

# Efficacy of Macrophage-activating Lipopeptide-2 Combined with Interferon- $\gamma$ in a Murine Asthma Model

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**Rationale:** The incidence and prevalence of allergic asthma, caused by Th2-mediated inflammation in response to environmental antigens, is increasing. Epidemiologic data suggest that a lack of Th1-inducing factors may play a pivotal role in the development of this disease. We have previously shown that dendritic cells treated with macrophage-activating lipopeptide-2 (MALP-2) combined with IFN- $\gamma$  modulate the Th2 response toward Th1 in an *in vitro* allergy model. **Objective:** To test *in vivo* efficacy of this regime, the effects of the substances were evaluated in a mouse model of allergic airway inflammation. **Methods:** Female Balb/c mice were sensitized to ovalbumin, whereas control animals were sham-sensitized with adjuvant only. After 4 weeks, MALP-2 and IFN- $\gamma$  or NaCl, respectively, were intratracheally instilled. After inhalational ovalbumin challenge, airway hyperreactivity (AHR) to inhaled methacholine was measured by head-out body plethysmography. The animals were subsequently killed to sample bronchoalveolar lavage fluid and lungs. **Results:** Sensitized NaCl-treated mice developed marked AHR compared with sham-sensitized animals. This coincided with eosinophilia as well as the amplification of eotaxin and the Th2 cytokines interleukin (IL)-5 and IL-13 in the bronchoalveolar lavage fluid. Treatment of sensitized mice with MALP-2 and IFN- $\gamma$  significantly reduced AHR compared with the sensitized, NaCl-treated positive control. Eosinophilia as well as Th2 cytokines were reduced to the levels of unsensitized animals. In contrast, IL-12p70 and neutrophils were markedly increased by treatment with both substances. **Conclusion:** These data demonstrate the *in vivo* efficacy of MALP-2 and IFN- $\gamma$  to reduce allergic inflammation and AHR in allergic asthma.

**Keywords:** immunotherapy; mouse; Th1/Th2 cells; Toll-like receptor

Allergic asthma is characterized by a Th2-driven eosinophilic airway inflammation associated with enhanced airway responsiveness to unspecific stimuli (airway hyperreactivity [AHR]). The prevalence of allergic asthma is increasing in urban but not in rural populations, coinciding with the germ-reduced living standard in cities. The inverse association of microbial load and Th2 disorders has led to the formulation of the hygiene hypothesis (1, 2). According to this hypothesis, constant Th1 triggering balances the immune system, and removal of these triggers skews the system toward Th2 (3).

On the basis of these observations, the idea of antagonizing Th2 responses by triggering the immune system with microbial components is regarded as therapeutic concept for allergic asthma. Th1-inducing stimuli could drive the response to allergen in the direction of Th1 responses and reduce Th2 cytokine production. This could decrease IgE production and eosinophilic influx and prevent allergic inflammation and AHR.

Factors to consider for such a therapeutic approach in asthma are subsumed as pathogen-associated molecular patterns. These consist of microbial molecules known as ligands of Toll-like receptors (TLRs). The TLRs represent a family of innate immunity receptors (4–6). The group of pathogen-associated molecular patterns consists of, for example, CpG-unmethylated DNA oligonucleotides (7), flagellin (8), or gram-negative LPS (9). Interaction of host cells with these mediators in general leads to the release of factors like interleukin (IL)-12p70, which are crucial for the generation of Th1 lymphocytes.

The impact of pathogen-associated molecular pattern-mediated signals in the pathogenesis of asthma has been emphasized in recently published studies by Eder and colleagues (10) and Tantisira and coworkers (11). They describe that genetic variations of TLR-2 and TLR-6 are major determinants of the susceptibility to asthma and allergies. Therefore, application of a standardized TLR-2 and TLR-6 agonist may be a promising approach for asthma therapy. Macrophage-activating lipopeptide-2 (MALP-2) is one of the pathogen-associated molecular patterns presently available. It is a 2-kD synthetic lipopeptide derived from *Mycoplasma fermentans* (12) and activates dendritic and other cells by signaling via TLR-2 and TLR-6 (9, 13, 14). MALP-2 is the N-terminal section of a mycoplasmal lipoprotein (15, 16) and can be synthesized according to good manufacturing practice regulations. Furthermore, it lacks the pyrogenic effects of bacterial LPS at effective doses (17). Therefore, MALP-2 is a substance that fulfils the criteria for therapeutic applications.

In a previous study using a human *in vitro* allergy model, we showed that the combination of IFN- $\gamma$  and MALP-2 shifts an existing allergen-dependent Th2 response toward Th1 (18). Therefore, the aim of this study was to investigate whether MALP-2 and IFN- $\gamma$  modulate Th2 responses *in vivo* and improve airway inflammation and AHR in a murine model of allergic asthma.

Some of the results of this study have been previously reported in the form of abstracts (19, 20).

## METHODS

### Animals

Female Balb/c mice (Charles River, Sulzfeld, Germany) were used at the age of 6 to 8 weeks. They were fed with ovalbumin (OVA)-free laboratory food and tap water *ad libitum* and held in regular 12-hour dark:light cycles at a temperature of 22°C. Mice were acclimatized for 7 days before starting the experiments. All animal experiments were performed in concordance with the German animal protection law

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under a protocol approved by the appropriate governmental authority (Bezirksregierung Hannover).

### Sensitization and Allergen Challenge of Mice

Animals were sensitized to and challenged with OVA as previously described (21). Details are provided in the online supplement. The study protocol is shown in Figure 1, and Table 1 provides details of the test groups.

### Treatment of Mice

To modulate the allergic response, mice were intratracheally treated with 20 ng MALP-2 (synthesized and purified as described in Reference 22),  $10^4$  U IFN- $\gamma$  (Strathman Biotec, Hamburg, Germany), or a combination of both in a total volume of 50  $\mu$ l saline on Day 27, 24 hours before the allergen challenge (*see* Figure 1). Control animals were treated with NaCl alone. Details are provided in the online supplement.

### Measurement of Lung Function

Twenty-four hours after the last allergen challenge, AHR to methacholine (MCh) was determined in nonanesthetized, spontaneously breathing animals using head-out body plethysmography, as described previously (21). Details are provided in the online supplement.

### Animal Dissection

After testing the lung function, mice were killed with an intraperitoneally injected overdose of pentobarbital Na (Merial, Hallbergmoos, Germany). Blood for counting of leukocytes and bronchoalveolar lavage (BAL) cells and for BAL fluid (BALF) was collected for differential cell counts and detection of cytokines; lungs were shock-frozen for histology. Details are provided in the online supplement.

### Differential Cell Count

Cytospins were stained according to the Pappenheim standard protocol. Differential cell counts were performed on all nucleated cells from blood and BAL. At least 300 cells of each sample were classified by light microscopy according to common morphologic criteria. Absolute cell counts of macrophages, lymphocytes, and eosinophilic and neutrophilic granulocytes were calculated.

### Histology

Cryosections of 5  $\mu$ m from frozen lungs were fixed in acetone (10 minutes at  $-20^\circ\text{C}$ ), washed in phosphate-buffered saline, and stained with hematoxylin–eosin according to standard procedures. Photographs were taken using an Axioskop 2 microscope equipped with an AxioCam CCD camera and Axiovision acquisition software (all Carl Zeiss GmbH, Jena, Germany).

### ELISA

For detection of IL-5, IL-13, IL-12p70, eotaxin, and regulated on activation, normal T-cell expressed and secreted (RANTES) in the BALF, Duo-Set ELISA kits (R&D Systems, Wiesbaden, Germany) were used according to the manufacturer's instructions.

### Statistical Analysis

To compare all groups to the OVA-sensitized and NaCl-treated positive control, Dunnett's test for multiple comparisons was used after analysis of variance showed significant differences of the means. An asterisk denotes *p* values of 0.05 or less (*see* Figures 2 and 3). Statistical analysis was performed using the Prism 3.0 software (GraphPad, San Diego, CA). Data are expressed as the mean of all animals in the accordant group (*see* Table 1)  $\pm$  SEM. Details are provided in the online supplement.

## RESULTS

### A Combination of MALP-2 and IFN- $\gamma$ Modulates the Allergen-induced Pattern of Cytokines and Chemokines

To test if local treatment with MALP-2 and IFN- $\gamma$  is associated with a modulation of the allergen-induced Th2 skew, the CC chemokines eotaxin and RANTES, and the cytokines IL-5, IL-13, and IL-12p70 were quantified in the BALF. Allergen sensitization and subsequent allergen challenge by inhalation resulted in upregulation of eotaxin and the Th2 cytokines IL-5 and IL-13 compared with the negative control, whereas RANTES and the Th1-inducing IL-12p70 were not affected (Figure 2). This cytokine profile corresponds to a clear shift toward Th2 in the OVA-sensitized and OVA-challenged animals, which is accompanied by increasing eotaxin levels. Local treatment with MALP-2 alone or with IFN- $\gamma$  alone was able to reduce all Th2 cytokines to the level of the sham-sensitized negative control, but did not amplify IL-12p70. Interestingly, eotaxin was even further amplified by each substance compared with the allergen-sensitized and allergen-challenged group at the time point analyzed, whereas RANTES induction was only seen in animals treated with IFN- $\gamma$ . MALP-2 alone had no effect on RANTES, but RANTES levels were decreased after animals had been treated with the combination of IFN- $\gamma$  and MALP-2. In contrast, the combination of MALP-2 with IFN- $\gamma$  did not induce higher eotaxin levels in BALF. The combination reduced Th2 cytokines and induced IL-12p70.

### Modulation of the Cellular Composition of BAL and Lung Tissue by Intratracheal Application of MALP-2, IFN- $\gamma$ , or Both

Next, we searched for the functional consequences of MALP-2 and IFN- $\gamma$  by determining cellular influx into the lung. Sensitization to OVA and subsequent OVA challenge by inhalation induced a highly significant influx of eosinophils into the BAL, as compared with the sham-sensitized negative control (Figure 3). Corresponding to the eotaxin concentrations in the BALF, either MALP-2 or IFN- $\gamma$  alone raised the eosinophil counts even further. Similarly, the absolute number of BAL lymphocytes was

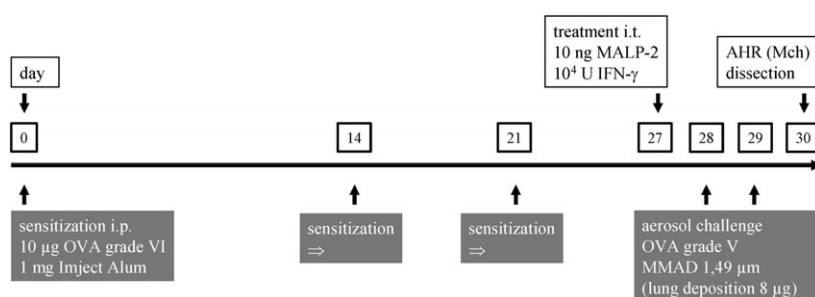


**Figure 1.** Schematic presentation of the study design. Female Balb/c mice were sensitized by intraperitoneal injection of ovalbumin (OVA) adsorbed to the adjuvant aluminum hydroxide (Alum) on Days 0, 14, and 21. Control animals were sham-treated with aluminum hydroxide alone. On Day 27, mice were intratracheally treated with NaCl, macrophage-activating lipopeptide-2 (MALP-2), IFN- $\gamma$ , or the combination of MALP-2 and IFN- $\gamma$ . All groups were challenged with OVA aerosol on Days 28 and 29. Twenty-four hours later, head-out body plethysmography was performed to measure airway hyperresponsiveness (AHR), followed by animal dissection. MCh = methacholine; MMAD = mean mass aerodynamic diameter.

TABLE 1. DESCRIPTION OF THE EXPERIMENTAL GROUPS

| Groups                    | –/NaCl | OVA/NaCl | OVA/MALP-2   | OVA/IFN-γ               | OVA/MALP-2 + IFN-γ                   |
|---------------------------|--------|----------|--------------|-------------------------|--------------------------------------|
| Sensitization             | NaCl   | OVA      | OVA          | OVA                     | OVA                                  |
| Challenge                 | OVA    | OVA      | OVA          | OVA                     | OVA                                  |
| Treatment (intratracheal) | NaCl   | NaCl     | 20 ng MALP-2 | 10 <sup>4</sup> U IFN-γ | 20 ng MALP-2 10 <sup>4</sup> U IFN-γ |
| No. animals               | 8      | 16       | 8            | 8                       | 16                                   |

Description of abbreviations: MALP-2 = macrophage-activating lipopeptide-2; OVA = ovalbumin.

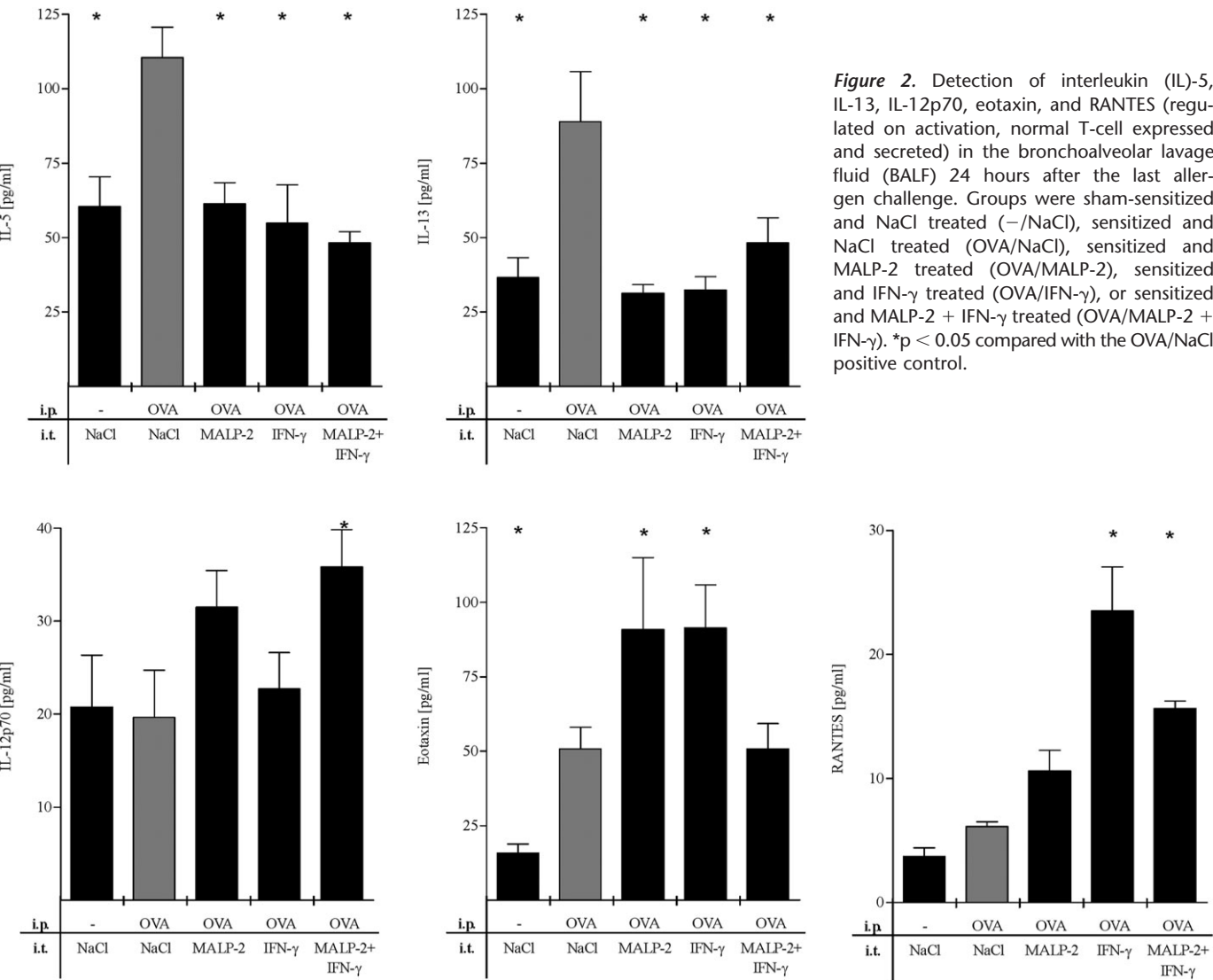


Figure 2. Detection of interleukin (IL)-5, IL-13, IL-12p70, eotaxin, and RANTES (regulated on activation, normal T-cell expressed and secreted) in the bronchoalveolar lavage fluid (BALF) 24 hours after the last allergen challenge. Groups were sham-sensitized and NaCl treated (–/NaCl), sensitized and NaCl treated (OVA/NaCl), sensitized and MALP-2 treated (OVA/MALP-2), sensitized and IFN-γ treated (OVA/IFN-γ), or sensitized and MALP-2 + IFN-γ treated (OVA/MALP-2 + IFN-γ). \*p < 0.05 compared with the OVA/NaCl positive control.

increased by both substances. However, the combination of both substances was able to abolish the allergen-induced eosinophil influx almost to the level of the sham-sensitized negative control. This was associated with the appearance of neutrophils and macrophages in the BALF (Figure 3). No systemic effects in terms of changes in the composition of blood leukocytes were detected (data not shown).

In accordance with the BAL cell counts, allergen sensitization and challenge induced a massive infiltration of mainly eosinophils in the peribronchial compartment 24 hours after the last allergen challenge (Figure 4B). This was not seen in sham-sensitized control animals (Figure 4A). The low influx of leukocytes in

the latter group might be due to an unspecific stimulus during instillation, because completely untreated animals did not show any infiltrates (data not shown). MALP-2 and IFN-γ treatment before allergen challenge switched the predominant cell type compared with the NaCl-treated group: tissue eosinophilia was reduced, and replaced by neutrophil infiltration. Granulocytes were also located peribronchially (Figure 4C).

A Combination of MALP-2 and IFN-γ Decreases AHR

To analyze the effect of MALP-2 and IFN-γ treatment on lung physiology, AHR was investigated. For this purpose, the MCh concentration-dependent decrease of midexpiratory airflow (EF<sub>50</sub>)

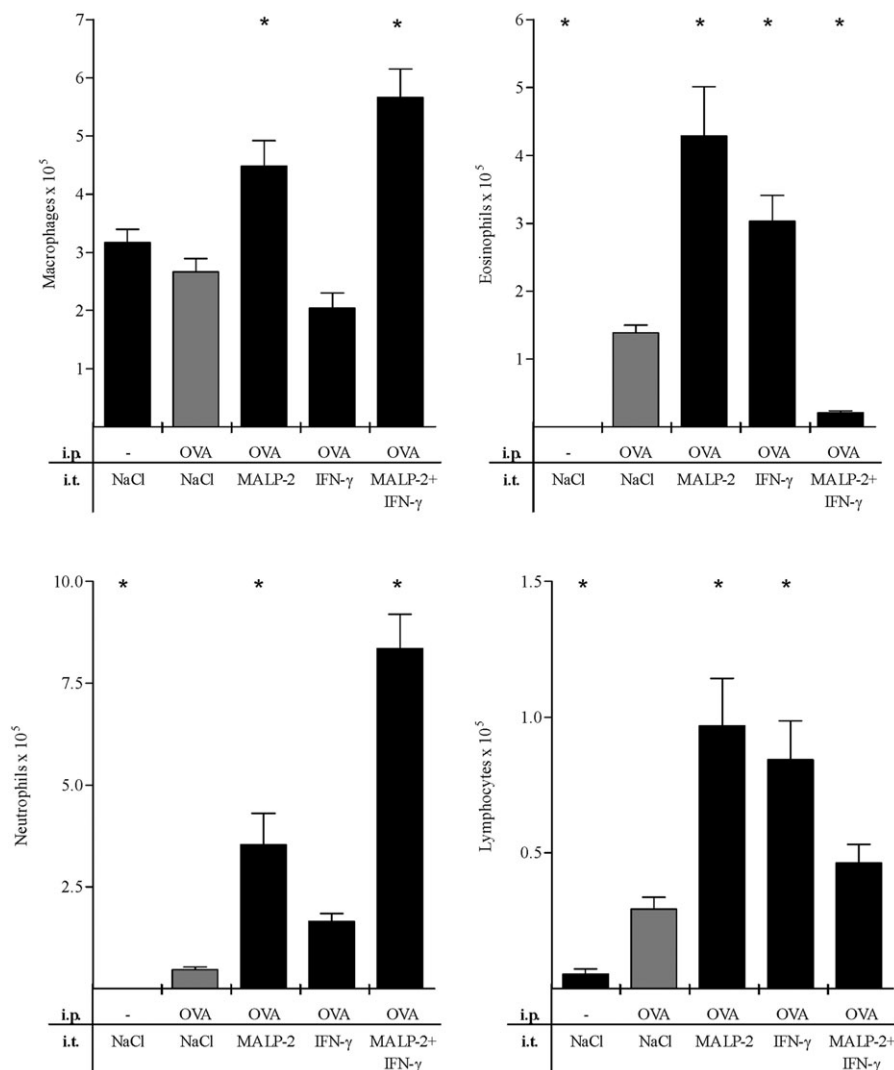


**Figure 3.** Cellular composition in the BALF 24 hours after the last allergen challenge. Groups were treated as described in Figure 1. \* $p < 0.05$  compared with the OVA/NaCl positive control.

was measured by head-out body plethysmography 72 hours after treatment and 24 hours after the last allergen challenge (Figure 5). There were no differences in  $EF_{50}$  baseline values between the groups (sham-sensitized animals,  $2.50 \pm 0.18$  ml; OVA-sensitized mice,  $2.43 \pm 0.19$  ml; MALP-2/IFN- $\gamma$ -treated animals,  $2.54 \pm 0.18$  ml). Inhalation of MCh decreased  $EF_{50}$  values in a dose-dependent manner. No differences between the different groups were observed at low doses of MCh. In contrast, application of moderate and high doses of MCh caused a more severe decrease in  $EF_{50}$  levels by equal concentrations in animals sensitized with OVA compared with sham-sensitized or MALP-2- and IFN- $\gamma$ -treated animals. From the dose-response curves, the  $EC_{50}$  (i.e., the concentration of MCh needed to decrease  $EF_{50}$  to 50% of baseline) was calculated, because changes in MCh sensitivity are often taken as the threshold values of response (e.g., dose required to produce a 20% fall in  $FEV_1$  in humans). As expected, mice sensitized with OVA showed AHR, indicated by a significantly lower  $EC_{50}$  as compared with sham-sensitized animals ( $16.58 \pm 1.61$  vs.  $29.92 \pm 3.73$  mg/ml MCh,  $p < 0.05$ ). Neither MALP-2 ( $EC_{50} = 20.13 \pm 1.36$  mg/ml MCh) nor IFN- $\gamma$  ( $EC_{50}$ ,  $17.67 \pm 3.05$  mg/ml MCh) alone significantly altered the allergen-induced changes in lung function. However, the combination of both substances showed an alleviated AHR with an  $EC_{50}$  of  $24.01 \pm 3.51$  mg/ml MCh ( $p < 0.05$  compared with OVA).

## DISCUSSION

Previously we have shown that the synthetic TLR-2/6 agonist MALP-2 combined with IFN- $\gamma$  modulates the Th2 skew toward Th1 in a human *in vitro* allergy model (18). This was achieved by induction of IL-12p70 and upregulation of costimulatory cell surface molecules on the dendritic cells used in the model. Either substance alone was not effective. The aim of this study was to investigate the *in vivo* relevance of the *in vitro* findings. For this purpose, we used a well-established murine model of allergic airway inflammation and AHR. Sensitization to OVA and subsequent OVA aerosol challenge induced AHR associated with tissue and BAL eosinophilia as well as amplification of eotaxin and the cytokines IL-5 and IL-13. Intratracheal instillation of MALP-2 with IFN- $\gamma$  resolved eosinophilia and the Th2-skewed cytokine milieu of sensitized and challenged mice, whereas an influx of neutrophils and induction of IL-12p70 and RANTES were observed. In addition, the combination of both substances improved lung function in response to MCh. Either substance alone did not significantly affect AHR. An additional, unsensitized control group that was treated with MALP-2 and IFN- $\gamma$  also developed neutrophilia associated with some infiltrating monocytes and lymphocytes, but no eosinophils. IL-5 and IL-13 levels remained unchanged, and AHR was not detectable. Each

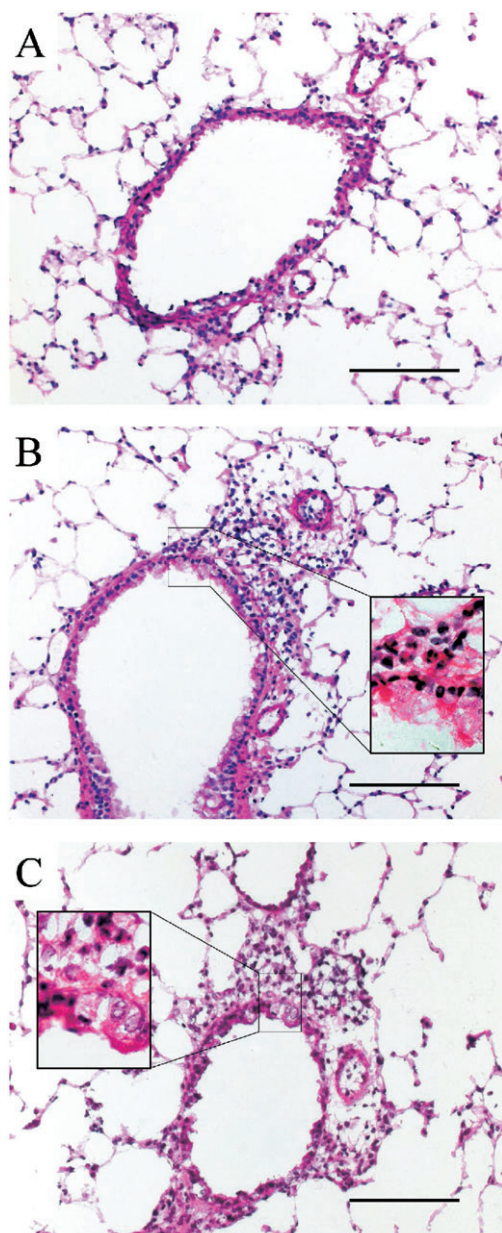


**Figure 4.** Lung histology 24 hours after the last allergen challenge. Cryostat sections 5- $\mu$ m thick were stained with hematoxylin-eosin. Mice were sham-sensitized and treated with NaCl (A), OVA-sensitized and treated with NaCl (B), or OVA-sensitized and treated with MALP-2 + IFN- $\gamma$  (C). Image acquisition was performed using a 20-fold objective and a resolution of 0.1  $\mu$ m<sup>2</sup>/pixel. The bar represents a 100- $\mu$ m segment.

parameter was of comparable magnitude to the equally treated sensitized group (data not shown).

The results demonstrate that the combination of MALP-2 and IFN- $\gamma$  had synergistic effects on airway inflammation as well as on lung function. The underlying mechanisms have recently been described by Dalpke and colleagues (23). According to the authors, TLR signaling by LPS or lipoteichoic acid leads to the phosphorylation of signal transducer and activator of transcription-1 by a p38 mitogen-activated protein kinase. This event can amplify the cascade of IFN- $\gamma$  signaling and is therefore suggested to be the biochemical basis for this synergism.

The results presented here are based on our above-mentioned

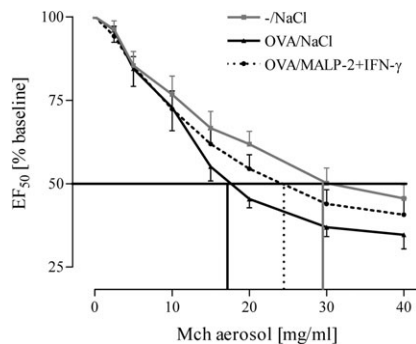


**Figure 5.** Lung function measurement 24 hours after the last allergen challenge. The midexpiratory airflow (EF<sub>50</sub>) is shown in dependence of different concentrations of MCh. Animals were sham-sensitized and treated (-/NaCl), OVA-sensitized and treated with NaCl (OVA/NaCl), or OVA-sensitized and

treated with MALP-2 + IFN- $\gamma$  (OVA/MALP-2 + IFN- $\gamma$ ). The vertical lines of each group give the MCh concentration that induces a 50% reduction of the EF<sub>50</sub>, the so-called mean effective concentration (EC<sub>50</sub>). Sensitization to OVA without treatment induces a significant ( $p < 0.05$ ) reduction of the EC<sub>50</sub> compared with the sham-sensitized, NaCl-treated control and with the sensitized MALP-2 + IFN- $\gamma$ -treated group.

*in vitro* experiments (18). Therefore, we propose that the substances modulate the function of local antigen-presenting cells, such as macrophages and dendritic cells in the lung. As a consequence, the production of IL-12 and of chemotactic mediators is induced, resulting in the influx of neutrophils. This response suggests the development of a Th1 reaction. A study by Luhrmann and coworkers (24) investigated the *in vivo* effects of MALP-2 after pulmonary application in the lung of healthy rats. Here, a chemotactic activity of the BALF was observed after intratracheal instillation or inhalation of MALP-2. Correspondingly, leukocytes, mainly neutrophils, massively infiltrated the bronchoalveolar space. Such treatment showed the tendency to increase dendritic cell numbers in the draining lymph nodes, further supporting our proposed mode of action.

However, the *in vivo* effects of locally administered MALP-2 in a Th2-driven inflammatory disorder, such as allergic asthma, have not yet been investigated. In our study, treatment with MALP-2 alone reduced the concentration of Th2 cytokines in the BAL, but increased eosinophil numbers. This discrepancy between cytokine regulation and cell influx into the lung might be explained by T-cell-independent direct and/or indirect effects of MALP-2. Although no data on the effect of MALP-2 on isolated eosinophils are yet available, MALP-2 may modulate eosinophil function directly. It has been shown that mRNA for TLR-2 and TLR-6 is expressed in eosinophils, and stimulation of these cells with TLR ligands increases expression of adhesion molecules, activates eosinophils, and promotes their survival (24, 25). It is rather unlikely, however, that the influence of MALP-2 on eosinophils reflects the predominant mechanism in our study, because, at the time point of treatment (24 hours before allergen challenge), eosinophils did not start to infiltrate the lung, and it is well described that the half-life of proteins and (lipo-)peptides in the enzymatic composition of the alveolar space is short. Thus, MALP-2 may cause the observed influx of eosinophils indirectly by induction of eotaxin synthesis and release in various cell types, such as epithelial cells and macrophages (25). This hypothesis is supported by the detection of enhanced eotaxin levels in the BALF after MALP-2 treatment in allergen-challenged mice of our study. Although the underlying mechanism leading to enhanced release of eotaxin by TLR agonists like MALP-2 as well as IFN- $\gamma$  remains unclear, it is noteworthy that the induction of eotaxin seems to be independent of a predominant Th2 pattern. In previous studies it could be shown that superimposing a Th1 on a Th2 immune response within the

airways does not downregulate chemokine expression (26–28). Contrary to eotaxin, we could not detect enhanced RANTES levels in BALF in OVA-sensitized animals. However, treatment with IFN- $\gamma$  clearly enhanced RANTES levels, whereas treatment with MALP-2 had no effect when applied alone. Increased RANTES levels are probably directly induced by IFN- $\gamma$ , as has been shown *in vitro* in eosinophils and bronchial epithelial cells (29–31) and *in vivo* by transfer of Th1 cells (28, 32). The combination of IFN- $\gamma$  and MALP-2 decreased RANTES levels compared with treatment with IFN- $\gamma$  alone. This effect can be explained, at least partly, by the decreased number of eosinophils that serve as a cellular source for RANTES (29, 30). Therefore, our results indicate that cytokines and CC chemokines may be differentially regulated by IFN- $\gamma$  and TLR agonists.

Other TLR agonists that have recently been shown to modulate allergic inflammation and AHR are CpG unmethylated DNA oligodeoxynucleotides (33, 34). These studies demonstrated a reduction of the asthmatic phenotype using OVA or *Schistosoma mansoni* antigens after intraperitoneal injection of CpG in an acute model and after several subcutaneous injections in a chronic model of asthma. The use of CpG as adjuvant, however, has experienced a drawback. Heikenwalder and colleagues (35) showed an altered morphology and functionality of mouse lymphoid organs and multifocal liver necrosis and hemorrhagic ascites after repeated injections of unmethylated oligodeoxynucleotides. Other groups described induction of autoimmunity after CpG application (36, 37). No such destructive effects are known for MALP-2. Indeed, MALP-2 is reported to have a low pyrogenicity (1,000-fold lower compared with LPS) (17) and is not expected to activate natural killer cells, as it needs TLR-2 for signaling, which is absent in those cells (38, 39). The induction of a Th1-driven inflammation, however, could also have some critical bystander effects. It was recently shown that Th1 cells can induce AHR, independent of the presence of granulocytes (40, 41). In addition, blocking of the Th1 cytokine IFN- $\gamma$  is able to prevent AHR in a murine model of chronic asthma (42). In contrast, other studies showed a clear beneficial effect of Th1 induction on allergic airway inflammation and AHR (43, 44). These conflicting results might be explained by differences in animal models and treatment protocols. However, the hygiene hypothesis gives clear evidence that missing Th1 induction in certain lifespans is critical for the development of the atopic diseases in humans. With the combination of MALP-2 and IFN- $\gamma$ , a new tool to direct immune response in a requested direction is at hand.

In conclusion, local treatment with MALP-2 and IFN- $\gamma$  was effective in alleviating the allergen-induced changes in Th2 cytokines and eosinophils, and in attenuating AHR in a short-time model of asthma. Because of the potential risk caused by the accompanying neutrophilic inflammation, the long-term safety and efficacy of this regime will be evaluated in a chronic model of asthma.

**Conflict of Interest Statement:** H.W. has a pending patent application concerning therapeutic strategies derived from the present study. C.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.F.M. is a former employee of the Gesellschaft für Biotechnologische Forschung (GBF), now retired, and is coinventor of the following two patent applications held by the GBF: (1) PCT/EP2004/005996, "Therapeutic composition containing dendritic cells and the use thereof," and (2) PCT/EP2001/11414, "Methods for treating lung infections and lung tumors and for treating and preventing metastases." N.K. is a coinventor of a patent for the treatment of human dendritic cells with MALP-2 and IFN- $\gamma$  for immunomodulatory cell therapy. A.B. has a pending patent application concerning therapeutic strategies derived from the present study.

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