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The *Slc11a1* (*Nramp1*) Gene Controls Efficacy of Mycobacterial Treatment of Allergic Asthma¹

Joost J. Smit,*[†] Henk Van Loveren,^{†‡} Maarten O. Hoekstra,[§] Khalil Karimi,* Gert Folkerts,^{2*} and Frans P. Nijkamp*

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 whether *Nramp1* (*Slc11a1*) alleles, which 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 in a mouse model. Treatment of OVA-sensitized *Nramp1*^s mice with *M. vaccae* suppressed airway hyperresponsiveness, airway eosinophilia, Ag-specific IgE, and IL-4 and IL-5 production after OVA aerosol challenge. In contrast, *M. vaccae* hardly affected these parameters in *Nramp1*^r mice. In addition, the *Nramp1* gene affected both T cell-mediated responses to *M. vaccae* in vivo and the level of macrophage activation after stimulation with *M. vaccae* in vitro. In conclusion, the efficacy of *M. vaccae* in preventing allergic and asthmatic manifestations in a mouse model is strongly affected by *Nramp1* alleles. These findings could have important implications for the future use of mycobacteria and their components in the prevention or treatment of allergic asthma. A new link is described between genes, the environment, and the development of allergy, in which the *Nramp1* gene fine tunes the hygiene hypothesis. *The Journal of Immunology*, 2003, 171: 754–760.

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 through the balance of Th1 and Th2 immune mechanisms. Because 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 that demonstrated that children that had been vaccinated with bacille Calmette-Guérin (BCG)³ 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 with 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 Ref. 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 *Mycobacterium tuberculosis*). More importantly, because the ability to mount a response to mycobacterial Ags 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 *Mycobacterium vaccae* is able to prevent and suppress features of allergy and asthma in a mouse model.⁴ Because *Nramp1* controls the immune

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³ Abbreviations used in this paper: BCG, bacille Calmette-Guérin; DIG, digoxigenin; DTH, delayed-type hypersensitivity; Penh, enhanced pause.

⁴ J. J. Smit, H. Van Loveren, M. O. Hoekstra, M. A. Schijf, G. Folkerts, and F. P. Nijkamp. *M. vaccae* administration during allergen sensitization or challenge suppresses asthmatic features. *Submitted for publication*.

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*⁺) and congenic C.D2-Vil6 (*Nramp1*⁻) mice were kindly provided by M. Potter (National Cancer Institute, Bethesda, MD). The C.D2-Vil6 mouse was originally derived from crossing a BALB/cAnPt and a DBA/2Npt (*Nramp1*⁻) mouse, which was crossed back to BALB/cAnPt 23 times (24). Animals were maintained under specific pathogen-free 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 wk 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 *M. vaccae* (SRL172, 10 mg or 10¹⁰ CFU/ml) were kindly provided by SR Pharma (London, U.K.). Diff-Quick staining solutions were purchased from Dade A.G. (Düdingen, Switzerland). OVA (grade V), acetyl- β -methylcholine chloride (methacholine), BSA, and *o*-phenylenediamine-dichloride substrate were purchased from Sigma-Aldrich (St. Louis, MO). Aluminum hydroxide (AlumInject) was obtained from Pierce (Rockford, IL). Digoxigenin (DIG), anti-DIG Fab coupled to HRP, and protease inhibitor were purchased from Roche Diagnostics (Basel, Switzerland). Anti-mouse IgE, biotinylated anti-mouse IgG1, and biotinylated anti-mouse IgG2a were obtained from BD PharMingen (San Diego, CA). Peroxidase-conjugated streptavidin (poly(HRP)) was purchased from CLB (Amsterdam, The Netherlands). ELISA buffer contained 0.5% BSA, 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, and 0.05% Tween 20. RPMI 1640 and DMEM medium was supplemented with 10% FCS, 4 mM L-glutamine, 5 $\times$ 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 μ g OVA adsorbed onto 2.25 mg aluminum hydroxide in 100 μ l saline on days 0 and 14. On days 35, 39, and 42, mice were challenged by inhalation of either OVA or saline aerosols in a Plexiglas exposure chamber for 20 min. The aerosols were generated by nebulizing an OVA solution (10 mg/ml) in saline or saline alone using a Pari LC Star nebulizer (Pari Respiratory Equipment, Richmond, VA) driven by compressed air at a flow rate of 6 L/min. *M. vaccae*-treated mice received a s.c. injection with 10⁷ CFU (0.01 mg) heat-killed *M. vaccae* in 100 μ l saline on days 0 and 14, immediately before the i.p. OVA/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, Sharon, CT). 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 min, 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 min. Aerosols were generated by a Pari LC Star nebulizer, and each aerosol was followed by 3 min 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 four times with 1 ml saline at 37°C. Lung lavage cells of each mouse were collected after centrifugation, pooled, and resuspended in 150 μ l 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 OVA-specific IgS

Blood was withdrawn by heart puncture 24 h after the last allergen challenge to prepare serum for determination of Ab levels in serum by ELISAs using microtiter plates from Nunc A/S (Roskilde, Denmark), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% Tween 20 for washing between incubations. To determine OVA-specific IgE levels, wells were coated overnight at 4°C with 1 μ g/ml of anti-mouse IgE in PBS, followed by blocking for 1 h and incubation of the wells with diluted serum samples and duplicate dilution series of an OVA-specific IgE reference serum, prepared as described previously (28), for 2 h. Hereafter, wells were incubated for 1 h with 1 μ g/ml of DIG-conjugated OVA, followed by incubation with anti-DIG Fab coupled to HRP, according to manufacturer's instructions.

To assess OVA-specific IgG1 or IgG2a levels, wells were coated with 10 μ g/ml OVA in PBS. After blocking, diluted serum samples or duplicate dilution series of a reference standard serum obtained from multiply OVA-boostered mice were added. Hereafter, wells were incubated with 1 μ g/ml of biotinylated anti-mouse IgG1, or 1 μ g/ml of biotinylated anti-mouse IgG2a for 2 h, followed by 1/10,000 diluted poly(HRP) for 1 h.

For color development, 0.4 mg/ml of *o*-phenylenediamine and 4 mM H₂O₂ in PBS were used, and the reaction was stopped by adding 4 M H₂SO₄. OD was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

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

Levels of IL-4, IL-5, and IL-10 in the lung lavage fluid were analyzed by sandwich ELISA using Ab pairs and standards purchased from BD PharMingen, according to manufacturer's instructions. Levels of TGF1- β 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- β was 8, 32, 10, and 60 pg/ml, respectively.

Measurement of delayed-type hypersensitivity (DTH)

For measurement of DTH to heat-killed *M. vaccae*, mice were injected s.c. in the neck with 10⁷ or 10⁸ CFU (0.01 or 0.1 mg) heat-killed *M. vaccae* in 100 μ l saline or saline alone on days 0 and 7. On day 21, mice were anesthetized by an i.m. injection of a mixture of xylazine and ketamine, and ear thickness of both ears was measured using a spring-loaded caliper (number 293-561; Mitutoyo, Veenendaal, The Netherlands). Accordingly, all mice were challenged intradermally with 15 μ g heat-killed *M. vaccae* in 20 μ l 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 h 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 D. Radzioch, McGill University, Montreal, Canada) are bone marrow-derived macrophage lines obtained from *Nramp1*⁺ and *Nramp1*⁻ congenic mice, respectively (29). These cells were cultured in DMEM, washed, and plated at 5 $\times$ 10⁵ cells/well in a 24-well plate (Costar, Cambridge, MA). Cells were incubated with medium alone or medium containing 10⁷, 10⁸, or 2.5 $\times$ 10⁸ CFU/ml *M. vaccae* for 48 h at 37°C with 5% CO₂. 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 h after stimulation was determined using a commercially available ELISA kit (Biosource), according to the instructions of the manufacturers. Nitrite (NO₂⁻) concentrations were measured using the Griess reaction (30).

Statistical analysis

All data are expressed as mean $\pm$ SEM. The airway response curves to methacholine and DTH responses were statistically analyzed by a general linear model of repeated measurements, followed by post hoc comparison

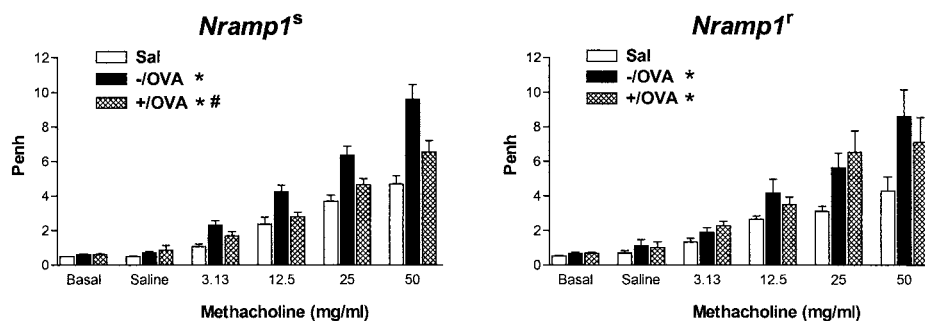


FIGURE 1. *Nramp1* affects the inhibition of airway hyperresponsiveness by killed *M. vaccae*. Airway response to increasing concentrations of nebulized methacholine of OVA-sensitized *Nramp1^s* or *Nramp1^r* mice, 24 h after final respiratory aerosol challenge with saline (Sal) or OVA. Mice were sensitized i.p. with OVA/alum on days 0 and 14 and challenged on days 35, 39, and 42. Mice of each strain were treated with 10^7 CFU heat-killed *M. vaccae* during OVA sensitization on days 0 and 14 (+/OVA). On day 43, unrestrained plethysmography measurements were performed for 3 min after each exposure to methacholine and expressed as Penh values. Data are represented as mean $\pm$ SEM, $n = 8$; *, $p < 0.05$ compared with the saline-challenged control groups. #, $p < 0.05$ compared with the -/OVA *Nramp1^s* group.

between groups. Data were $\log_{10}$ 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 OVA-sensitized mice to increasing concentrations of methacholine 24 h after final OVA 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 OVA-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 OVA 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.

Second, we examined the influx of inflammatory cells into the lungs after OVA challenge. Cell counts in the lung lavage fluid obtained 24 h after final challenge showed that in both strains OVA challenge induced a significant increase in the total number of cells in the lavage fluid compared with 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 nontreated OVA-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.

These results clearly demonstrate that *Nramp1* affects the efficacy of *M. vaccae* in reducing asthmatic manifestations, as characterized by airway hyperresponsiveness and eosinophilia.

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, Ab levels of sera prepared from blood collected 24 h before (data not shown) or after the final saline challenge of OVA-sensitized *Nramp1^s* and *Nramp1^r* mice appeared similar (Fig. 3). OVA challenge, however, markedly increased serum levels of OVA-specific IgE (Fig. 3A) compared with the saline-challenged animals, but to a significantly lesser extent in the *Nramp1^r* mice compared with the *Nramp1^s* mice. Treatment with *M. vaccae* decreased the levels of IgE after OVA challenge only in the *Nramp1^s* mice. OVA challenge of nontreated mice induced a comparable increase in the levels of OVA-specific IgG1 and IgG2a in both strains, compared

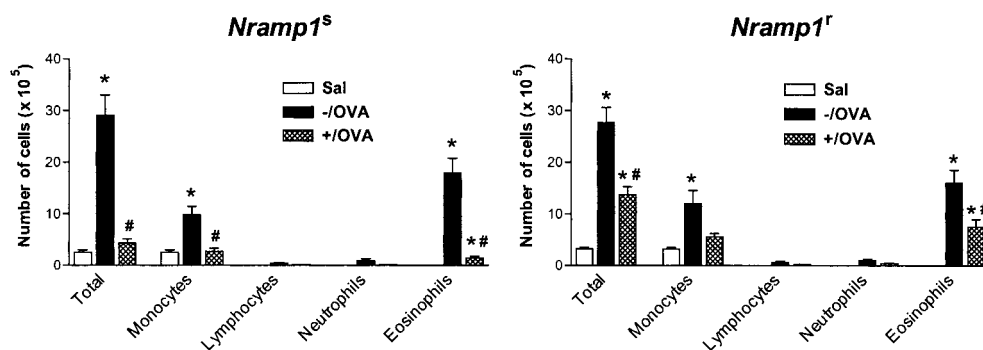


FIGURE 2. *Nramp1* influences the inhibition of airway eosinophilia by killed *M. vaccae*. Number of cells in lung lavage fluid obtained from OVA-sensitized *Nramp1^s* or *Nramp1^r* mice, 24 h after final respiratory aerosol challenge with saline (Sal) or OVA. Mice were sensitized i.p. with OVA/alum on days 0 and 14 and challenged on days 35, 39, and 42. Mice of each strain were treated with 10^7 CFU heat-killed *M. vaccae* during OVA 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 $\pm$ SEM, $n = 8$. *, $p < 0.01$ compared with the saline-challenged control groups. #, $p < 0.05$ compared with the -/OVA group of each strain.

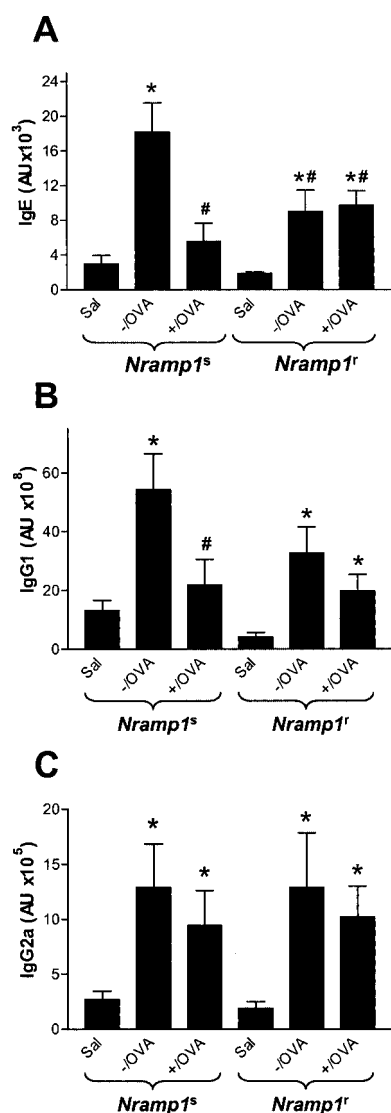


FIGURE 3. The inhibition of IgE and IgG1 levels by *M. vaccae* treatment is influenced by *Nrampl1*. Levels of OVA-specific IgE (A), IgG1 (B), and IgG2a (C) measured by ELISA in serum OVA-sensitized *Nrampl1^s* or *Nrampl1^r* mice, 24 h after final respiratory aerosol challenge with saline (Sal) or OVA. Mice were sensitized i.p. with OVA/alum on days 0 and 14 and challenged on days 35, 39, and 42. Mice of each strain were treated with 10^7 CFU heat-killed *M. vaccae* during OVA sensitization on days 0 and 14 (+OVA). Vertical bars indicate mean arbitrary units (AU) $\pm$ SEM, $n = 8$, $p < 0.01$ compared with the saline-challenged control groups. #, $p < 0.05$ compared with the -OVA *Nrampl1^s* group.

with the saline-challenged animals (Fig. 3, B and C). *M. vaccae* treatment decreased the IgG1 response after challenge only in the *Nrampl1^s* mice. No effect of *M. vaccae* was observed on the serum levels of IgG2a after challenge.

Because 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- β . Lung lavage fluid of OVA-sensitized mice contained no detectable levels of IL-4, IL-5, TGF1- β , and IL-10 after saline challenge (Fig. 4). OVA challenge caused a significant increase in the levels of these cytokines in both strains as compared with the saline-challenged controls. Lung lavage fluid of *Nrampl1^r* mice, however, contained one-half to one-third the amount of IL-4, IL-5, and IL-10 as found in lavage fluid

of *Nrampl1^s* mice. In addition, *M. vaccae* treatment of *Nrampl1^s* mice significantly reduced the levels of IL-4 and IL-5 and TGF1- β . No effect of *M. vaccae* treatment on these cytokines was observed in *Nrampl1^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). To summarize, these data show that *Nrampl1* strongly influences the reduction of Th2 responses and subsequent allergic responses in the lung after *M. vaccae* treatment.

Nrampl1 affects the T cell-mediated responses to *M. vaccae*

Next, to examine whether *Nrampl1* influenced T cell-mediated responses to heat-killed *M. vaccae*, we measured DTH responses to these killed bacteria. Challenge with *M. vaccae* in the ear induced a small background swelling in nonsensitized mice ($<25 \mu\text{m}$). A significantly stronger ear swelling, however, was observed in *M. vaccae*-sensitized mice compared with nonsensitized mice in both strains (Fig. 5) that peaked at 24 h and resolved at 216 h. 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 *Nrampl1^r* mice compared with the *Nrampl1^s* mice. So, we demonstrated that T cell-mediated, cellular immune responses were significantly affected by *Nrampl1*.

M. vaccae strongly activates macrophages in vitro, which is affected by *Nrampl1*

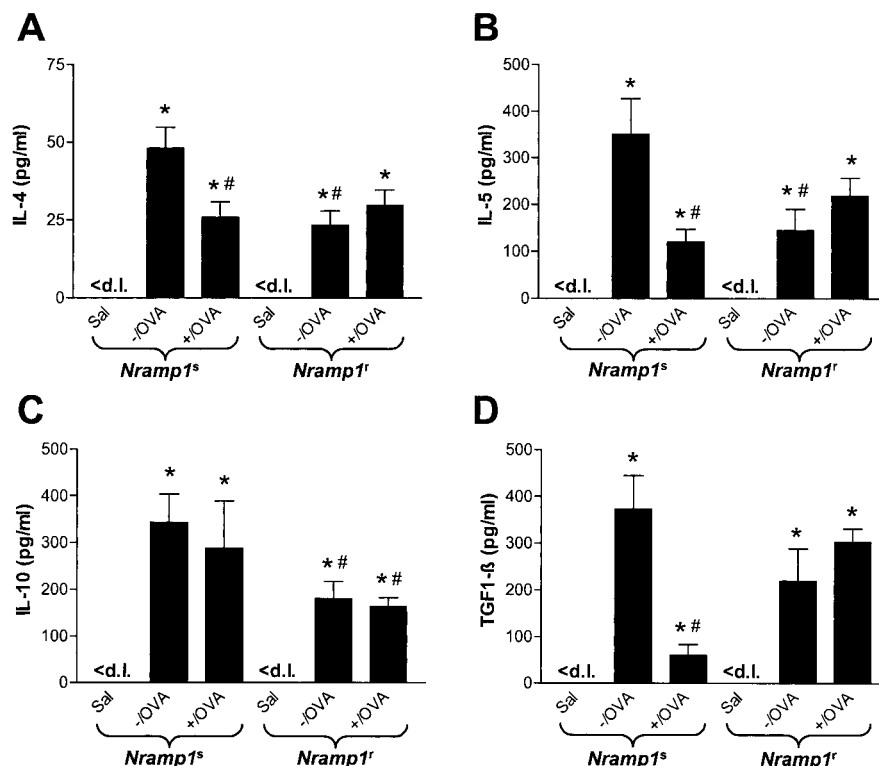
Finally, because *Nrampl1* is almost exclusively expressed in macrophages, we investigated the effect of heat-killed *M. vaccae* on macrophage function in vitro. *Nrampl1^r* and *Nrampl1^s* macrophages were stimulated for 48 h with increasing concentrations of *M. vaccae*. *M. vaccae* dose dependently activated *Nrampl1^r* macrophages, as indicated by the production of NO (measured by NO_2 concentrations) and TNF- α (Fig. 6). Interestingly, the production of NO and TNF- α was considerably less in the *Nrampl1^s* compared with the *Nrampl1^r* macrophages. Therefore, it was demonstrated that *Nrampl1* controls the capacity of macrophages to become activated by *M. vaccae*.

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 *Nrampl1^s* mice. As treatment of *Nrampl1^r* mice with *M. vaccae* did not lead to any reduction in these manifestations of allergic asthma, with the exception to a certain extent of airway eosinophilia, we concluded that *Nrampl1* strongly influences the efficacy of *M. vaccae* in treatment of allergic asthma in a mouse model.

The present data are 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 down-regulate the allergic Th2 response (13). Most likely, other mechanisms for the mode of action of *M. vaccae* must be accounted for. Zuany-Amorim et al. (31) clearly demonstrated that vaccination with heat-killed *M. vaccae* in mice induced allergen-specific CD4^+ cells, in particular T regulatory $\text{CD4}^+\text{CD25}^+\text{CD45RB}^{\text{low}}$ cells, which diminished allergic inflammation. DTH reactions have shown to be absolutely dependent on the presence of CD4^+ T cells (32, 33). By inducing a DTH reaction after *M. vaccae* sensitization and challenge, we investigated the effect of *Nrampl1* on the development of the CD4^+ T cell-mediated response to *M. vaccae*. We demonstrated that the

FIGURE 4. The inhibition of Th2 responses after allergen challenge by *M. vaccae* is influenced by *Nramp1*. Levels of IL-4 (A), IL-5 (B), IL-10 (C), and TGF1- β (D) measured by ELISA in lung lavage fluid obtained from OVA-sensitized *Nramp1^S* or *Nramp1^r* mice, 24 h after final respiratory aerosol challenge with saline (Sal) or OVA. Mice were sensitized i.p. with OVA/alum on days 0 and 14 and challenged on days 35, 39, and 42. Mice of each strain were treated with 10^7 CFU heat-killed *M. vaccae* during OVA sensitization on days 0 and 14 (+OVA). Data are represented as mean pg/ml $\pm$ SEM, $n = 8$. *, $p < 0.01$ compared with the saline-challenged control groups. #, $p < 0.05$ compared with the -/OVA *Nramp1^S* group. <d.l., Below detection limit.



lower DTH response 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, *Nramp1^r* mice have lower Th2 responses, as measured by Th2 cytokines and allergen-specific IgE, to merely OVA. *Nramp1* generally affects the T cell-mediated immunity to several Ags, including OVA, 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 a significant increase in regulatory CD4⁺CD25⁺CD45RB^{low} cells in *Nramp1^S* mice after *M. vaccae* immunization compared with nonimmunized mice, while *M. vaccae* vaccination did not increase the number of this cell type in *Nramp1^r* mice (data not shown). Although the inhibition of asthmatic responses in mice by regulatory T cells was reported to be mediated through IL-10 and TGF- β (31), we did not observe an up-regulation of both IL-10 and TGF- β after *M. vaccae* treatment. In contrast, TGF- β levels were decreased after *M. vaccae* treatment. However, both cytokines are indicative of Th2 feedback mechanisms after OVA-induced inflammation (35). Because 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 Ags such as killed *M. vaccae* (38). Therefore, to provide a mechanism for the observed phenomena caused by *Nramp1*, the effect of *Nramp1* on activation of macrophages by *M. vaccae* was investigated. 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 NO and TNF- α . How *Nramp1* affects the macrophage activity is demonstrated by several other investigators. The *Nramp1* protein is a divalent cation (Fe²⁺, Zn²⁺, and Mn²⁺) transporter, which mediates metal ion homeostasis in macrophages (39). *Nramp1^S* macrophages are less capable of releasing iron than *Nramp1^r* macrophages (40, 41), and in this way, high cytoplasmic iron levels in *Nramp1^S* macrophages cause the reduced capacity of *Nramp1^S* macrophages to become activated (42–44).

Because activation of macrophages, as mediated by *Nramp1*, is necessary for clearance of mycobacteria or their components (23,

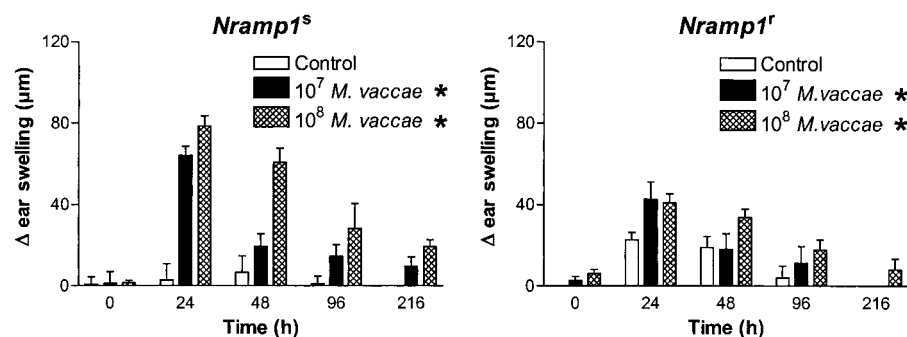


FIGURE 5. *Nramp1* influences the DTH responses to *M. vaccae*. *Nramp1^S* and *Nramp1^r* mice were injected with 10^7 or 10^8 CFU heat-killed *M. vaccae* or saline (Control) on days 0 and 7. On day 21, ear thickness of both ears was measured and mice were challenged with heat-killed *M. vaccae* in the ear. Hereafter, thickness of both ears was measured 24, 48, 96, and 216 h after challenge. Data are depicted as mean swelling of the left ear compared with the control right ear $\pm$ SEM ($n = 6$). *, $p < 0.01$ compared with the control group of both strains.

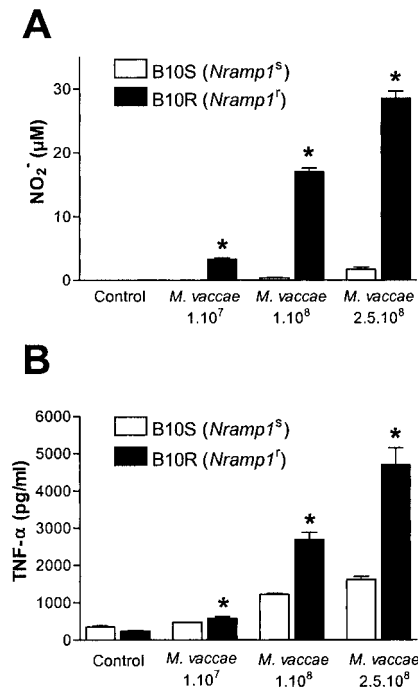


FIGURE 6. *Nramp1* affects NO and TNF-α production after *M. vaccae* stimulation. B10R (*Nramp1*^r) and B10S (*Nramp1*^s) macrophages were incubated for 48 h with medium (control) or medium with increasing concentrations of heat-killed *M. vaccae* (1×10^7 , 1×10^8 , and 2.5×10^8 CFU/ml). After incubation, the supernatant of the different cell cultures was collected and analyzed for nitrite (NO₂⁻) concentrations (A), using the Griess reaction, or TNF-α (B) concentrations by ELISA. Data are represented as mean μM or pg/ml ± SEM, respectively, $n = 4$. *, $p < 0.01$ compared with B10S macrophages.

37), it is likely that clearance of *M. vaccae* is inferior and delayed in *Nramp1*^s mice. The natural defense to mycobacteria or their components mediated through macrophages can be either impaired (as in *Nramp1*^s animals) or overcome by a high infectious dose. At that stage, the acquired immunity becomes the main effector mechanism (45). After *M. vaccae* vaccination in *Nramp1*^s mice, the inferior clearance of *M. vaccae* in *Nramp1*^s macrophages probably resulted in a higher antigenic load available for professional presenting cells, such as dendritic cells. Subsequently, the T cell-mediated immune response was stronger in *Nramp1*^s mice than *Nramp1*^r mice, as demonstrated by the higher DTH response to *M. vaccae*. Consequently, it is likely that more regulatory T cell responses develop after *M. vaccae* vaccination in *Nramp1*^s mice. The antiasthmatic effect of *M. vaccae* is dependent on the induction of regulatory T cells (31); the higher induction of these cells in *Nramp1*^s mice may explain the higher efficacy of *M. vaccae* to reduce the allergic and asthmatic responses in these mice. To summarize, we hypothesize that the low capacity of *Nramp1*^s macrophages to become activated results in 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 possibly down-regulate the allergic and asthmatic response. *Nramp1*^r macrophages have a high capacity to become activated, which results in good *M. vaccae* clearance. Subsequently, little or no T cell-mediated responses will develop in *Nramp1*^r mice, including no induction of protective regulatory T cells.

Other investigators already suggested that polymorphisms in the genomic region of *NRAMP1* 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 and 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 the key to the hygiene hypothesis, providing a link between genes, the (bacterial) environment, and allergy or asthma.

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