DIG-Fab fragments coupled to horse radish peroxidase, according to manufacturer's instructions.

To assess ovalbumin-specific IgG1 or IgG2a levels, wells were coated with 10 µg/ml ovalbumin in PBS. After blocking, diluted serum samples or duplicate dilution series of a reference standard serum obtained from multiply ovalbumin-boosted mice were added. Hereafter, wells were incubated with 1 µg/ml of biotinylated anti-mouse IgG1, or 1 µg/ml of biotinylated anti-mouse IgG2a for 2 hours, followed by 1:10,000 diluted Poly-HRP for 1 hour.

For color development, 0.4 mg/ml of OPD and 4 mM H_2O_2 in PBS was used and the reaction was stopped by adding 4 M H_2SO_4. OD was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

Determination of IL-4, IL-5, IL-10 and TGF1-ß in lung lavage fluid

Levels of IL-4, IL-5 and IL-10 in the lung lavage fluid were analyzed by sandwich ELISA using antibody pairs and standards purchased from Pharmingen, according to manufacturer's instructions. Levels of TGF1-ß were analyzed using an ELISA kit (Biosource, Etten-Leur, the Netherlands), according to the instructions of the manufacturer. The detection limit of the ELISA for IL-4, IL-5, IL-10 and TGF1-ß was 8, 32, 10 and 60 pg/ml, respectively.

Measurement of Delayed-Type Hypersensitivity (DTH)

For measurement of delayed-type hypersensitivity (DTH) to heat-killed *M. vaccae*, mice were injected subcutaneously in the neck with 10^7 or 10^8 CFU (0.01 or 0.1 mg) heat-killed *M. vaccae* in 100 µl saline or saline alone on day 0 and 7. On day 21, mice were anaesthetized by an intramuscular injection of a mixture of xylazin and ketamin and ear thickness of both ears was measured using a spring loaded caliper (No. 293-561, Mitutoyo, Veenendaal, The Netherlands). Accordingly, all mice were challenged intradermally with 15 µg heat-killed *M. vaccae* in 20 µl saline in the left ear and saline alone in the right ear. Ear thickness of both ears was measured after anesthesia as described above, 24, 48, 96 and 216 hours after ear challenge. The ear swelling was defined as the difference in thickness between the left and right ear.

Culture and stimulation of murine macrophages

B10S and B10R cell lines (kindly donated by Danuta Radzioch, McGill University, Montreal, Canada) are bone marrow-derived macrophage lines obtained from $Nramp1^s$ and $Nramp1^r$ congenic mice, respectively (29). These cells were cultured in DMEM, washed, and plated at 5×10^5 cells/well in a 24-well plate (Costar, Cambridge, MA, USA). Cells were incubated with medium alone or medium containing 10^7, 10^8 or 2.5 $\times 10^8$ CFU/ml *M. vaccae* for 48 hours at 37°C with 5% CO_2. After stimulation, the supernatant of the different cultures was collected and frozen at -20°C until use.

Determination of TNF-α and nitrite in culture supernatants

TNF-α in the cell culture supernatant 48 hours after stimulation was determined using a commercially available ELISA kit (Biosource), according to the instructions of the manufacturers. Nitrite (NO_2^-) concentrations were measured in microtiter plates (Greiner Bio-One, Alphen a/d Rijn) using the Griess reaction (30).

Statistical Analysis

All data are expressed as mean ± SEM. Airway response curves to methacholine and DTH responses were statistically analyzed by a general linear model of repeated measurements followed by post-hoc comparison between groups. Data were log10 transformed before analysis to equalize variances in all groups. Cell counts were statistically analyzed using the Mann-Whitney U test. All other analyses were performed using Student's *t* test. A probability value *p* < 0.05 was considered significant.

Results

Nramp1 influences the inhibition of asthmatic features by M. vaccae.

First, we investigated the airway responses of ovalbumin-sensitized mice to increasing concentrations of methacholine 24 h after final ovalbumin or saline challenge (Fig. 1). Saline-challenged *Nramp1*[s] and *Nramp1*[r] control mice showed similar shallow increases in Penh in response to increasing doses of aerosolized methacholine. The dose response curves to methacholine of ovalbumin-challenged mice of both *Nramp1*[s] and *Nramp1*[r] mice were significantly higher than those of the saline-challenged control mice. Treatment with 10^7 CFU heat-killed *M. vaccae* during ovalbumin sensitization was able to decrease the airway hyperresponsiveness significantly in the *Nramp1*[s] mice. In contrast, treatment with *M. vaccae* did not reduce airway hyperresponsiveness in the *Nramp1*[r] mice.
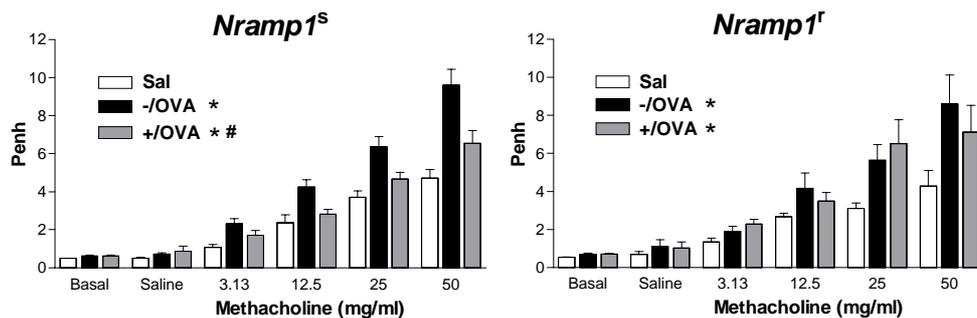
Figure 1. *Airway response to increasing concentrations of nebulized methacholine of ovalbumin-sensitized Nramp1*[s] *or Nramp1*[r] *mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Mice of each strain were treated with 10^7 CFU heat-killed M. vaccae during ovalbumin sensitization on days 0 and 14 (+/OVA). On day 43, unrestrained plethysmography measurements were performed as Penh-values. Data are represented as mean ± SEM, n=8, * p < 0.05 compared to the saline-challenged control groups. # p <0.05 compared to the -/OVA Nramp1*[s] *group.*

Secondly, we examined the influx of inflammatory cells into the lungs after ovalbumin challenge. Cell counts in the lung lavage fluid obtained 24 hours after final challenge showed that in both strains ovalbumin challenge induced a significant increase in the total number of cells in the lavage fluid compared to saline challenge (Fig. 2). This increase in total number was mainly caused by an increase in the number of eosinophils. No significant differences in eosinophil numbers were observed between the non-treated ovalbumin-challenged *Nramp1^s* and *Nramp1^r* mice. Heat-killed *M. vaccae* drastically diminished the number of eosinophils in the *Nramp1^s* mice, and to a lesser extent in the *Nramp1^r* mice. Similar results were obtained in both strains for the other cell types including monocytes and lymphocytes.

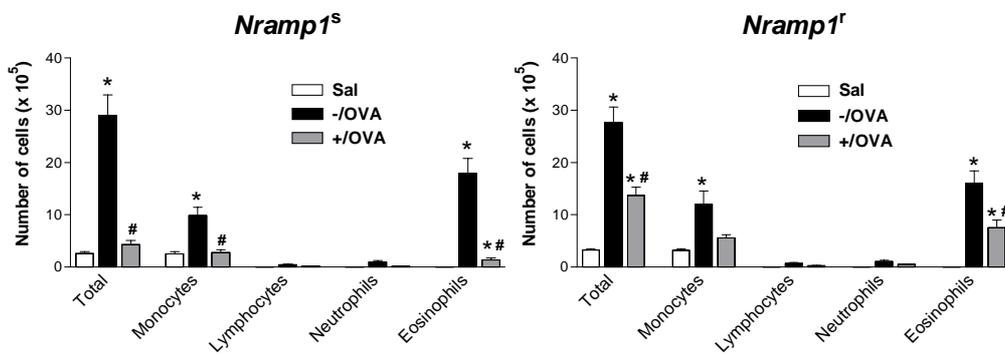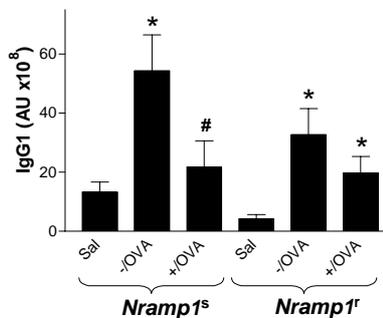**Figure 2.** *Number of cells in lung lavage fluid obtained from ovalbumin-sensitized Nramp1^s or Nramp1^r mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Mice of each strain were treated with 10^7 CFU heat-killed M. vaccae during ovalbumin sensitization on days 0 and 14 (+/OVA). Cells were isolated by lung lavage on day 43 and differentiated in monocytes, lymphocytes, neutrophils and eosinophils. Data are represented as mean cell number ± SEM, n=8. * p < 0.01 compared to the saline-challenged control groups. # p <0.05 compared to the -/OVA group of each strain.*

Nramp1 affects the inhibition of the allergic immune response by M. vaccae.

We next examined whether *Nramp1* influenced the inhibition of Th2-mediated allergic manifestations by *M. vaccae* as well. First, Antibody levels of sera prepared from blood collected 24 hours before (data not shown) or after the final saline challenge of ovalbumin-sensitized *Nramp1^s* and *Nramp1^r* mice appeared similar (Figs. 3 and 4). Ovalbumin challenge, however, markedly increased serum levels of ovalbumin-specific IgE (Fig. 3) compared to the saline-challenged animals, but to a significantly lesser extent in the *Nramp1^r* mice compared with the *Nramp1^s* mice. Treatment with *M.*

Figure 3. *Levels of ovalbumin-specific IgE measured by ELISA in serum of ovalbumin-sensitized Nramp1^s or Nramp1^r mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Mice of each strain were treated with 10^7 CFU heat-killed M. vaccae during ovalbumin sensitization on days 0 and 14 (+/OVA). Vertical bars indicate mean Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control groups. # p <0.05 compared to the -/OVA Nramp1^s group*

vaccae decreased the levels of IgE after ovalbumin challenge only in the *Nramp1^s* mice. Ovalbumin challenge of non-treated mice induced a comparable increase in the levels of ovalbumin-specific IgG1 and IgG2a in both strains, compared to the saline-challenged animals (Fig. 4). *M. vaccae* treatment decreased the IgG1 response after challenge only in the *Nramp1^s* mice. No effect of *M. vaccae* was observed on the serum levels of IgG2a after challenge.
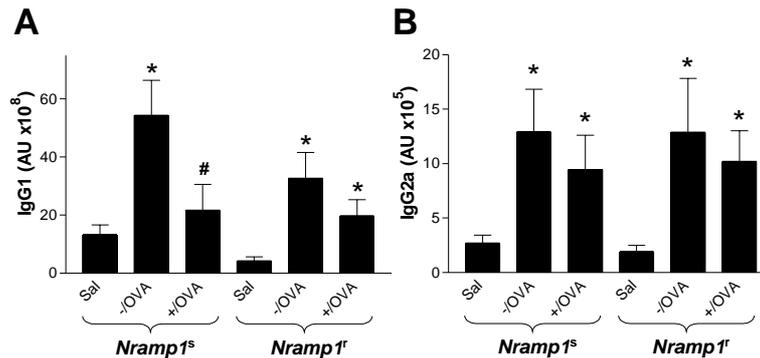

Figure 4. Ovalbumin-specific IgG1 (A) and IgG2a (B) levels in serum of ovalbumin-sensitized Nramp1^s or Nramp1^r mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Mice of each strain were treated with 10^7 CFU heat-killed M. vaccae during ovalbumin sensitization on days 0 and 14 (+/OVA). Vertical bars indicate mean Arbitrary Units (AU) ± SEM, n=8. * p < 0.01 compared to the saline-challenged control groups. # p <0.05 compared to the -/ OVA Nramp1^s group.

Since Th2 type responses are crucial in the development and maintenance of allergy and allergic asthma, we looked at the expression of Th2 cytokines in the lung and examined the levels of the regulatory cytokines IL-10 and TGF1-ß. Lung lavage fluid of ovalbumin-sensitized mice contained no detectable levels of IL-4, IL-5, TGF1-ß and IL-10 after saline challenge (Fig. 5). Ovalbumin challenge caused a significant increase in the levels of these cytokines in both strains as compared to the saline-challenged controls. Lung lavage fluid of *Nramp1^r* mice, however, contained half to one third the amount of IL-4, IL-5 and IL-10 as found in lavage fluid of *Nramp1^s* mice. In addition, *M. vaccae* treatment of *Nramp1^s* mice significantly reduced the levels of IL-4 and IL-5 and TGF1-ß. No effect of *M. vaccae* treatment on these cytokines was observed in *Nramp1^r* mice. Moreover, no effect of *M. vaccae* treatment was observed on the levels of IL-10 in both strains. IFN-γ was not detectable in the lavage fluid, irrespective of treatment and mouse strain (detection limit ELISA: 10 pg/ml, data not shown).

Nramp1 affects the T cell-mediated responses to M. vaccae.

To examine whether *Nramp1* influenced T cell-mediated responses to heat-killed *M. vaccae*, we measured delayed-type hypersensitivity (DTH) responses to these killed bacteria. Challenge with *M. vaccae* in the ear induced a small background swelling in non-sensitized mice (< 25 µm). A significantly stronger ear swelling, however, was observed in *M. vaccae*-sensitized mice compared to non-sensitized mice in both
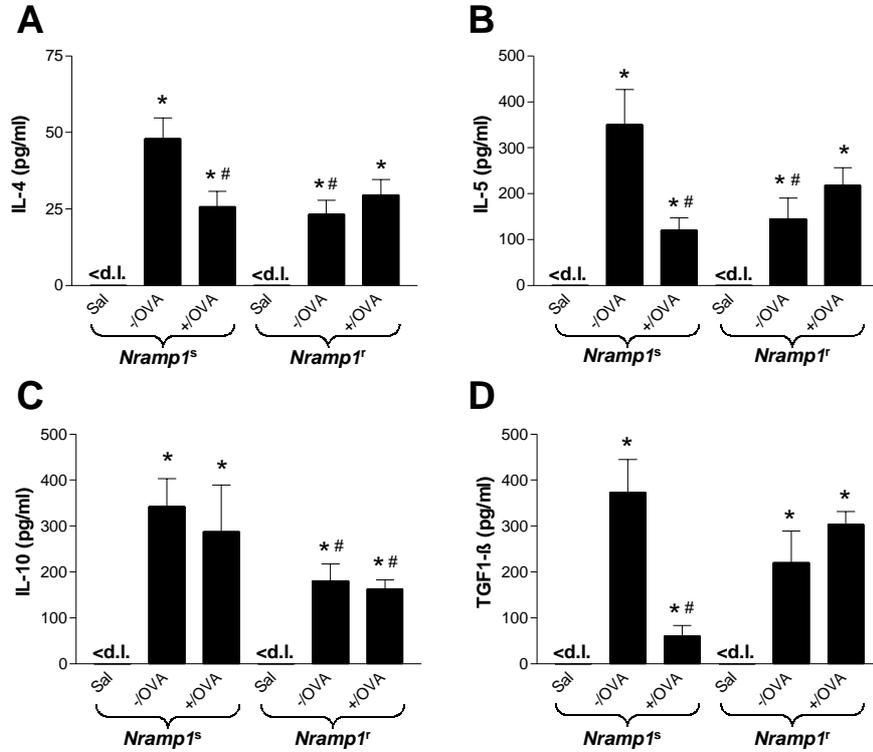
Figure 5. *Levels of IL-4 (**A**), IL-5 (**B**), IL-10 (**C**) and TGF1-ß (**D**) in lung lavage fluid obtained from ovalbumin-sensitized Nramp1ˢ or Nramp1ʳ mice, 24 hours after final respiratory aerosol challenge with saline (Sal) or ovalbumin (OVA). Mice of each strain were treated with 10⁷ CFU heat-killed M. vaccae during ovalbumin sensitization on days 0 and 14 (+/OVA). Data are represented as mean pg/ml ± SEM, n=8. * p < 0.01 compared to the saline-challenged control groups. # p <0.05 compared to the -/OVA Nramp1ˢ group. <d.l. below detection limit*
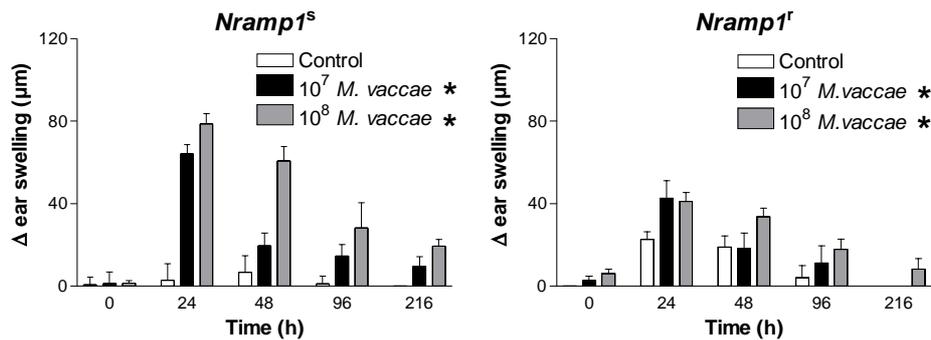
Figure 6. *DTH responses to M. vaccae in Nramp1ˢ and Nramp1ʳ mice. Mice were injected with 10⁷ or 10⁸ CFU heat-killed M. vaccae or saline (Control) on day 0 and 7. On day 21, ear thickness of both ears was measured and mice were challenged with heat-killed M. vaccae in the ear. Hereafter, thickness of both ears was measured 24, 48, 96 and 216 hours after challenge. Data are depicted as mean swelling of the left ear compared to the control right ear ± SEM (n=6). * p < 0.01 compared to the control group of both strains.*

strains (Fig. 6) that peaked at 24 hours and resolved at 216 hours. Sensitization with 10^8 CFU *M. vaccae* resulted in a stronger DTH response compared with 10^7 CFU *M. vaccae*. The DTH response was markedly less in *Nramp1^r* mice compared with the *Nramp1^s* mice.

***M*. vaccae strongly activates macrophages in vitro, which is affected by Nramp1.**
Since *Nramp1* is almost exclusively expressed in macrophages, we investigated the effect of heat-killed *M. vaccae* on macrophage function *in vitro*. *Nramp1^r* and *Nramp1^s* macrophages were stimulated for 48 hours with increasing concentrations of *M. vaccae*. *M. vaccae* dose-dependently activated *Nramp1^r* macrophages, as indicated by the production of NO· (measured by NO_2^- concentrations), and TNF-α (Fig. 7). Interestingly, the production of NO· and TNF-α was considerably less in the *Nramp1^s* compared to the *Nramp1^r* macrophages.
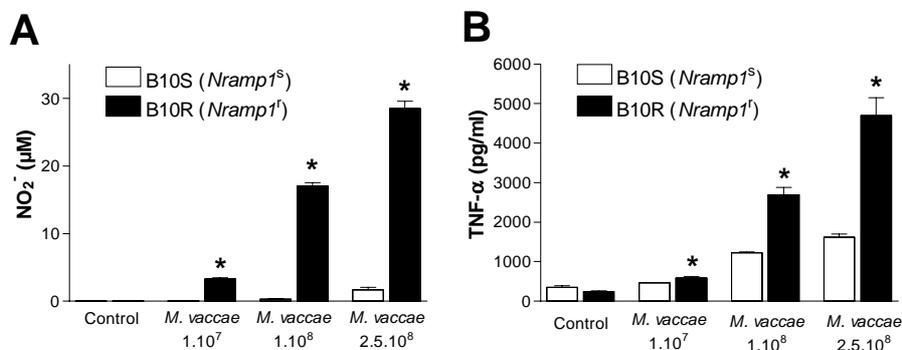
Figure 7. *NO· (**A**) and TNF-α (**B**) production by B10R (Nramp1^r) and B10S (Nramp1^s) macrophages. Cells were incubated for 48 hours with medium (control) or medium with increasing concentrations of heat-killed M. vaccae (1 x 10^7, 1 x 10^8 and 2.5 x 10^8 CFU/ml). After incubation, the supernatant of the different cell cultures was collected and analyzed for nitrite (NO_2^-) concentrations, using the Griess reaction, or TNF-α by ELISA. Data are represented as mean µM or pg/ml ± SEM, respectively, n=4. * p < 0.01 compared to B10S macrophages.*

Discussion

In the present study, we demonstrated that heat-killed *M. vaccae*, when administered during sensitization, is able to inhibit the induction of important hallmarks of allergic asthma such as airway hyperreactivity, airway eosinophilia, allergen-specific IgE, and lung lavage levels of IL-4 and IL-5 in *Nramp1^s* mice. As treatment of *Nramp1^r* mice with *M. vaccae* did not lead to any reduction in these manifestations of allergic asthma, with the exception of airway eosinophilia, we concluded that *Nramp1* strongly influences the efficacy of *M. vaccae* in treatment of allergic asthma.

The present data is in line with previous studies, in which mycobacterial components diminished the asthmatic response in animal models (10-13). However, *M. vaccae* does not induce allergen-specific Th1 responses, which downregulate the allergic Th2 response (chapter 4,13). Therefore, other mechanisms for the mode of action of *M. vaccae* must be accounted for. Zuany-Amorim and coworkers clearly

demonstrated that vaccination with heat-killed *M. vaccae* in mice induced allergen-specific CD4+ cells, in particular T regulatory CD4$^+$CD25$^+$CD45RB^{lo} cells, which diminished allergic inflammation (31).

DTH reactions have shown to be absolutely dependent on the presence of CD4+ T cells (32, 33). Therefore, we investigated the effect of *Nramp1* on the development of the CD4+ T cell-mediated response to *M. vaccae* by inducing a DTH reaction after *M. vaccae* sensitization and challenge. We demonstrated that this DTH response, and therefore most likely T cell-mediated immunity to *M. vaccae* as well, was significantly lower in the *Nramp1^r* mice compared with *Nramp1^s* mice. The lower T cell-mediated response to *M. vaccae* most likely explains the inferior capacity of *M. vaccae* in reducing allergic manifestations in *Nramp1^r* mice. Interestingly, we demonstrated that *Nramp1^r* mice have lower Th2 responses, as measured by Th2 cytokines and allergen-specific IgE, to merely ovalbumin. *Nramp1* generally affects the T cell-mediated immunity to several antigens, including ovalbumin, as suggested by other investigators (34).

Accordingly, we hypothesized that *Nramp1* affects the development of regulatory T cell responses after immunization with *M. vaccae* as well. Analysis of splenocytes revealed an significant increase in regulatory CD4$^+$CD25$^+$CD45RB^{lo} cells in *Nramp1^s* mice after *M. vaccae* immunization compared with non-immunized mice, while *M. vaccae* vaccination did not increase the number of this cell type in *Nramp1^r* mice (data not shown). This inhibition of asthmatic responses in mice by regulatory T cells was mediated trough IL-10 and TGF1-ß (31). Therefore, both these cytokines were measured in lavage fluid after ovalbumin challenge in our model. Nonetheless, we did not observe an upregulation of both IL-10 and TGF1-ß after *M. vaccae* treatment. In contrast, TGF1-ß levels were decreased after *M. vaccae* treatment. On the other hand, both cytokines are indicative of feedback mechanisms after ovalbumin-induced inflammation and can be produced by monocytes and B-cells as well (35). Since the induction of Th2 responses was generally reduced in *Nramp1^s* mice after treatment, reduced feedback mechanisms are likely to occur. Besides, the cytokine levels in the lung lavage fluid may not account for cell-cell interactions and temporal responses.

Nramp1 is almost exclusively expressed in primary macrophages and in granulocytic lineages (36, 37). In addition, macrophages are primarily responsible for uptake and clearance of particulate antigens like killed *M. vaccae* (38). Therefore, in order to provide a mechanism for the observed phenomena caused by *Nramp1*, we investigated the effect of *Nramp1* on activation of macrophages by *M. vaccae*. After incubation with *M. vaccae*, *Nramp1^r* macrophages were strongly activated while *Nramp1^s* macrophages show significantly less prominent activation, as indicated by production of nitric oxide and TNF-α. How *Nramp1* affects the macrophage activity is demonstrated by several other investigators. It was shown that the Nramp1 protein is a divalent cation (Fe^{2+}, Zn^{2+} and Mn^{2+}) transporter, which mediates metal ion homeostasis in macrophages (39). It was found that *Nramp1^s* macrophages are less capable of releasing iron than *Nramp1^r* macrophages (40, 41). In this way, high

cytoplasmic iron levels in *Nramp1ˢ* macrophages may have caused the reduced capacity of *Nramp1ˢ* macrophages to become activated (42-44).

Since activation of macrophages, as mediated by *Nramp1*, is necessary for clearance of mycobacteria or their components (23, 37), we presume that clearance of *M. vaccae* was inferior and delayed in *Nramp1ˢ* mice. The natural defense to mycobacteria or their components, mediated through macrophages can be either impaired (as in *Nramp1ˢ* animals) or overcome by a high infectious dose. At that stage the acquired immunity becomes the main effector mechanism (45). After *M. vaccae* vaccination of *Nramp1ˢ* mice, the inferior clearance of *M. vaccae* in *Nramp1ˢ* macrophages will probably result in a higher antigenic load available for professional presenting cells, such as dendritic cells. Subsequently, the T cell-mediated immune response was stronger in *Nramp1ˢ* mice than *Nramp1ʳ* mice, as demonstrated by the higher DTH response to *M. vaccae*. Consequently, it is likely that that a higher regulatory T cell response develops after *M. vaccae* vaccination in *Nramp1ˢ* mice. Since the anti-asthmatic effect of *M. vaccae* is dependent on the induction of these regulatory T cells (31), the higher induction of regulatory T cell responses in *Nramp1ˢ* mice may lead to the observed higher efficacy of *M. vaccae* to reduce the allergic and asthmatic responses in these mice.

Other investigators already suggested that polymorphisms in the genomic region of the human *NRAMP1* gene are associated with risk of atopy in BCG vaccinated children (20). The current controversy about the efficacy of mycobacteria and their components in the treatment of allergic asthma in humans (46, 47) can be explained by the strong influence of genetic factors, such as the *NRAMP1* gene, on the inverse relationship between infection and the development of allergic disease. In conclusion, we have demonstrated that *Nramp1* clearly affects the efficacy of heat-killed *M. vaccae* in diminishing the allergic asthmatic response in a mouse model. These findings could have important implications for the future use of mycobacteria and their components in the prevention or treatment of allergic asthma. Moreover, *Nramp1* may be a key to the hygiene hypothesis, providing a link between genes, the (bacterial) environment, and allergy or asthma.

Acknowledgement

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Chapter 7

General discussion

Introduction

Allergic asthma is a chronic disease characterized by airway hyperresponsiveness to a variety of specific and non-specific stimuli, by chronic pulmonary inflammation with eosinophilia and by IgE mediated immune responses (1). The underlying mechanisms of the development of atopic asthma have been revealed over the past decades. At the same time, the prevalence of asthma has steadily increased. The specific cause for this rise is not known, but an explanation which has gained much attention is the "hygiene hypothesis". It suggests that the increase in allergic diseases is caused by a cleaner environment and fewer childhood infections (2, 3).

As a mechanism for the hygiene hypothesis, originally the concept of a disbalance in Th1 and Th2 cells in allergic disease served as a basis. Most bacterial and viral infections induce Th1-mediated reactions, which can downregulate the allergic Th2 response (4). For that reason, a reduction in the infectious burden will result in declining Th1 responses and subsequently unrepressed Th2 responses, which may result in an increased risk for developing allergic disease (5, 6). This mechanism, however, is challenged recently by observations that Th2-mediated helminth infections are negatively associated with allergy (7) and that the prevalence of Th1-mediated autoimmune diseases is increasing the last decades as well (8). Therefore, in order to explain the hygiene hypothesis, other mechanisms should be taken into account. In this regard, it is important to realize that either allergy and asthma or autoimmune diseases are a consequence of poorly regulated and as a result exaggerated immune responses. Most of the regulation of the immune system is done by different regulatory cells, including the specialized regulatory T cells (9). These cells prevent the activity of effector T cells and other inflammatory cells, by secreting inhibitory cytokines such as IL-10 and TGF1-ß, but also by direct contact with effector T cells or antigen presenting cells (10). Possibly, the decreased infectious pressure of westernized lifestyles is depriving the population of important counter-regulatory feedback. For that reason, the hygiene hypothesis may be mechanistically explained by employing a balance between regulated and disregulated immune responses (11, 12). Infection or colonization with various microbial agents (whether Th1 or Th2 stimulatory) may lead to an upregulation of regulatory feedback, which results in the suppression of the underlying predisposition to allergy and asthma.

Since the proposal of the hygiene hypothesis in 1989 (13), mycobacteria have drawn a lot of attention as microorganisms which may influence the development of allergic diseases. Experimental proof for the candidature of mycobacteria in the hygiene hypothesis was derived from experiments in animal models, in which bacillus Calmette-Guérin (BCG) or *Mycobacterium vaccae* reduced allergic and asthmatic manifestations (14-17). However, retrospective studies in humans are conflicting about the possible correlation between BCG vaccination during early childhood and the subsequent development of atopy or asthma (18-21). The age at

vaccination, the frequency of administration of BCG, the BCG strain used, and the varying natural exposure to mycobacteria (including *M. tuberculosis*) may have influenced these conflicting results (22). Crucial in all these studies, however, may have been a genetic contribution to the possible inverse association between mycobacterial infection and the development of allergy and asthma, since the ability to mount a response to mycobacterial antigens is highly influenced by the genotype of the individual (23, 24).

Importantly, resistance to intracellular bacteria, such as mycobacteria, is under control of the natural-resistance-associated macrophage protein 1 gene in mice (*Nramp1*, recently designated *Slc11a1*) (24-26). In humans, association or linkage of *NRAMP1* with susceptibility to several infectious diseases has been demonstrated as well. Additionally, linkage of *NRAMP1* to atopy and autoimmune disease has been demonstrated (27-29). The murine polymorphism of *Nramp1* is apparent as either high (*Nramp1^r*) or low (*Nramp1^s*) resistance to the growth of intracellular organisms (24). This difference in resistance has been related to the observation that *Nramp1^r* macrophages display faster and superior activation in response to intracellular bacteria, bacterial products and IFN-γ than *Nramp1^s* macrophages (30, 31).

In the present thesis, we investigated whether vaccination with mycobacteria may suppress the development of allergic and asthmatic manifestations in the mouse. Since the immune response to intracellular bacteria, including mycobacteria, is under control of the *Nramp1* gene, we hypothesized that *Nramp1* affects the efficacy of mycobacterial vaccines in the treatment of allergic asthma. Therefore, in this thesis, we addressed the following questions:

1. *Is heat-killed Mycobacterium vaccae able to prevent and/or treat allergic and asthmatic manifestations in a mouse model of allergic asthma?*
2. *Does the Nramp1 gene, which controls the resistance to intracellular bacteria, influence sensitivity to induction of allergic asthma in mice?*
3. *Does the Nramp1 gene affect the efficacy of heat-killed M. vaccae in suppressing the allergic and asthmatic response in mice?*

1. Is heat-killed M. vaccae able to prevent and/or treat allergic and asthmatic manifestations in a mouse model of allergic asthma?

In line with the hygiene hypothesis it has been suggested that mycobacteria can be used as an "anti-asthma" vaccine (32). Although this idea is still debated and controversial in the human situation (21, 33-35), studies in animal models are fairly conclusive. In several experiments, vaccination with mycobacteria such as BCG or *M. vaccae* inhibited the allergic and asthmatic response in rodents (16, 36-38). In this thesis, a heat-killed preparation of *M. vaccae* (SRL172) was used, a environmental, saprophytic, fast growing mycobacterium, first described in isolates from

the environment of cattle (39). The latter might be relevant in the light of the studies which showed that a farming environment might be protective in the development of asthma (40, 41). Heat-killed *M. vaccae* has undergone extensive toxicological and immunological testing (42) and may be effective in preventing and treating both allergic and autoimmune diseases (32, 43). We took into account that possible treatment of allergic asthma can be applied during two time periods: Before the onset of allergic disease (prophylaxis), or after the onset of allergic disease (therapy).

In **chapter 2**, it was investigated whether prophylactic treatment with 10^8 CFU *M. vaccae* in a mouse model, two to three weeks before ovalbumin sensitization was able to inhibit the asthmatic response after challenge. Prophylactic *M. vaccae* treatment did not lead to an alteration in airway hyperreactivity, airway eosinophilia or levels of ovalbumin-specific immunoglobulins after ovalbumin challenge. Nevertheless, heat-killed *M. vaccae* was able to elicit a strong delayed-type hypersensitivity (DTH) response, showing that *M. vaccae* generates a strong T cell-mediated immune response, as has been demonstrated before (44). In addition, previous studies showed that administration of both 10^7 and 10^8 CFU heat-killed *M. vaccae* was capable of eliciting a strong Th1 response, while 10^9 CFU elicited mixed Th1 and Th2 responses in spleen cell cultures (44, 45). Therefore, it was concluded that the time and dose of *M. vaccae* used was sufficient to elicit a relatively pure and strong Th1 response. This strong Th1 reaction, however, did not result in an inhibition of Th2-mediated allergic and asthmatic reactions.

In **chapter 3**, it was investigated whether therapeutic treatment with *M. vaccae* would be more effective. After ovalbumin sensitization, mice received 10^7 or 10^8 CFU *M. vaccae*, three and two weeks before ovalbumin challenge. Since the use of ovalbumin/alum as sensitizing agent might induce too strong allergic and asthmatic responses, which cannot be reversed by treatment, a mild, non-adjuvant model for allergic asthma was used as well. Results clearly showed that allergic and asthmatic manifestations measured were indeed drastically higher in the severe model compared with the mild model. Treatment with *M. vaccae* failed to decrease the allergic and asthmatic manifestations in both the mild and the severe model of allergic asthma. Therefore, the lack of effect of *M. vaccae* in our experiments is not a consequence of the model used.

There are several explanations for the lack of a beneficial prophylactic (chapter 2) or a therapeutic (chapter 3) effect of *M. vaccae* in both mouse models of allergic asthma. First, in chapter 3, *M. vaccae* was given therapeutically, a long period after sensitization, while most other studies administered mycobacteria before, during or shortly after sensitization (15, 16, 36, 46). In a murine non-adjuvant ovalbumin model for allergic disease it was found that after sensitization, Th2 memory was present even after more than 400 days (47). It is possible that long after sensitization the allergic and asthmatic response is irreversible, and cannot be reversed by *M. vaccae* treatment.

Secondly, the site of administration of *M. vaccae* could be important. Treat-

ment with mycobacteria in allergic asthma models was most effective when these mycobacteria were administered locally by the intranasal route (15, 37). Nonetheless, although largely neglected, intranasal application of live BCG or *M. vaccae*, and heat-killed BCG as well, was accompanied by serious inflammation in the airways of mice (37, 46). Therefore, subcutaneous administration, as used in our studies, is safest and probably the only acceptable route of administration of mycobacteria or their components.

Finally, the time of vaccination with *M. vaccae* is important in the treatment of allergic asthma. In chapter 2 and 3, *M. vaccae* was administered in the absence of allergen exposure. Other investigators hereafter suggested (48, 49) that mycobacteria is most efficient in treating allergy and asthma when present during the time of allergen exposure. Since *M. vaccae* in contrast to BCG is relative rapidly cleared after administration(50), there is a limited period of simultaneous presence of *M. vaccae* and the allergen.

Accordingly, in **chapter 4**, it was investigated whether treatment with *M. vaccae* is dependent on allergen exposure. Therefore, mice were treated with *M. vaccae* either during sensitization or challenge with ovalbumin. Interestingly, *M. vaccae* treatment during sensitization suppressed the airway hyperresponsiveness, eosinophilia, serum levels of IgE, and the Th2 cytokines IL-4 and IL-5 in the lung lavage fluid considerably after ovalbumin challenge. This effect was dose-dependent, 10^7 CFU was the optimal dose for *M. vaccae* treatment, while 10^8 CFU appeared less and 10^6 CFU not effective in inhibiting the allergic and asthmatic response. Furthermore, therapeutic treatment with 10^7 CFU *M. vaccae* during challenge inhibited the airway hyperresponsiveness and airway eosinophilia after challenge, which was accompanied by a lower level of IL-5 in the lavage fluid. These data suggest that timing and dose of *M. vaccae* administration is crucial: *M. vaccae* should probably be present during allergen exposure and given in an optimal dose of 10^7 CFU to be effective.

The reduced Th2 response after treatment with *M. vaccae* either during sensitization or challenge could explain the observed effects of *M. vaccae* on allergen-induced eosinophilia and airway hyperresponsiveness (both downstream manifestations of the asthma process). In addition, a low dose of *M. vaccae* (10^7 CFU) was more effective in reducing Th2 responses than a high dose (10^8 CFU). Interestingly, a low dose of *M. vaccae* (10^7 CFU) primes a Th1 response in the absence of a Th2 component, while higher doses (10^8 and 10^9 CFU) induced mixed Th1/Th2 response in the spleen of mice (51). The differences in cytokine secretion related to the *M. vaccae* immunizing dose may well explain the different effects of different doses of *M. vaccae* on Th2 responses.

However, in spite of the inhibition of Th2 responses, in both treatment regimes no upregulation of Th1 responses was observed after ovalbumin challenge, as characterized by the levels of IFN-γ in the lung lavage fluid or the Th1-related IgG2a antibodies in serum. The lack of a Th1 response suggests that mechanisms other than simple changes in the balance between Th1 and Th2 are responsible for

the observed effects of *M. vaccae*. A more likely mechanism for the beneficial effects of *M. vaccae* comes from recent studies by Zuany-Amorim and co-workers (36). Treatment with *M. vaccae* in a model for allergic asthma induced allergen-specific CD4⁺CD45RBˡᵒ T regulatory cells, which conferred protection against airway inflammation, mediated through the cytokines IL-10 and TGF1-ß. The role of regulatory T cells and their released cytokines in the observed effects of *M. vaccae* in our model remains unclear, since we did not observe an upregulation in the levels of IL-10 or TGF1-ß (data not shown). However, we did not analyze induction of regulatory T cells after *M. vaccae* treatment in this model. Moreover, direct cell-cell interactions or temporal regulatory responses after *M. vaccae* treatment may be important regulatory mechanisms as well (52).

As shown in chapter 4, administration of *M. vaccae* suppresses allergic and asthmatic responses during, but also after, the onset of allergic disease. Importantly, *M. vaccae* must be administered during allergen exposure to be effective. This may have important implications for the application of *M. vaccae* in humans. The contradictive results in clinical trails using *M. vaccae* (35, 53, 54) but also BCG (21, 55) may be due to the duration and/or the level of exposure to allergens during the treatment period.

2. Does the Nramp1 gene, which controls the resistance to intracellular bacteria, influence sensitivity to induction of allergic asthma-like disease in mice?

In **chapter 5**, we hypothesized that genes controlling the resistance to bacterial infections can influence the development of allergic diseases as well. The *Nramp1* gene is undoubtedly one of the best characterized genes controlling resistance to intracellular infections (24). Therefore, it was studied whether *Nramp1* alleles, that determine resistance (*Nramp1ʳ*) or susceptibility (*Nramp1ˢ*) to intracellular bacteria (24, 26, 56), affect the sensitivity to the induction of allergic asthma. After ovalbumin sensitization and challenge, both *Nramp1ʳ* and *Nramp1ˢ* mice developed an equal degree of airway hyperresponsiveness and airway eosinophilia. Interestingly, manifestations of allergy, including levels of IgE, mast cell degranulation and levels of Th2 cytokines were significantly lower in *Nramp1ʳ* mice. Hence, the *Nramp1* gene affects the development of allergy, but not the development of airway hyperresponsiveness and eosinophilia.

This finding confirms the complex relation between allergy and asthma (57) and confirms the role of various mechanisms in the induction of asthmatic manifestations (1, 58). Airway macrophages are activated after respiratory ovalbumin challenge (59, 60). Therefore, the level of macrophage activation, which is strongly influenced by *Nramp1*, could explain the observed effects of *Nramp1* on the development of allergy. That the degree of macrophage activation controls the level of the antibody response, including IgE, was shown previously with the well-known Biozzi mice. Macrophages of Biozzi high antibody responder mice displayed decreased constitutive and bactericidal activity and deceased antigen degradation than macrophages of Biozzi low antibody responder mice (61). Similarly, *Nramp1ʳ* macrophages

show faster and superior activation after stimulation with various stimuli than $Nramp1^s$ macrophages (26, 29, 31).

Therefore, the lower IgE response in the $Nramp1^r$ mice is likely to be caused by the higher macrophage activation in this strain. In this respect, the secretion of nitric oxide (NO·), which is directly related to the degree of macrophage activation and influenced by the *Nramp1* gene (62, 63), might be of importance. NO· is the primary mediator by which alveolar macrophages downregulate the presentation of allergens by pulmonary dendritic cells (64). Consequently, alveolar macrophages inhibited the IgE response (65). In addition, NO· inhibits the proliferation of both Th1 as Th2 cells (66, 67). Accordingly, we presume that the elevated NO· production by sensitized $Nramp1^r$ mice after allergen challenge resulted in inhibition of the T cell response, as demonstrated by the lower IL-4, IL-5, IL-10 and IL-13 production in these mice compared with $Nramp1^s$ mice. Conceivably, the lower levels of IL-4 and IL-13 could have led to the observed reduced production of IgE in $Nramp1^r$ mice.

Together with the reduction in IgE production, we demonstrated that $Nramp1^r$ mice displayed a lower level of mast cell degranulation, as measured by MMCP-I levels in the lavage fluid. PCR analysis showed that *Nramp1* is not expressed in mast cells and therefore a direct effect of *Nramp1* on mast cell degranulation is unlikely. The decreased mast cell degranulation could have been caused by the lower IgE levels in $Nramp1^r$ mice, which may have led to less IgE dependent mast cell activation and degranulation in these mice. Secondly, since IL-4 and IL-10 are potent stimulators of mast cell proliferation and degranulation (68, 69), the decreased production of these cytokines in $Nramp1^r$ mice could have reduced the mast cell degranulation in these mice as well (Fig. 1).
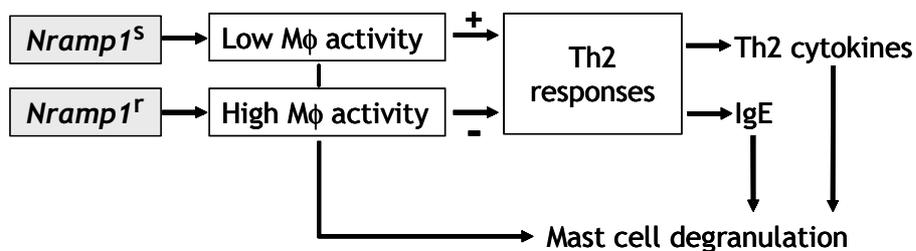
Figure 1. *Schematic diagram of the hypothesized action of the Nramp1 gene on the development of Th2 manifestations and mast cell degranulation. See text for explanation. Mφ, macrophage*

In our opinion, *Nramp1* may now be considered as a potential locus for susceptibility for allergy and allergic disease. Moreover, *Nramp1* could provide a link between genes, the (bacterial) environment and the development of allergy. *Nramp1* shares some similarity with some other genes in this respect. For instance, *Tim1*, a mouse homolog of a human gene encoding a receptor for hepatitis A virus, regulated the Th2 responses and development of airway hyperresponsiveness in allergic mice (70). In addition, a genetic variation in the regulation for CD14, the endotoxin re-

ceptor was strongly associated with IgE responses (71, 72). Finally, the genes for IL-12 (73), nitric oxide synthase 1, tumour necrosis factor-α (TNF-α) and IFN-γ, important mediators of resistance to infection are candidate genes for asthma (72). The association between these genes, including *Nramp1,* and the development of allergic diseases, suggests that genes controlling the Th1-mediated immune response to infections may influence the development of Th2-mediated allergic diseases as well.

3. Does the Nramp1 gene affect the efficacy of heat-killed M. vaccae in suppressing the allergic and asthmatic response?

Heat-killed *M. vaccae* prevented and suppressed features of allergy and asthma in a mouse model (chapter 4). Since *Nramp1* controls the immune response to intracellular microorganisms such as *M. vaccae*, *Nramp1* may be involved in treatment of allergic disease with heat-killed *M. vaccae* as well. Therefore, in **chapter 6**, it was investigated whether *Nramp1* affected the efficacy of heat-killed *M. vaccae* treatment of allergic asthma. *M. vaccae*, administered during sensitization, inhibited the induction of important hallmarks of allergy and asthma such as airway hyperreactivity, airway eosinophilia, allergen-specific IgE, and Th2 cytokine levels in the lung lavage in *Nramp1^s* mice. In contrast, treatment of *Nramp1^r* mice with *M. vaccae* did not lead to any reduction in these manifestations of allergic asthma, with the exception of airway eosinophilia. So, *Nramp1* strongly influences the efficacy of *M. vaccae* in treatment of allergic asthma. Next, it was investigated whether the *in vivo* T cell response to *M. vaccae* is affected by *Nramp1*, by measuring the DTH response to *M. vaccae*. This DTH response was significantly reduced in the *Nramp1^r* mice compared with *Nramp1^s* mice.

To explain these phenomena, it has to be noted that the *Nramp1* gene is important in the natural resistance of macrophages to microorganisms. The *Nramp1^r* mice are resistant to most intracellular pathogens without the need for a T cell-mediated cellular immune response. In contrast, the susceptible *Nramp1^s* mice are only resistant to bacteria when they acquire a specific cellular, T cell-mediated, immune response (74). Furthermore, the degree of antigenic load that develops in the infected host during the initial phase of natural resistance is the critical issue in the development of immunity to antigens like *M. vaccae*. *Nramp1^s* macrophages were significantly less activated *in vitro* after *M. vaccae* incubation when compared with *Nramp1^r* macrophages. Since the clearance of (myco) bacteria is highly dependent on macrophage activity, including the secretion of NO· and TNF-α (75, 76), it is likely that a higher antigenic load was allowed to develop in macrophages of *Nramp1^s* mice. Consequently, the high antigenic load in *Nramp1^s* mice induced T cell-mediated immunity, as indicated by the high DTH response in these mice. In contrast, in the *Nramp1^r* mice, *M. vaccae* can be rapidly cleared, moreover since the *M. vaccae* preparation used was heat-killed prior to vaccination. Little or no T cell-mediated immunity to *M. vaccae* is likely to develop in *Nramp1^r* mice, resulting in a lower DTH to this agent in these mice. It cannot be excluded that the

Nramp1^r mice will eventually develop cellular T cell response to *M. vaccae*, when infected with a higher, unclearable dose of bacteria, or with life bacteria like BCG (77).

So far, the mechanism of the observed effects of *Nramp1* on the efficacy of *M. vaccae* is not precisely known, but some pieces of the puzzle can be put together (Fig. 2). It was demonstrated that *Nramp1* affects the cellular, T cell-mediated immunity to *M. vaccae*. This may be caused by the effects of *Nramp1* on macrophage activity and clearance of mycobacteria and their components. Therefore, the regulatory T cell responses after *M. vaccae* vaccination could have been affected by *Nramp1* as well. We observed an increase in regulatory CD4+CD25+CD45RB^{lo} T cells in
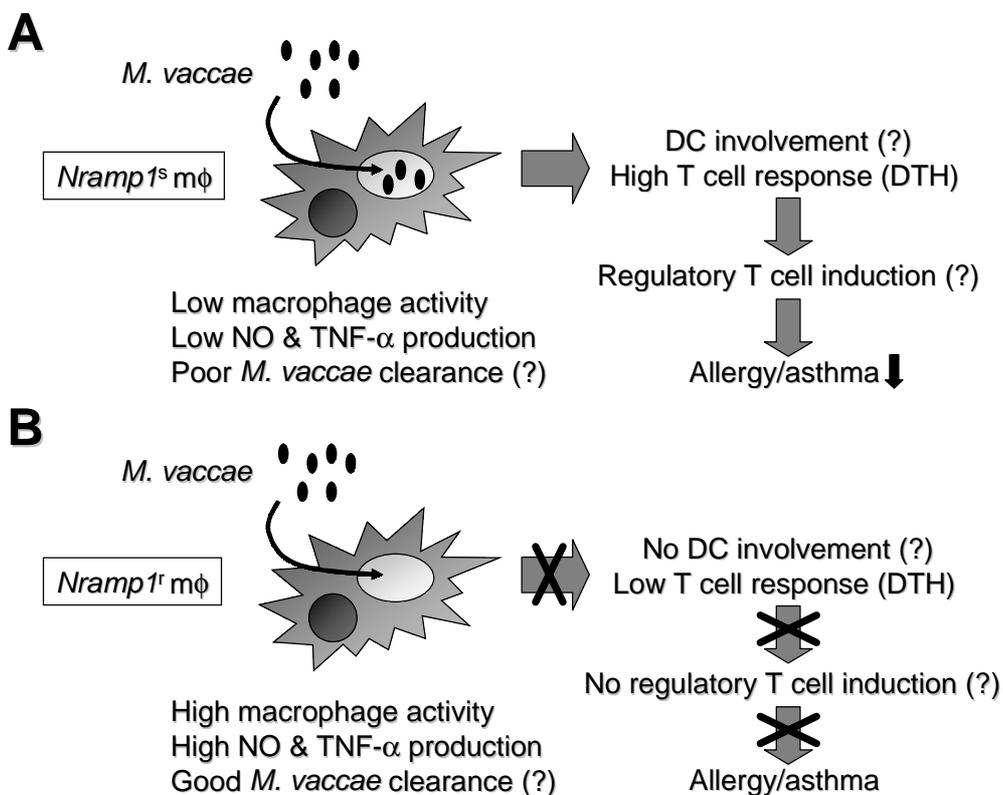
Figure 2. Schematic diagram of the hypothesized action of the Nramp1 gene on the efficacy of M. vaccae.
A. Nramp1^s mice. Phagocytosis of M. vaccae in Nramp1^s macrophages results in low macrophage activity, as indicated by low NO· and TNF-α production, and therefore poor or prolonged M. vaccae clearance. This may lead to the involvement of dendritic cells, which subsequently will induce T cell-mediated responses. The stimulation of a T cell response may include the induction of regulatory T cells, which have possibly to downregulate the allergic and asthmatic response.
B: Nramp1^r mice. Phagocytosis of M. vaccae in Nramp1^r macrophages results in high macrophage activity (high levels of NO· and TNF-α), which results in good M. vaccae clearance. Subsequently, little or no T cell-mediated responses will develop, including no induction of protective regulatory T cells.
Mφ, macrophage; NO, Nitric Oxide; DC, dendritic cell; DTH, delayed-type hypersensitivity

the spleen of *M. vaccae* vaccinated *Nramp1*[s] mice, while *M. vaccae* did not increase the number of these cells in *Nramp1*[r] mice. Interestingly, these CD4^{+}CD25^{+}CD45RB^{lo} T cells were reported to transfer the protective effect of *M. vaccae* in an allergic asthma model (36). Possibly, regulatory T cells, as generated by *M. vaccae* treatment, have an essential role in restoring the immune balance in allergic disease (33). *M. vaccae* induced a higher cellular, T cell-mediated, immune response in *Nramp1*[s] mice, which possibly includes a higher regulatory T cell response as well. As a result, treatment with *M. vaccae* is more efficient in *Nramp1*[s] mice when compared with *Nramp1*[r] mice, since the downregulation of the allergic and asthmatic response is dependent on the induction of regulatory T cells.

These findings have important implications for human application of *M. vaccae*, but also for application of other mycobacteria like BCG in the treatment of allergic asthma. Several polymorphisms in the *Nramp1* gene have been described in humans (78) and other investigators already suggested that polymorphisms in the genomic region of human NRAMP1 are associated with the risk of atopy in BCG vaccinated children (28). Polymorphisms in human NRAMP1, and possibly other infection related genes, could contribute to the suggested inverse correlation between (myco) bacterial infection and allergic asthma. This might also explain the observed discrepancy of mycobacterial treatment of allergic asthma in humans.

Final conclusions

First, in this thesis it was demonstrated that *M. vaccae* inhibits allergic and asthmatic responses in mice. However, in our studies, *M. vaccae* must be administered in an optimal dose, and during allergen exposure to be effective, since treatment weeks before allergen sensitization or challenge was not effective in suppressing the allergic and asthmatic response in our model. In addition, there was no shift from allergen-induced Th2 towards Th1 type responses observed after *M. vaccae* treatment in our model. Most likely, as suggested by others, other mechanisms, such as the induction of regulatory T cells, explain the downregulatory effects of *M. vaccae* in our allergic asthma model.

Secondly, the macrophage bacterial resistance gene, *Nramp1*, significantly affects the development of allergic manifestations, as demonstrated by lower levels of IgE, lower degree of mast cell degranulation and decreased Th2 cytokine response in the lung in the *Nramp1*[r] mice compared to the *Nramp1*[s] mice. In contrast, the *Nramp1* gene does not influence the development of asthmatic manifestations, such as airway hyperresponsiveness or eosinophilia, in the mouse. The strong influence of *Nramp1* on macrophage activation most likely explains the selective effects of *Nramp1* on Th2-mediated allergy in our allergic asthma model.

Finally, *Nramp1* significantly affects the efficacy of *M. vaccae* in reducing the allergic and asthmatic response. While treatment of *Nramp1*[s] mice with *M. vaccae* reduced airway hyperresponsiveness, eosinophilia, levels of IgE and the IL-4 and IL-5 production, treatment of *Nramp1*[r] mice hardly reduced these parameters.

Nramp1 affected the macrophage activation after *M. vaccae* treatment and as a result, *Nramp1* affected probably the cellular, T cell-mediated immune response to *M. vaccae*. This effect of *Nramp1* on the T cell response to *M. vaccae* may include effects on regulatory T cells that probably mediate the inhibition of asthmatic responses after *M. vaccae* treatment.

In summary, this thesis shows that the macrophage, which function is determined by *Nramp1*, may play a crucial role in the downregulation by mycobacteria of allergic and asthmatic responses. The macrophage, whose activation is regulated by *Nramp1*, seems to be a key cell connecting innate, natural immunity with the adaptive, cellular immunity, and therefore might be an important target for the treatment of asthma.

Possible future directions for research

Concerning treatment of allergic asthma with *M. vaccae*, the role of *Nramp1* in the development of asthma and the role of *Nramp1* in *M. vaccae* therapy, some questions remain to be answered:

Do allergen-specific regulatory T cells mediate the inhibition of the allergic and asthmatic response in our model?
It remains to be investigated whether the induction of regulatory T cells is the only mechanism by which *M. vaccae* inhibits the allergic response. Interestingly, many of the observed effects of *M. vaccae* bear a resemblance to the inhibition of allergic asthma symptoms after glucocorticoid treatment. There are suggestions that (myco) bacterial infections directly, or indirectly, via neural pathways, activate the hypothalamus-pituitary gland-adrenal gland and hereby induce the secretion of cortisone/cortisol (79, 80), which subsequently may reduce allergic inflammation.

Which component of M. vaccae binds to which receptor?
Interactions of ligands of *M. vaccae* with several receptors on host cells play a crucial role in the immune response to *M. vaccae* and its efficacy in treatment. Mycobacteria express a whole range of ligands that trigger signalling through pattern recognition receptors (mannose receptor, Toll-Like Receptor (TLR) 2 and/or TLR 4) on host phagocytes and other cells, resulting in activation of phagocytes and the release of pro- or con-inflammatory mediators. *M. vaccae* express mycobacterial heat shock proteins (81), which have an important role in the regulation of the immune system during inflammation (82). However, the predominant non-protein antigen on mycobacteria is lipoarabinomannan (LAM). While slow-growing mycobacteria such as BCG and *M. tuberculosis* express mannose-capped LAM (manLAM), fast-growing mycobacteria express arabinofuransyl-terminated LAM (araLAM) which potently induces cytokine release from macrophages (83). Untill now, it is unknown which type of LAM *M. vaccae* expresses. This might be of importance since DC-SIGN,

an important recently discovered receptor for LAM binds only manLAM (84). Both CD14 and the macrophage mannose receptor serve as LAM receptors as well. In addition, the TLR 2 and 4 are capable of LAM (83) and heat shock protein signalling. Induction of Toll-like receptors and DC SIGN influences not only the induction of inflammatory responses but also to the development of antigen-specific adaptive immunity, including regulatory T cell development (84, 85).

Is Nramp1 expressed in dendritic cells and does Nramp1 affect the function of these cells?
Dendritic cells are important in both the induction of the allergic cascade and in the induction of cellular T cell-mediated response to bacteria. However, only limited, rather incomplete studies looked at the expression of *Nramp1* in dendritic cells (74, 86).

The Nramp1 gene encodes a divalent ion pump, therefore, what is the role of iron, or other divalent metal ions, in the influence of Nramp1 on development of allergy and the influence of Nramp1 on the efficacy M. vaccae?

What is the role of NO· in the observed influence of Nramp1 on allergic disease?

Is clearance of M. vaccae indeed affected by Nramp1?

How does the macrophage affect the cellular, T cell mediated response?

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Samenvatting

Allergie, astma, mycobacteriën en Nramp1.

Astma is een een chronische ziekte die gekenmerkt wordt door een chronische ontsteking van de luchtwegen en door overgevoeligheid van de luchtwegen voor specifieke en niet-specifieke prikkels (hyperreactiviteit). In een groot deel van de patienten ontstaat astma na ontsporing van het immuun systeem (allergie) tegen bepaalde stoffen (allergenen), men spreekt dan van allergisch asthma. Een allergie kan ook huidaandoeningen (eczeem), of darmklachten (voedselallergie) veroorzaken. De laatste jaren is er sprake van een toename van allergische ziekten in Westerse landen. Het percentage mensen met een luchtwegallergie lijkt in Nederland te zijn verdubbeld tot zo'n 10 %. In Engeland heeft zelfs 15 % van alle kinderen last van een luchtwegallergie, terwijl dit percentage in Albanië maar 3 % is. Waarschijnlijk wordt de snelle toename in allergische ziekten veroorzaakt door een verandering in de omgeving. Een toename in luchtvervuiling, een toename in de blootstelling aan allergenen door isolatie van huizen of veranderingen in het voedsel en de samenstelling van de darmflora zijn mogelijk van invloed op het ontstaan van allergieën.

Een verklaring voor de toename van allergische ziekten die veel aandacht heeft gekregen is de "hygiëne hypothese". Deze hypothese stelt dat door de verbeterde hygiëne en door vaccinaties en antibiotica, kinderen in een westerse maatschappij minder infecties doormaken. Dit verminderde aantal infecties zou het risico op de ontwikkeling van een allergische ziekten kunnen verhogen. Er zijn diverse studies die deze hypothese ondersteunen. Zo ontwikkelen kinderen die op een boerderij worden opgevoed minder snel een allergie, mogelijk door blootstelling aan allerlei bacteriën en hun componenten. Italiaanse soldaten die in contact zijn geweest met het hepatitis A virus hadden minder vaak allergieën dan hun leeftijdsgenoten. Allergie komt minder vaak voor bij kinderen die in een gezin leven met meerdere oudere kinderen en bij kinderen die veel in een crèche verbleven, en die dus meer infecties via andere kinderen konden oplopen.

Desondanks is er nog steeds onduidelijkheid en controverse over het verband tussen infecties en allergische ziekten. Inmiddels zijn er echter twee mechanismen die de hygiene hypothese proberen te verklaren. De eerste gaat uit van een balans binnen Thelper 1 en Thelper 2 lymfocyten (witte bloedcellen). Thelper cellen zijn de regisseurs van het immuun systeem en sturen andere cellen aan die de afweer tegen ziekteverwekkers verzorgen. Thelper 1 cellen spelen een rol bij infecties met bacteriën en virussen. Ze activeren bijvoorbeeld macrofagen, die vervolgens de bacterie kunnen opruimen. Thelper 2 cellen zijn belangrijk in de allergische reactie. Ze produceren stoffen (cytokines) die onder andere B lymphocyten aanzetten tot de productie van immunoglobuline E (IgE), een belangrijke antistof in de allergische reactie. Contact van allergenen met IgE zorgt voor activatie van mestcellen die hierna stoffen uitscheiden die een luchtwegvernauwing veroorzaken.

Th1 cellen kunnen de ontwikkeling van Th2 cellen remmen. Voor en vlak na de geboorte overheersen in een kind van nature de Thelper2 cellen maar na de

geboorte worden door infecties (Thelper 1 stimuli) de Thelper 1 en Thelper 2 cellen in evenwicht gebracht. Door een afname in de hoeveelheid infecties neemt echter de Thelper 1 stimulatie af en worden Thelper 2 cellen niet meer geremd, wat vervolgens het risico op het ontwikkelen van een allergie doet toenemen.

Het tweede mechanisme (figuur 1), dat momenteel de meeste bijval krijgt, gaat er vanuit dat allergie, maar ook bijvoorbeeld autoimmuniteit (een immuunreactie tegen de eigen lichaamscellen), een uiting is van een verstoorde regulatie van het immuunsysteem. Het immuunsysteem staat in nauw contact met zowel het eigen weefsel als de omgeving. Het is dus van belang om géén immuun reactie te ontwikkelen tegen (onschadelijke) stimuli en het eigen weefsel. Er is dus een vorm van regulatie nodig. Dit wordt grotendeels gedaan door een speciale subset van T cellen, de regulatoire T cellen. Deze cellen zorgen voor een remming van Thelper 1 en Thelper 2 reponsen door het uitscheiden van onderdrukkende cytokines als IL-10 en TGF1-ß en/of door direct contact tussen cellen te maken. Regulatoire cellen worden geïnduceerd bij infecties met bacteriën of parasitaire wormen. Hygiëne, antibiotica en vaccinaties hebben het aantal van deze infecties in kinderen gereduceerd, waardoor erdus ook minder regulatoire T cellen aangemaakt worden. Hierdoor krijgen ongecontroleerde immuunresponsen als allergie en autoimmuniteit een kans. In het geval van een ongecontroleerde Thelper 1 reactie spreekt men van autoimmuniteit, in het geval van een ongecontroleerde Thelper 2 reactie is er sprake van allergie.
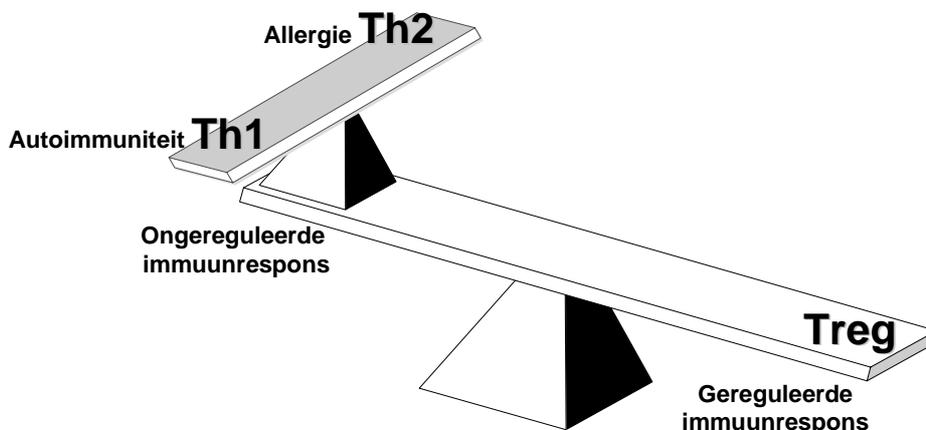
Figuur 1. *De balans tussen een gereguleerde immuun respons (door regulatoire T cellen, Treg) en een ongereguleerde immuun respons. De uitkomst van een ongereguleerde immuun respons hangt af van de balans tussen Thelper1 (Th1, autoimmuniteit) of Thelper 2 (Th2, allergie).*

Op basis van de hygiëne hypothese zou het dus mogelijk kunnen zijn om bacteriën en hun componenten te gebruiken om allergische ziekten te voorkomen of behandelen. Dit potentiële "anti-allergie of anti-astma" vaccin zou dan wel aan een aantal voorwaarden moeten voldoen. Allereerst moet het vaccin bestaan uit niet-pathogene bacteriën, moet het uiteraard weinig bijwerkingen veroorzaken, en toch een sterke Thelper1 of regulatoire T cel reactie kunnen induceren. Daarnaast zou een voordeel zijn als het vaccin bestaat uit een bacterie die normaliter voorkomt in

de omgeving, maar de laaste jaren mogelijk verdwenen is uit het Westerse milieu. Belangrijke kandidaten zijn dan de groep bacteriën die bekend staan als mycobacteriën. Naast de bekende ziekteverwekkers *Mycobacterium tuberculosis* (tuberculose) en *Mycobacterium leprae* (lepra) bestaat deze groep organismen vooral uit soorten die in de bodem en het water voorkomen. Blootstelling aan mycobacteriën wordt dus bepaald door de omgeving.

In het kader van de hygiëne hypothese hebben diverse studies al onderzocht of mycobacteriële vaccins, zoals BCG (het vaccin tegen tuberculose), allergie en astma kunnen voorkomen of behandelen. De eerste studies uitgevoerd in Japan en Guinee Bissau lieten zien dat kinderen die vlak na hun geboorte waren gevaccineerd met BCG, op latere leeftijd aantoonbaar minder allergische en astmatische verschijnselen hadden dan niet-gevaccineerde kinderen. Voorwaarde was echter wel dat het BCG vaccin was aangeslagen en een immuunreactie opwekte. Eenzelfde soort studies in Europa konden deze correlatie echter niet aantonen. Een mogelijk verband tussen BCG vaccinatie en de preventie van allergie en astma is dus nog controversieel. Mogelijk speelt hier de genetische achtergrond van kinderen een rol.

Het staat vast dat in het ontstaan van allergie en astma niet alleen omgevingsfactoren, zoals bovengenoemd, maar ook genetische factoren een cruciale rol spelen. Een kind van niet-allergische ouders heeft ongeveer 10 % kans om een allergie te ontwikkelen.Heeft het een allergisch broertje of zusje, dan wordt deze kans 20 %. Een kind met één allergische ouder heeft 40 % kans op het ontwikkelen van een allergie, terwijl deze kans bij een kind met twee allergische ouders maar liefst 60 % is. Om de genen op te sporen die voor deze aanleg verantwoordelijk zijn, zijn in de afgelopen jaren veel studies uitgevoerd, waaruit blijkt dat astma een genetisch complexe ziekte is met koppelingen naar veel verschillende genen. Om het genetisch onderzoek te vereenvoudigen maakt men veel gebruik van de muis als genetisch model. Bestaat er het vermoeden dat een bepaald gen een rol speelt in een ziekte dan spreekt men van een kandidaat-gen.

Eén van de kandidaat-genen voor het onwikkelen van allergie en astma zou het *Nramp1* gen kunnen zijn. *Nramp1* codeert voor een eiwit dat aanwezig is in met name fagocyterende cellen (cellen die bacteriën of virussen actief kunnen opnemen en doden), waaronder macrofagen. Dit eiwit bevindt zich in het gedeelte van deze cellen dat zorgt voor de ruiming van bacteriën. Het Nramp1 eiwit is waarschijnlijk een metaalionenpomp die er voor zorgt dat ofwel bacteriën sterven door een tekort aan metalen of dat metaalionen enzymen kunnen activeren die de bacteriën aanvallen. In muizen bestaan er twee variëteiten (allelen) van *Nramp1*. Het ene allel (*Nramp1^s*) codeert voor een defect *Nramp1* eiwit en maakt hierdoor muizen gevoelig voor een aantal bacteriën, waaronder interessant genoeg ook de mycobacteriën. Het andere allel (*Nramp1^r*) codeert voor een normaal *Nramp1* eiwit en daarom voor resistentie tegen deze bacteriën. Zoals hierboven genoemd zouden bacteriële infecties mogelijk een rol kunnen spelen in het ontstaan van allergische ziekten. Het *Nramp1* gen, dat de reactie op veel van deze infecties controleerd, zou dus een kandidaat-gen kunnen zijn voor allergie en astma

In het allergie en astma onderzoek wordt de muis veel gebruikt als model voor allergisch astma. Muismodellen voor astma hebben al veel bijgedragen aan de huidige kennis van de onderliggende mechanismen van deze ziekte. Het muismodel zoals gebruikt in dit proefschrift kent twee fasen. De eerste fase is het eerste contact, de sensibilisatie, met het allergeen, meestal het eiwit ovalbumine. In de tweede fase dient men het allergeen een aantal keren toe in de luchtwegen en er vindt een allergische respons plaats. Er ontwikkelen zich nu in de muis verschillende astmatische verschijnselen zoals luchtweghyperreactiviteit, luchtwegontsteking en een stijging in het bloed van de hoeveelheid IgE.

Doel van dit proefschrift

Mycobacteriën zouden mogelijk allergisch astma kunnen helpen voorkomen of behandelen. Aangezien het *Nramp1* gen de mate van immuniteit tegen mycobacteriën bepaalt, zou dit gen ook wel eens de effectiviteit van mycobacteriën in de behandeling van allergisch astma kunnen beïnvloeden. In de studies beschreven in dit proefschrift hebben we gebruik gemaakt van een hitte-gedood preparaat van *Mycobacterium vaccae*, een mycobacterie die voor het eerst beschreven werd in de omgeving van boerderijen en vee. Dit is interessant in het licht van de studies die lieten zien dat deze omgeving juist beschermend werkt in het ontstaan van allergie en astma. Ook maakten we gebruik van *Nramp1* congene muizen, genetisch identieke muizen die uitsluitend verschillen in het *Nramp1* gen dat ze bezitten. De ene muizenstam is *Nramp1*[r] (resistent) en de ander *Nramp1*[s] ("susceptible", gevoelig).

In de studies beschreven in dit proefschrift hebben we getracht de volgende vragen te beantwoorden:

1. Kan gedode *Mycobacterium vaccae* allergisch astma in de muis voorkomen of behandelen?
2. Beïnvloedt het *Nramp1* gen, dat de resistentie tegen mycobacteriën controleert, ook de mate van gevoeligheid voor allergisch astma in de muis?
3. Beïnvloedt het *Nramp1* gen de effectiviteit van gedode *Mycobacterium vaccae*?

1. Kan gedode Mycobacterium vaccae allergisch astma in de muis voorkomen of behandelen?

We veronderstelden eerst dat mogelijke behandeling van astma in ons model grofweg op twee momenten kan gebeuren: voor het ontstaan van allergie en astma (prophylaxis) of erna (therapie). In hoofdstuk 2 onderzochten we of prophylactische behandeling met gedode *M. vaccae*, 2 of 3 weken voor het begin van de sensibilisatie met ovalbumine, in staat is het ontstaan van astmatische verschijnselen in de muis te voorkomen. De *M. vaccae* behandeling leidde echter niet tot verlaging van bijvoorbeeld de mate van ontsteking in de long en luchtweghyperreactiviteit. Wel kon aangetoon worden dat *M. vaccae* een sterke T cel gemedieerde immuunreactie opwekt. Hieruit en uit studies van anderen concludeerden we dat het tijdstip van *M. vaccae* toediening en de gebruikte dosis voldoende was om een sterke Thelper 1 reactie op te wekken. Echter, deze sterke Thelper 1 reactie was dus niet in staat de allergische Thelper2 reactie te onderdrukken.

In hoofdstuk 3 onderzochten we of therapeutische behandeling van astma met *M. vaccae*, na sensibilisatie en 2 en 3 weken voor het begin van de ovalbumine challenge, wel effectief was. Dit experiment werd uitgevoerd in een mild en een ernstig model voor allergisch astma om er zeker van te zijn dat het effect van *M. vaccae* niet afhankelijk is van de ernst van het gebruikte astma model. In beide modellen was de behandeling met *M. vaccae* echter niet in staat om de allergische en astmatische verschijnselen te verlagen. Wel bleek dat het veronderstelde ernstige model voor astma inderdaad sterkere allergische en astmatische reacties veroorzaakte dan het milde model.

Het tijdstip van *M. vaccae* toediening is mogelijk van belang, en hangt misschien af van allergeen blootstelling. Daarom werden in de studies beschreven in hoofdstuk 4 muizen gedurende ovalbumine sensibilisatie of challenge behandeld met *M. vaccae*. Deze behandeling, mits *M. vaccae* gegeven werd in een bepaalde dosis, bleek nu wel effectief in het verlagen van de astmatische verschijnselen: de mate van luchtweghyperreactiviteit, luchtwegontsteking, de hoeveelheden IgE en Thelper 2 cel cytokines waren aanzienlijk verlaagd. Zelfs behandeling tijdens de ovalbumine challenge was in staat astmatische verschijnselen te verlagen. Deze bevindingen laten zien dat het tijdstip en de dosis van *M. vaccae* behandeling belangrijk is. In ons model moet *M. vaccae* gedurende allergeen blootstelling gegeven worden. Andere studies hebben laten zien dat *M. vaccae* in staat is regulatoire T cellen op te wekken, die de allergische reactie onderdrukken. Waarschijnlijk is dit het mechanisme waardoor *M. vaccae* in staat is de verstoorde immuun balans te herstellen.

2. Beïnvloedt het Nramp1 gen, dat de resistentie tegen mycobacteriën controleert, ook de mate van gevoeligheid voor allergisch astma in de muis?

In de studies beschreven in hoofdstuk 5 onderzochten we of het *Nramp1* gen, dat de resistentie tegen mycobacteriën controleert, ook de ontwikkeling van allergie en astma beïnvloedt. In de muis zijn er twee allelen (genetische variëteiten) van *Nramp1*: het *Nramp1*[r] allel en het *Nramp1*[s] allel. Na ovalbumine sensibilisatie en challenge

vertoonden *Nramp1^s* en *Nramp1^r* muizen een gelijke mate van hyperreactiviteit en ontsteking van de luchtwegen. De hoeveelheden IgE en Thelper 2 cytokines en de mate van mestcelactivatie waren echter wel veel lager in de *Nramp1^r* muizen vergeleken met de *Nramp1^s* muizen. Het lijkt er dus op dat in de muis *Nramp1* wel de mate van allergie, maar niet de mate van astma beïnvloedt. Een verschil in macrofaagactivatie, veroorzaakt door *Nramp1*, is waarschijnlijk de oorzaak van de effecten van *Nramp*1 op de ontwikkeling van allergie. De macrofaag is in de luchtwegen een cel die veel immuun responsen in de long remt en onder controle houdt, onder andere door de stof nitric oxide (NO·). De lagere activiteit van *Nramp1^s* macrofagen vergeleken met *Nramp1^r* macrofagen resulteert dan waarschijnlijk ook in een verminderde remming van de Thelper 2 en IgE-gemedieerde reacties. Door het controleren van de activatie van de macrofaag, een cel die ook in allergische en astmatische reacties een rol speelt, kan *Nramp1* de ontwikkeling van allergie beïnvloeden. Het *Nramp1* gen kan dus worden beschouwd als een nieuw kandidaat gen voor het onstaan van allergie.
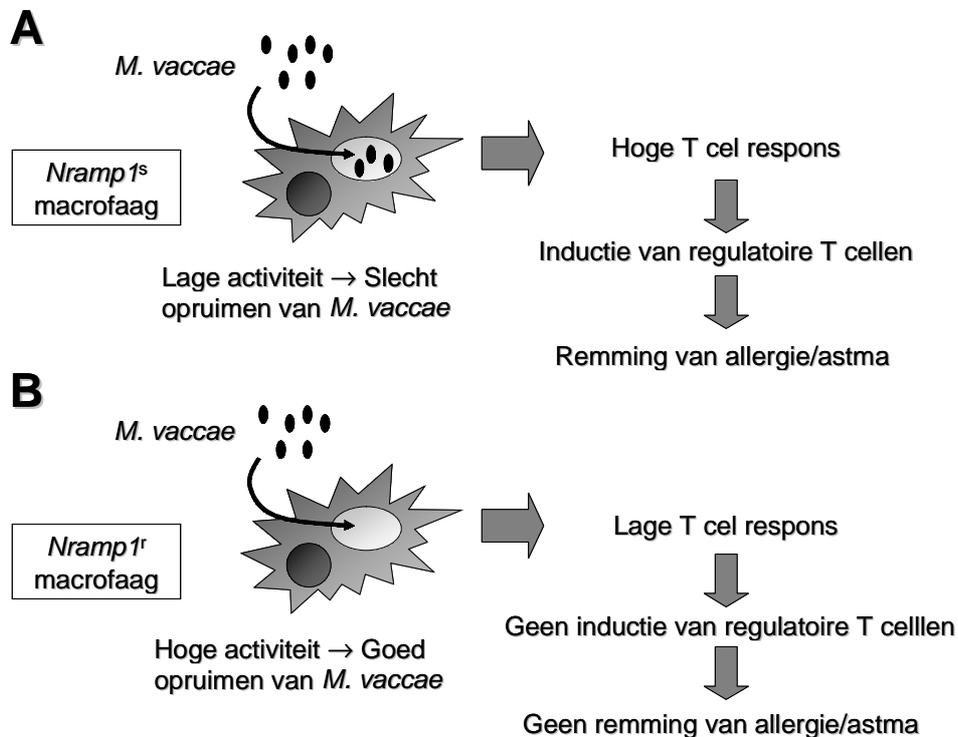
3. Beïnvloedt het Nramp1 gen de effectiviteit van Mycobacterium vaccae?

Hierboven is beschreven dat *M. vaccae,* onder bepaalde omstandigheden in staat is om allergische en astmatische verschijnselen in de muis te voorkomen en te behandelen. Ook toonden we aan dat het *Nramp1* gen de mate van allergische (maar niet van astmatische) verschijnselen beïnvloedt. De volgende stap was te onderzoeken of allelen van *Nramp1* de effectiviteit van *M. vaccae* beïnvloeden. De experimenten beschreven in hoofdstuk 6 laten zien dat *M. vaccae* effectief is in het verlagen van allergie en astma in *Nramp1^s* muizen, maar niet in *Nramp1^r* muizen. Ook toonden we aan dat in *Nramp1^s* muizen *M. vaccae* een sterkere T cel gemedieerde reactie induceerde in vergelijking met de *Nramp1^r* muizen. Er lijkt dus een verband te zijn tussen het *Nramp1* gen, een gestoorde immuunrespons op *M. vaccae* en een verminderde effectiviteit van *M. vaccae* in het voorkomen van allergie en astma.

Hoe *Nramp1* de respons op *M. vaccae* beïnvloed is nog niet helemaal duidelijk. We toonden wel aan dat *Nramp1^s* macrofagen minder geactiveerd werden na stimulatie met *M. vaccae* dan *Nramp1^r* macrofagen. Mogelijk leidt deze verminderde activatie van *Nramp1^s* macrofagen tot het relatief slecht kunnen opruimen van *M. vaccae* in deze cellen. Om *M. vaccae* toch op te kunnen opruimen heeft de *Nramp1^s* macrofaag hulp van T cellen nodig. We toonden inderdaad aan dat er een sterkere T cel reactie op *M. vaccae* in *Nramp1^s* muizen ontwikkelt. Dit kan tot gevolg hebben dat er ook een sterkere regulatoire T cel respons in *Nramp1^s* muizen ontwikkelt, die weer de ontwikkeling van allergische en astmatische verschijnselen remt (Figuur 2).

De effecten van *Nramp1* op de effectiviteit van *M. vaccae* zoals beschreven in hoofdstuk 6 zouden belanrijke gevolgen kunnen hebben voor het gebruik van *M. vaccae* en andere mycobacteriële vaccins in de behandeling van astma. Variaties in het humane NRAMP1 gen zouden wel eens een deel van de verklaring kunnen zijn voor de controverse rond het verband tussen BCG vaccinatie en allergie en astma.

A

M. vaccae

Nramp1ˢ
macrofaag

Hoge T cel respons

Inductie van regulatoire T cellen

Remming van allergie/astma

Lage activiteit → Slecht
opruimen van *M. vaccae*

B

M. vaccae

Nramp1ʳ
macrofaag

Lage T cel respons

Geen inductie van regulatoire T celllen

Geen remming van allergie/astma

Hoge activiteit → Goed
opruimen van *M. vaccae*

Figuur 2. *Hypothetisch schema over de invloed van het Nramp1 gen op de effectiviteit van M. vaccae in allergie/astma. A: Nramp1ˢ muizen, B: Nramp1ʳ muizen*

Conclusies

In dit proefschrift laten we zien dat gedode *M. vaccae* in staat is om allergische en astmatische reacties te voorkomen en te behandelen. *M. vaccae* moet echter wel tijdens allergeen blootstelling en in een specifieke dosis gegeven worden. Ten tweede laten we zien dat het *Nramp1* gen, dat de resistentie tegen mycobacteriën controleert, de mate van allergie, maar niet de mate van astma beïnvloedt. Als laatste laten we zien dat het *Nramp1* gen ook de effectiviteit van *M. vaccae* in ons allergisch astma model beïnvloedt. Het lijkt dus mogelijk om *M. vaccae* te gebruiken ter preventie of als behandeling van astma. Op dit moment worden verschillende klinische studies uitgevoerd waarin het effect van *M. vaccae* in allerlei allergische ziekten in de mens, waaronder astma bestudeert wordt. Naar onze mening moet er echter wel rekening gehouden worden met complicerende factoren zoals de mate van allergeen blootstelling en de genetische opmaak van het individu. Met name genetische variëteiten van het *Nramp1* gen zouden een rol kunnen spelen. Dit gen kan ook gezien worden als een nieuw kandidaat-gen voor allergie. Wellicht is *Nramp1* een sleutel in de relatie tussen (bacteriële) infecties en allergische ziekten.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 25 februari 1975 te Ermelo. Na het behalen van het VWO diploma aan het Nassau Veluwe College in Harderwijk in 1994, begon hij aan de studie Biologie aan de Universiteit Utrecht. In de docteraalfase van deze studie werden twee onderzoeksstages voltooid. De eerste stage vond plaats bij de afdeling Immuno-, Inhalatie- en *In Vitro* Toxicologie, divisie Toxicologie van TNO voeding onder leiding van Dr. Léon Knippels. Hier deed hij ter validatie van een rattenmodel voor voedselallergie onderzoek naar immuungemedieerde effecten in dit model. De tweede stage werd uitgevoerd bij de afdeling Voeding, Faculteit Diergeneeskunde van de Universiteit Utrecht onder leiding van Dr. Thomas Schonewille en Prof. Anton Beynen. In deze stage deed hij onderzoek naar de effecten van een hoge kalium concentratie in de voeding op de plasma vitamine B_{12}-concentraties in Ouessant schapen. Vervolgens was hij vanaf januari 1999 werkzaam als assistent-in-opleiding bij de afdeling Farmacologie en Pathofysiologie van de faculteit Farmaceutische Wetenschappen van de Universiteit Utrecht en het Laboratorium voor Pathologie en Immunobiologie in het RIVM. Daar werkte hij aan het onderzoeksproject "Asthma a disease due to the lack of bacterial infections in child-hood" gefinancierd door het Astma Fonds en het NWO/ZonMw onder leiding van Frans Nijkamp, Henk van Loveren en Gert Folkerts. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

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