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Helminth infection modulates the development of allergen-induced airway inflammation

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Abstract

It has been proposed that infections with helminths can protect from the development of allergic diseases. However, epidemiological and experimental studies have yielded conflicting results. Therefore we investigated if an infection with *Nippostrongylus brasiliensis* influenced the development of allergen-induced Th2 cell responses in mice. We found a decrease in allergen-induced airway eosinophilia and Eotaxin levels in the airways when mice were infected with the helminths 8 weeks, and especially 4 weeks, but not 1 or 2 weeks before ovalbumin (OVA)-airway challenge. While OVA-specific IgG1 and IgE serum levels and cutaneous hypersensitivity reactions were not reduced by the helminth infection, there was a reduction in OVA-specific IgG1 and IgE levels in bronchoalveolar lavage fluid of mice. Suppression of allergen-induced airway eosinophilia and reduction of Eotaxin production was not observed in IL-10 deficient mice. In addition, we found that helminth-induced airway eosinophilia and Eotaxin production was strongly increased in IL-10 deficient mice infected with the helminths in comparison to control mice. Taken together, these results show that infection with *N. brasiliensis* suppresses the development of allergen-induced airway eosinophilia and that this effect may be mediated by IL-10. Our results support the view that helminth infections can contribute to the suppression of allergies in humans.

Introduction

The two most important prerequisites for the development of atopic disorders such as asthma, hay fever, eczema or allergic rhinitis are allergen exposure followed by the generation of allergen-specific $CD4^+$ Th2 cells. Once generated, effector Th2 cells secrete IL-4, IL-5 and IL-13, which in turn induce the production of allergen-specific IgE by B cells, the development and recruitment of eosinophils, smooth muscle contraction and mucus production. Degranulation of eosinophils and mast cells (via IgE cross-linking) are the two major factors leading to most clinical manifestations of allergic diseases (1). Although relatively much is known about the immunological processes leading to the development of atopy it is still not completely understood why only a minority of individuals which are exposed to allergens develop allergic disorders. Genetic factors clearly play a major role; however, the current dramatic increases in both the incidence and severity of atopic disorders cannot be explained by genetic factors alone.

It has been proposed that environmental and not genetic factors are responsible for this development. In particular, childhood infections inducing Th1 responses have been directly associated with the inhibition of allergic Th2 responses (2). Other infectious diseases that might also have an impact on the development of atopic disorders are caused by helminths. In contrast to most other types of infections, helminths induce strong Th2-type responses. For this reason it could be expected that helminths promote allergic disorders through the induction of IL-4 leading to increased development of allergen-specific Th2 cells. Supporting this view are the findings that helminth infections directly induce airway eosinophilia and hyper-responsiveness in mice and that allergic manifestations occur more often in children

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seropositive for Toxocara or Ascaris than in seronegative children (3–8). However, numerous other epidemiological studies have found an inverse correlation between helminth infections and atopy, suggesting a possible protective effect of helminth infections on the development of atopy (9–13). Two mechanisms have been implicated to possibly mediate this effect. Firstly, the large amounts of polyclonal IgE induced by the helminth infection may saturate the number of Fcε receptors on mast cells and thus interfere with the binding of allergen-specific IgE to the mast cells, resulting in the inhibition of allergen-mediated mast-cell degranulation ('IgE blocking hypothesis') (14). The findings that protective effects of helminth infections on allergic reactivity were associated with high levels of IgE in the serum supports this view (15–18). However, a recent study found that the inverse association between the degree of infection with Schistosomes and positive skin-prick test was not associated with increased total serum IgE levels but with the amount of IL-10 present in the serum of the patients (11). This result has lead to the suggestion that IL-10 and/or transforming growth factor-β secreted by antigen presenting cells (APCs) or T regulatory (Tr) cells in response to a chronic helminth infection directly interferes with allergic effector mechanisms by inhibiting mast cell degranulation or inhibiting Th2 cell proliferation (19).

Although this is a very compelling hypothesis, it cannot be ruled out that patients suffering from atopy may be less susceptible to helminth infections as suggested by some investigators (20,21). Furthermore, animal experiments addressing this question have also yielded somewhat conflicting results (22–24). To clarify this issue further we investigated if infections with the helminth *Nippostrongylus brasiliensis* had an effect on the development of ovalbumin (OVA)-induced allergic Th2 responses in a murine model. Mice were infected i.p. with third stage larvae of a mouse-adapted strain of *N. brasiliensis*. The larvae migrate to the lungs, where they remain for 24–72 h, are coughed up, swallowed, and develop into mature adults in the gut where they produce eggs. Once infected, mice totally expel the worms after 2–3 weeks and are protected from subsequent re-infection. During this life cycle the larvae and the adult worms induce strong Th2 responses first in the lung and then in the gut, which are responsible for the elimination of the parasites (25). Here we report that infection with *N. brasiliensis* inhibited the development of allergen-induced airway eosinophilia with the effect being dependent on the time point of infection. This effect was not observed in mice deficient for IL-10.

Methods

Animals

C57BL/6 and BALB/c mice were purchased from Charles River (Sulzfeld, Germany) and IL-10 deficient mice (C57BL/6 genetic background) were generously provided by Werner Müller (GBF, Braunschweig, Germany) (26). All animals used for the experiments were between 5 and 7 weeks of age and housed in a conventional animal facility. All animal experiments were performed according to the guidelines of the local and national authorities.

Infection of mice with N. brasiliensis

Naive mice and mice immunized with OVA were infected with 1000 L3 larvae of *N. brasiliensis* i.p. 1, 4, 8 or 12 weeks prior to the single intra-nasal (i.n.) challenge with OVA as described previously (27). A mouse-adapted strain of *N. brasiliensis* was maintained and passed in Lewis rats at the University of Würzburg.

OVA immunization protocol

Mice were injected i.p. with 2 μg of OVA (Sigma, St Louis, MO) in 200 μl alum adjuvant (Serva, Heidelberg, Germany) on day 0 and boosted again i.p. with 2 μg OVA/alum on day 14. Ten days after the second i.p. immunization mice were anesthetized by an i.p. injection of a mixture of Ketamine and Xylazine (Sigma) and were treated i.n. with 100 μg OVA in 50 μl PBS. Mice were analyzed 6 days after the i.n. application of OVA.

Detection of different cell types in the bronchoalveolar lavage (BAL)

Mice infected with *N. brasiliensis* and/or immunized with OVA were sacrificed at the time points indicated in the figure legends. The trachea was then cannulated, and a BAL was performed by flushing lung and airways five times with 1 ml PBS. BAL cells were counted and spun onto glass slides using a cytocentrifuge (Shandon Southern Products Ltd., Asmoor, UK) and afterwards stained with Dif-Quik according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Percentages of macrophages, lymphocytes, neutrophils and eosinophils were determined microscopically using standard histological criteria.

Culture conditions of cells

Single-cell suspensions from the mediastinal lymph nodes (MLN), mesenteric lymph nodes (MESLN) or spleen (2×10^6 cells/ml) of the different groups of mice were prepared and cultured in RPMI medium (Sigma) supplemented with sodium bicarbonate (3.024 g/l), 10 μg/ml streptomycin, 10 U/ml penicillin, 50 μM 2-mercaptoethanol, and 10% fetal calf serum. The cell preparations were stimulated with a mAb to CD3 (145-2C11, 25 μg/ml; PharMingen, San Diego, CA) together with 200 U/ml recombinant human IL-2 (Norvatis, Basel, Switzerland) or 40 μg/ml OVA (Sigma). After 48 h the culture supernatants were harvested and tested for the presence of cytokines by ELISA. As controls cells were also cultured in medium alone.

Detection of cytokines

For the detection of IL-4, IL-5, IL-13, IFN-γ, and Eotaxin, sandwich ELISAs were performed as described previously (28). For the detection of IL-10, biotinylated rat anti-mouse IL-10 (JES5-2A5) and unconjugated rat anti-mouse IL-10 (SXC 1) were used (PharMingen). The BAL fluid was concentrated 4-fold on columns (Millipore ultrafree 15; Sigma) to detect IL-5, IL-13, and Eotaxin. No IL-10 or IL-4 was detected in the concentrated BAL fluid in any of the samples and no cytokines or Eotaxin was detected in the concentrated BAL fluid of non-treated control mice (data not shown).

Detection of CD4+ T cells producing IL-4 and/or IL-10 and expressing CD25 or CTLA-4

Two-colour cell labeling was used to identify IL-4-producing CD4+ T cells in BAL fluid and MLN from mice infected with the helminths and/or immunized with OVA. Furthermore, three-colour cell labeling was performed to detect IL-10- and IL-4-producing CD4+ cells or CD4+ T cells secreting IL-10 and expressing CD25 or CTLA-4 in BAL fluid and MLN. The analyses were performed with a FACScan™ (Becton Dickinson, Mountain View, CA). BAL cells were spun down and single cell suspensions were prepared from the MLN. The cells were then stimulated with phorbol ester (5 ng/ml) and calcium ionophore (0.5 μM) for 6 h (both reagents from Sigma). Brefeldin A (2 μg/ml; Sigma) was added for the last 2 h of the *in vitro* culture period. The intracytoplasmic cell labeling with antibodies were performed according to the instructions from Pharmingen (using a saponin based permeabilization buffer). Briefly, after the 6 h stimulation, cells were washed and stained with FITC-labeled anti-CD4 mAb (L3T4) (for two-colour cell labeling) or Cy-Chrome™-labeled anti-CD4 mAb (for three-colour cell labeling). The cells were then fixed with 4% formalin in PBS for 20 min and later incubated with anti-CD16/CD32 mAb (2.4G2; Fc Block®, 5 μg/ml). After 30 min cells stained with FITC-labeled anti-CD4 were stained with PE-anti-IL-4 mAb (11B11) (two-colour cell labeling). For the three-colour cell labeling, Cy-Chrome™-labeled anti-CD4 mAb stained cells were incubated with either FITC-labeled anti-IL-10 mAb (JES5-16E3) and PE-labeled anti-CTLA-4 mAb (UC10-4F10-11) or with FITC-labeled anti-IL-10 mAb and PE-labeled anti-IL-4 mAb or a combination of FITC-labeled anti-IL-10 mAb and PE-labeled anti-CD25 mAb (3C7). Specificity of Ab binding was controlled by cell labeling with irrelevant isotype matched control Ab (rat IgG1-PE-, rat IgG2b-FITC-, rat IgG2b,κ-PE or Armenian Hamster IgG-PE-labeled). All mAb were purchased from PharMingen.

Determination of total and OVA-specific IgG1 and IgE levels

The amounts of total IgG1 and IgE in the serum of the different groups of mice were determined by sandwich ELISAs as described previously. Microtiter plates were coated with anti-mouse IgG1 or anti-mouse IgE, both at 10 μg/ml in coating buffer (0.05 M $NaCO_3$, pH 9.5) for 24 h, washed with 0.1% Tween 20 (Sigma) in PBS and incubated for 2 h with 10% BSA. Serial dilutions of serum were put into the wells and incubated at room temperature for 2 h, washed and incubated with biotinylated anti-mouse IgG1 or biotinylated anti-mouse IgE mAbs for 1 h. The ELISAs were developed as described above. The amounts of IgG1 and IgE present in the serum were determined by including serial dilutions of purified murine IgG1 and IgE in the ELISA assays. For quantification of the isotypes present in the serum of the mice, titrations were performed with murine IgG1 and IgE. OVA-specific Ig levels were determined in the serum and BAL as described previously (28). (As a reference, the serum of untreated control mice was also included in the ELISA assays.) OVA-specific IgG1 and IgE titers are shown as the inverse of the dilution that yielded an OD >0.2 (the OD of a 1/10 serum dilution from control mice was <0.1 in both the OVA-specific IgG1 and IgE ELISAs).

Cutaneous anaphylaxis

Active cutaneous anaphylaxis was tested in *N. brasiliensis*/OVA or OVA-only treated mice or control mice (10 days after OVA-airway challenge) as described previously, by s.c. injecting 50 μl PBS containing 10, 1 or 0.1 μg/ml OVA (29). The degree of bluing as an indicator for the intensity of mast cell degranulation was scored by two independent observers (0 = no bluing, 1 = slight bluing, 2 = strong bluing, 3=very strong bluing).

Histological analysis

Tissues from helminth-infected and/or OVA-immunized mice were fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin wax. Sections (2–3 μm) were cut and stained using standard histological protocols with hematoxylin and eosin or periodic acid schiff reaction. The stained sections were visualized by light microscopy.

Statistical analysis

Statistical significance was analyzed by unpaired Student's *t*-test.

Results

Infection with N. brasiliensis induces strong transient type-2 responses

In order to determine how an infection with the helminth *N. brasiliensis* affects the development of an allergen-specific Th2 response in mice, we felt it important to characterize the kinetics of the helminth-induced Th2 response. For this purpose, mice were infected i.p. with third-stage larvae for 1, 2, 4 or 8 weeks. Figure 1(A) shows that infection with *N. brasiliensis* leads to an increase in macrophages, lymphocytes and especially eosinophils in the airways. Maximal airway eosinophilia was detected 2 weeks after infection ($P < 0.05$ compared to the values found in control mice and mice infected with the helminths at other time points), and is almost not detectable 4 weeks after infection. Infection with the helminth also leads to a strong blood eosinophilia with a similar kinetics (data not shown). After restimulation with anti-CD3 and IL-2 T, cells from the MLN, MESLN and spleen of infected mice secrete strongly increased amounts of IL-4, IL-5, IL-10 and IL-13 in comparison to T cells from uninfected mice (Fig. 1C–F, respectively). IFN-γ levels were not increased (Fig. 1B) except after 4 weeks of infection, particular in the spleen. None of the cytokines were detected in the non-concentrated BAL fluids at any time point (data not shown). These results clearly indicate that the helminth infection induces strong Th2 responses in the lung, spleen and gut. Maximal Th2 cytokine secretion was detected 1 week after infection in all the cultures ($P < 0.05$ compared to the values found in control mice and mice infected with the helminths at other time points). Importantly, judging by the amounts of Th2 cytokines secreted in the *in vitro* cultures, the strongest Th2 response was mounted in the lung. The strongest increase in serum IgG1 and IgE levels was detected 2 weeks after infection. Although the Th2 response was substantially lower after 8 weeks of infection in comparison to 1, 2 and 4 weeks, it did not reach the same low levels detected in uninfected mice.

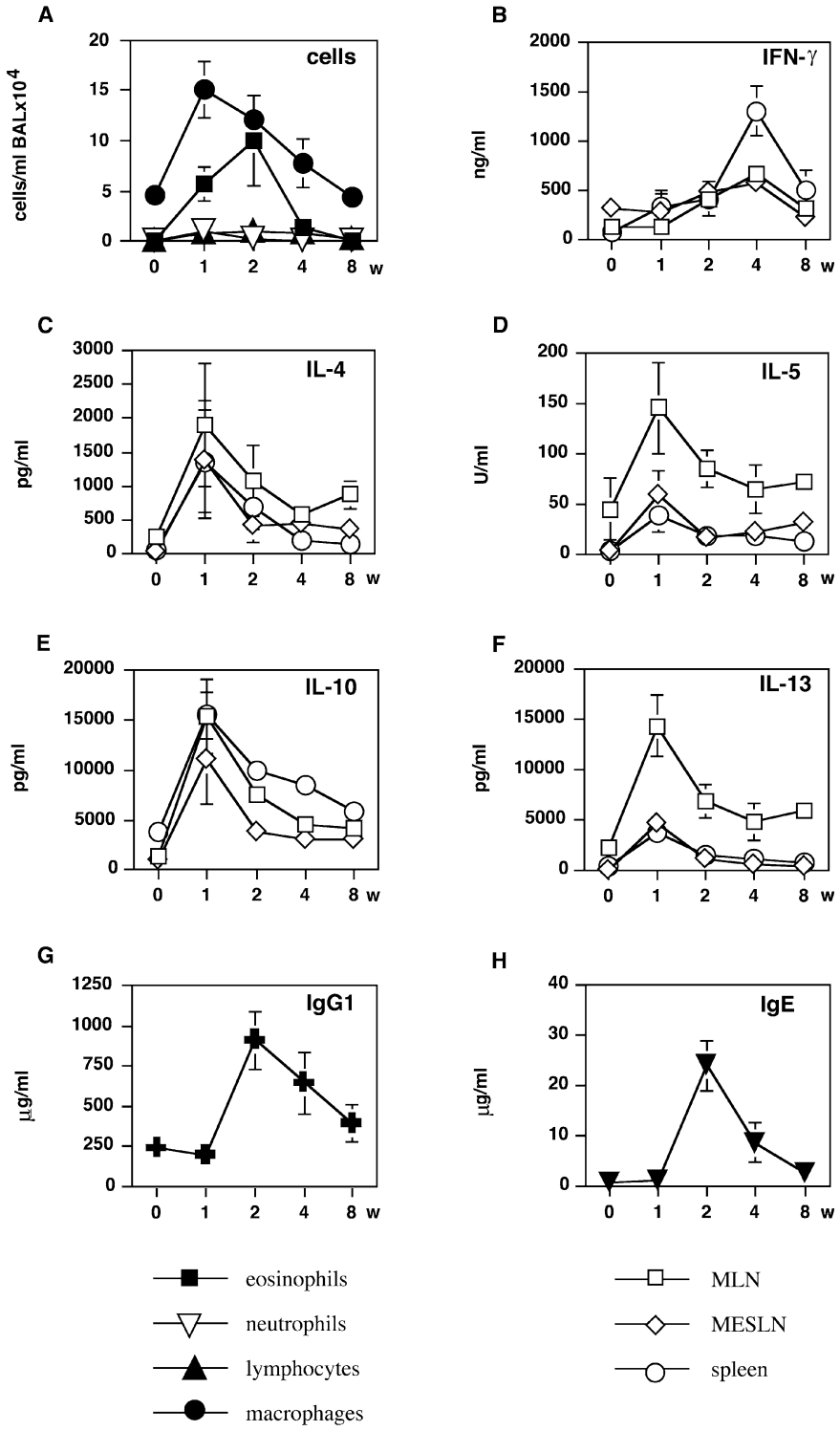

Fig. 1. Infection with *N. brasiliensis* induces a transient Th2 response in mice. At 1, 2, 4 and 8 weeks after infection with 1000 L3 larvae, mice were sacrificed and the Th2 response was analysed. Uninfected mice were also included as controls (0 weeks). Shown are the average numbers of eosinophils, macrophages, neutrophils and lymphocytes present in the BAL fluid after the different timepoints of infection (A). Single cell suspensions were prepared from MLN (MESLN and spleens and stimulated *in vitro* for 48 h on anti-CD3 bound plates in the presence of IL-2). Shown are the amounts of IFN-γ, IL-4, IL-5, IL-10 and IL-13 present in the supernatants and determined by ELISA (B–F). In addition, serum was prepared and the total amount of IgG1 and IgE present in the serum of infected and uninfected mice was determined by ELISA (G and H). Shown are the average numbers of cells present in the BALs, the average amounts of cytokines present in the cell culture supernatants and the average amounts of IgG1 and IgE present in the serum of 5–6 mice/group with SEM (calculated from individual mice).

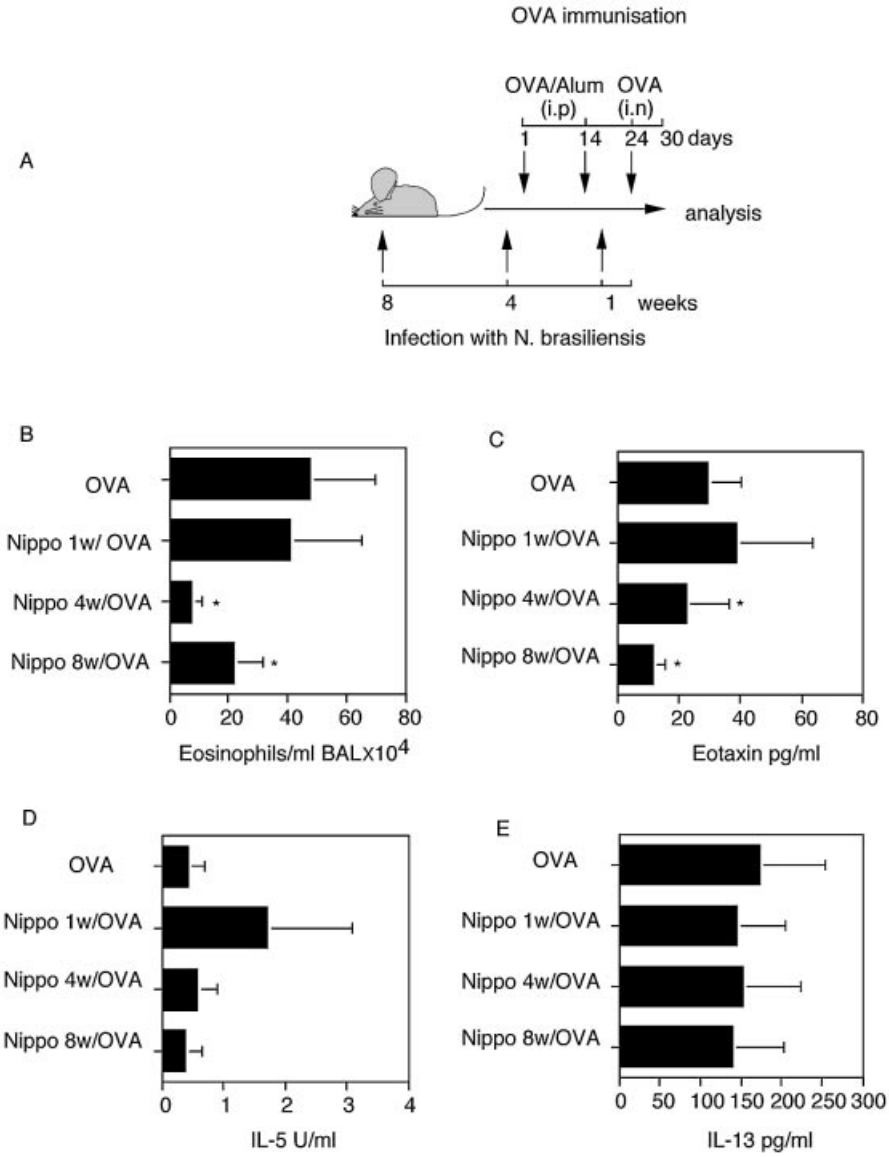

Fig. 2. Helminth infection reduces allergen-induced airway eosinophilia and Eotaxin production. Mice were infected 1, 4 and 8 weeks prior to OVA i.n. challenge as described in Methods (A). One group of mice was treated with OVA only as controls. Six days after the i.n. application of OVA airway challenge BALs were prepared and the number of eosinophils determined. At the same time point the amounts of Eotaxin, IL-5 and IL-13 present in the BALs of the different groups of mice were determined by ELISA (B–E). Shown are the mean amounts of eosinophils and cytokines + SEM determined in 10–12 individual mice per group (mice analyzed were from two separate experiments) (B, D and E). In (C) the mean amounts of eosinophils and cytokines + SEM from 5–7 individual mice/group (from one experiment) are shown and the experiment repeated once with similar results. *$P < 0.05$, compared to values obtained in OVA-only treated mice.

The results shown reflect the kinetic of the T cell response when the cells were polyclonally restimulated. We cannot rule out that restimulating antigen specifically might yield somewhat different results.

Allergen-induced Th2 responses in the lung of mice infected with N. brasiliensis

In order to address the question if helminth infections modulate the development of allergic responses we infected mice with the helminth *N. brasiliensis* and subjected the mice to an OVA immunization protocol (Fig. 2A). Figure 2(B) shows that mice infected with *N. brasiliensis* 8 and especially 4 weeks before OVA-airway challenge showed a significant decrease in eosinophil numbers in the airways in comparison to OVA-only immunized mice. However, when the mice were infected with the helminths 1 week (or 2 weeks, data not shown) before OVA i.n. challenge, eosinophil numbers were not reduced. The observed reduction in eosinophil numbers correlated with significantly reduced amounts of Eotaxin present in the BAL fluid (Fig. 2C). The amount of IL-5 and IL-13 present in the airways was not reduced by the helminth infection (Fig. 2D and E). T cells from the MLN of mice infected with the helminths 4 weeks before OVA i.n. challenge also secreted similar amounts of IL-5 after *in vitro* stimulation with anti-CD3/IL-2 or

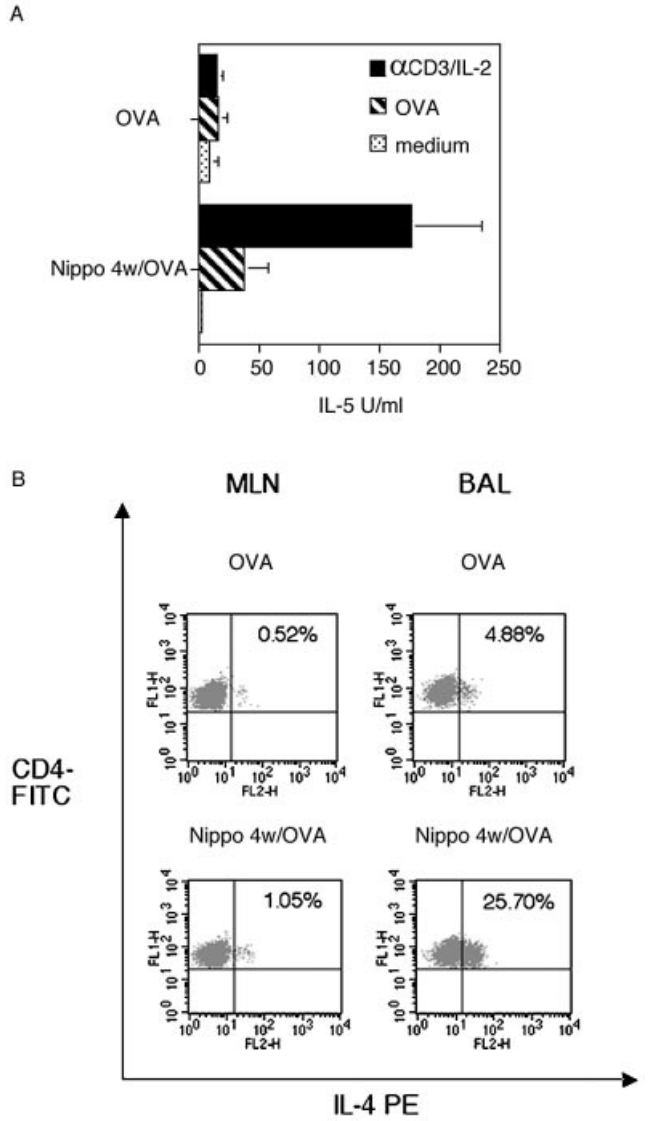

Fig. 3. Th2 cell responses in mice infected with *N. brasiliensis* and immunized with OVA. Mice were infected with *N. brasiliensis* and then subjected to an OVA-immunization protocol (as described in the legend for Fig. 2A) or treated with OVA only. Mice were infected 4 weeks before OVA-airway challenge. Six days after OVA-airway challenge single cell suspensions of MLN were prepared and BALs were performed. MLN cells were prepared and stimulated *in vitro* (see Methods). The level of IL-5 present in the supernatants was determined by ELISA. Shown are the mean values of six mice/group + SEM. The experiment was repeated twice with similar results (A). In addition the isolated cells were stimulated and stained with a combination of PE-labeled anti-IL-4 and anti-CD4-FITC mAb as described in the Methods (B). Shown are cells labeled with antibody FACS-stainings and gated on $CD4^+$ T cells representative of six mice per group. The percentage of $CD4^+$ T cells positively labeled for IL-4 production is also indicated. This experiment was repeated once with similar results.

OVA, respectively, in comparison to T cells from OVA-only treated mice (Fig. 3A). Furthermore, the numbers of Th2 cells detected in the BAL of OVA-immunized mice increased 5-fold, when the mice had been previously infected with the helminths. (Fig. 3B). However, no IL-4 or IL-10 could be detected in any of the samples. Taken together, these results indicate that the reduced amounts of eosinophils and Eotaxin detected in the mice infected with the helminths cannot be explained by a reduction of allergen-specific IL-4-producing $CD4^+$ cells present in the lungs. Histological analysis of the lung from mice (six per group) infected with the helminths 1, 4 or 8 weeks before OVA airway challenge revealed that goblet cell metaplasia and mucus production induced by the OVA immunization was also not influenced by the helminth infections (data not shown).

Cutaneous hypersensitivity and allergen-specific IgG1/IgE serum levels are not affected by infection with N. brasiliensis

Infection with *N. brasiliensis* leads to strongly increased Th2 cell responses (see above). It may be expected that the increased amounts of IL-4 present after the infection with the helminths would also increase the production of allergen-specific IgG1 and IgE in mice. Figure 4(A and B) shows that the OVA-specific IgG1 and IgE serum levels were not affected by the helminth infection. Furthermore, cutaneous anaphylaxis was also not reduced or increased by the helminth infections (Fig. 4C), although the helminth infection leads to strongly increased amounts of IgG1 and in particular IgE in the serum (see Fig. 1G and H) (potentially interfering with allergen-induced mast cell degranulation). These results clearly show that systemic allergen-specific B cell and mast cell responses are not influenced by infection with *N. brasiliensis*. However, when we determined OVA-specific IgE and IgG1 levels in the BAL, we found that a helminth infection 4 weeks before OVA i.n. challenge reduced these Ig levels detected in the BAL fluid (Table 1).

Helminth-induced suppression of airway eosinophilia was not observed in IL-10 deficient mice

Recent publications suggest that helminth-induced modulation of allergic responses may be associated with IL-10-mediated downregulation of Th2 effector mechanisms (11). Furthermore, we found that T cells from the MLN, MESLN and spleen from mice infected with *N. brasiliensis* secreted easily detectable amounts of IL-10 after *in vitro* restimulation (see Fig. 1E). For this reason we analysed if an infection with *N. brasiliensis* could also suppress the development of OVA-induced airway eosinophilia in IL-10 deficient mice. Figure 5 shows that helminth infection-associated suppression of allergen-induced airway eosinophilia and reduction of Eotaxin levels was not observed in IL-10 deficient mice. This suggests that this effect may be dependent upon the presence of IL-10 induced by the helminth infection. Supporting this view is our finding that IL-10 deficient mice develop stronger airway eosinophilia and Th2 responses in MLN of the lung 2, 3 and 4 weeks after infection with *N. brasiliensis* in comparison to control mice (Fig 6). Although IL-4 and IL-5 levels were clearly enhanced in the MLN cell cultures of IL-10 deficient mice, in comparison to wild-type mice IL-13 levels were similar (data not shown). Interestingly, the Th2 response initiated in the lung 1 week after infection was not increased in IL-10 deficient mice. This suggests that IL-10 may be involved in the downregulation of already established Th2 responses and not their initiation.

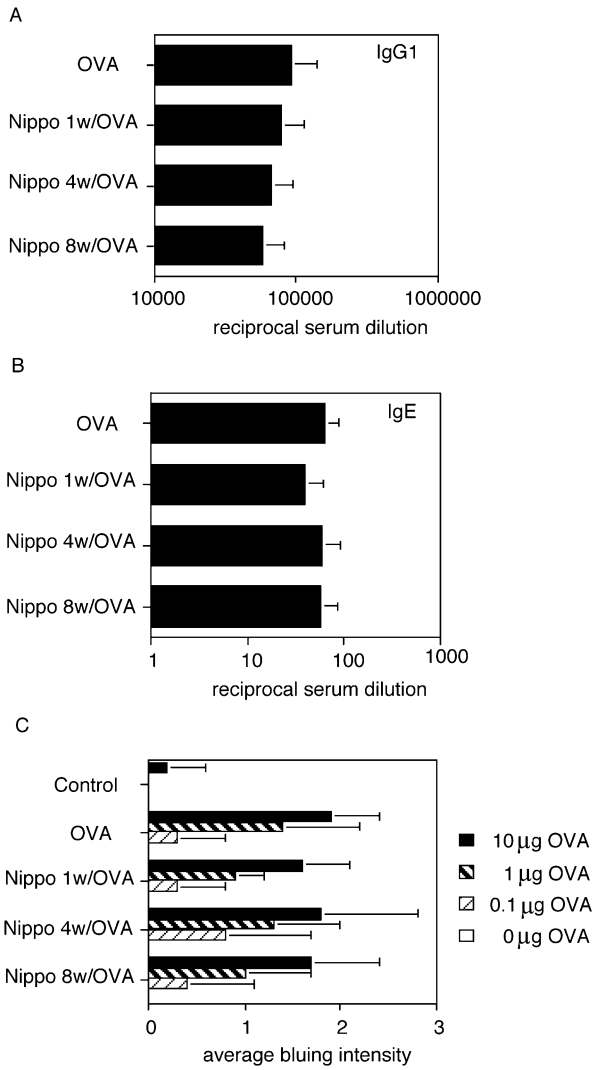

Fig. 4. OVA-specific IgG1 and IgE serum levels and cutaneous hypersensitivity reactions in mice infected with *N. brasiliensis*. Mice were treated as described in the legend to Fig. 2(A). Serum was prepared 6 days after OVA airway challenge and OVA-specific IgG1 and IgE antibody levels were determined by ELISA as described in Methods. Values represent mean serum titers + SEM of two separate experiments with 13–15 mice per group (A and B). The experiments were repeated once with similar results. Cutaneous hypersensitivity was determined by the degree of bluing intensity after i.d. application of OVA as described in the Methods (C). (0 = no bluing, 1 = slight bluing, 2 = strong bluing, 3 = very strong bluing.) Shown is the average bluing intensity with SEM of the different groups of mice (8–12 mice per group) of two separate experiments.

Table 1. OVA-specific IgG1 and IgE levels in the BAL of mice infected with *N. brasiliensis* 4 weeks before OVA i.n. challenge

	OVA-specific	
	IgG1	IgE
OVA	4318 ± 2790	12 ± 4
Nippo 4w/OVA	501 ± 163	4 ± 1

BALs were prepared and the BAL fluid was concentrated as described in Methods. The amount of antigen-specific IgG1 and IgE was determined by ELISA. Shown are the mean values ± SEM of six mice per group. The experiment was repeated once with similar results.

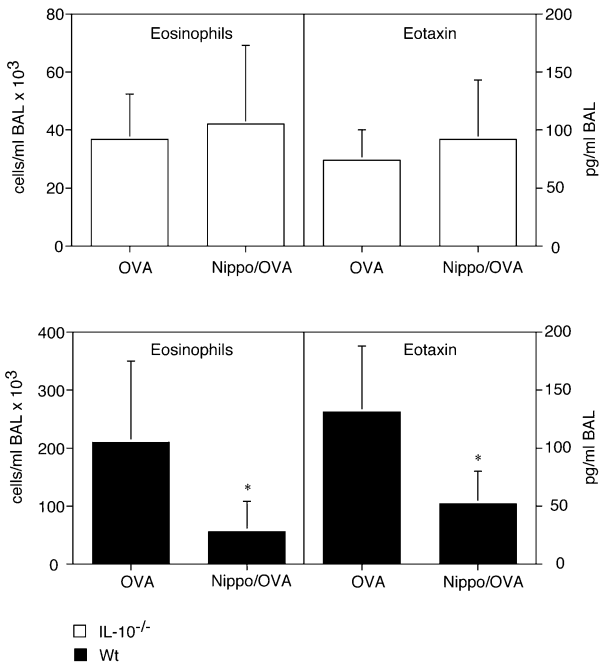

Fig. 5. Eosinophilia and Eotaxin levels in the lung of IL-10 deficient mice infected with *N. brasiliensis* and immunized with OVA. IL-10 deficient mice (IL-10$^{-/-}$) and wildtype mice (Wt) were immunized with OVA and infected with *N. brasiliensis* 4 weeks before OVA airway challenge or were immunized with OVA only. BALs were prepared 6 days after OVA i.n. challenge and the amount of eosinophils present in the airways of the groups of mice was determined The amount of Eotaxin present in the BALs was determined by ELISA. Shown are the mean values + SEM of two separate experiments from 12 mice per group (IL-10$^{-/-}$) or 7 mice per group (Wt).

We also investigated which cells produced IL-10 in the airways after helminth infection. Figure 7(A) shows that CD4$^+$ T cells secreting IL-10 could be detected in the BAL fluid of mice infected with *N. brasiliensis*. After 1 week of infection 12% of the CD4$^+$ T cells produced IL-10. Figure 7(B) shows that 73% of the CD4$^+$IL-10$^+$ cells also produced IL-4, 94% expressed CD25, and 99% produced CTLA-4 after 1 week of infection. Similar results were also found 2 weeks after infection. Four weeks after infection only 0.49–0.86% of the CD4$^+$ T cells produced IL-10; 44–48% of the IL-10-secreting CD4$^+$ T cells also produced IL-4, 23–31% CD25 and 55–67% CTLA-4 (data not shown). This suggests that ~20% of the IL-10-secreting CD4$^+$ T cells have a phenotype characteristic of Tr cells. However, the vast majority of IL-10 producers have a phenotype characteristic of Th2 cells. Furthermore, CD4$^+$ T cells were the only cells detected in the BAL fluid which produced significant amounts of IL-10 above background cell labeling using isotype control mAb (data not shown).

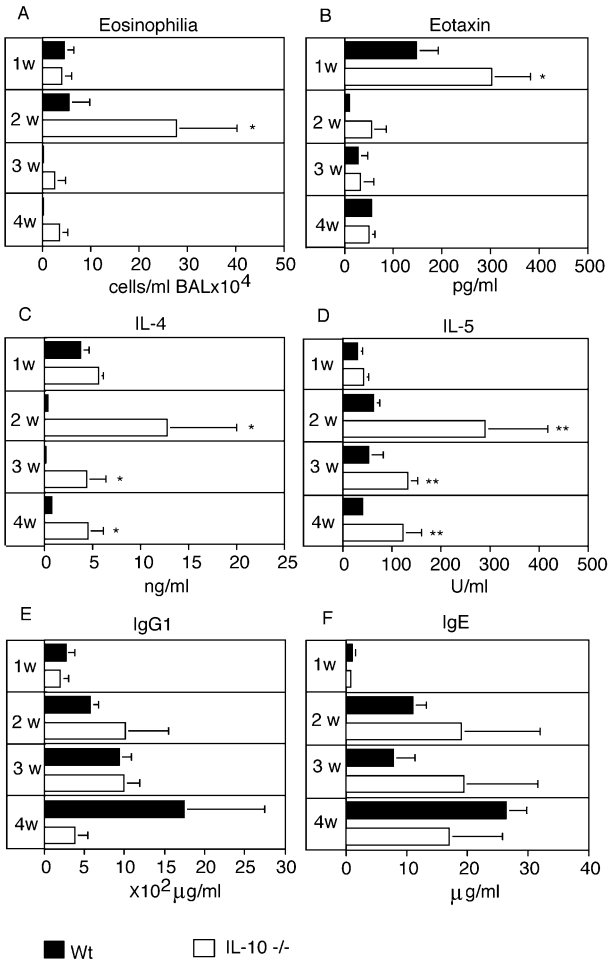

Fig. 6. IL-10 deficient mice infected with *N. brasiliensis* show enhanced Th2 responses. Wild type (Wt) and IL-10 deficient mice (IL-10$^{-/-}$) were infected with *N. brasiliensis* for 1, 2, 3 and 4 weeks. At the indicated time points BALs were prepared and the number of eosinophils and the amount of Eotaxin present in the BALs was determined (A and B). MLN cells were prepared and stimulated *in vitro* by αCD3/IL-2 for 48 h. Shown are the amounts of IL-4 and IL-5 present in the cultures (C and D). Serum was prepared and the amount of IgG1 and IgE present in the serum was determined by ELISA (E and F). Shown are mean values + SEM from six mice per group. $^{*}P < 0.05$, $^{**}P < 0.001$, compared to values obtained in control mice.

Discussion

The results presented in this report clearly demonstrate that infection with the helminth *N. brasiliensis* suppresses the development of allergen-induced airway eosinophilia and significantly reduces the production of Eotaxin in the airways. This effect was observed when the infection was performed 4 and 8 weeks but not 1, 2 and 12 (data not shown) weeks before OVA i.n. challenge, indicating that the time point of infection is critical for the suppressive effect. However, the pulmonary OVA-specific Th2 cell response was not negatively influenced by the helminth infection at any of the timepoints.

Recent reports have demonstrated that the application of IL-10 or adenovirures expressing granulocyte macrophage colony stimulating factor together with IL-10 into the airways of mice lead to a reduction in allergen-induced airway eosinophilia (30–32). Moreover, recent publications have led to the hypothesis that infections of humans with helminths induce the production of IL-10, which interferes with Th2 cell effector mechanisms (19). For these reasons, we investigated if *N. brasiliensis*-induced suppression of airway eosinophilia was dependent upon IL-10. We found that the suppressive effect on airway eosinophilia and the reduction of Eotaxin in the airways of OVA/helminth co-treated mice was not observed in IL-10 deficient mice. This result suggests that infection with *N. brasiliensis* induces the production of IL-10, which in turn may lead to the suppression of allergen-induced airway eosinophilia and reduces the production of Eotaxin.

How can the presence of IL-10 selectively reduce eosinophil numbers in the BAL fluid? Ohkawara *et al.* (33) recently reported that IL-10 inhibits the expression of CD40 on human eosinophils, leading to increased apoptosis. It is possible that this IL-10 effect on eosinophils was responsible for our observations. A further explanation of our findings is that IL-10 reduces the amount of Eotaxin in the airways which in turn results in reduced amounts of eosinophils being recruited into the airways, since Eotaxin is needed for the efficient recruitment of eosinophils into the airways (34). How and if IL-10 directly mediates this effect is not clear. We cannot rule out that the reduced airway inflammation was responsible for the reduced amounts of Eotaxin present in the airways. However, helminth-induced down regulation of Eotaxin production may be a general phenomenon since mice immunized with OVA and infected with *Strongyloides stercoralis* also showed a decrease in Eotaxin levels in the BAL fluid (22).

The source of the IL-10 possibly responsible for helminth-induced suppression of airway eosinophilia is also not clear. Numerous cells including macrophages, dendritic cells, B cells and T cells have been shown to produce IL-10 (32). Our results point at CD4^{+} T cells being a source of IL-10 since we found that T cells from the MLN, MESLN and spleen from mice infected with *N. brasiliensis* secreted IL-10 after *in vitro* restimulation with anti-CD3 and IL-2. We could also detect CD4^{+} T cells producing IL-10 in the BAL and MLN of helminth-infected mice. Some of these cells had a phenotype characteristic of Th2 cells (IL-10 and IL-4 coproducers) and others of Tr cells (CD25^{+}CTLA4^{+}IL-10^{+}IL-4^{-}) (35). It is possible that the helminth infection induces the generation of Th2 and/or Tr cells which secrete IL-10 as part of an anti-inflammatory network. This may result in the shutting down of the inflammatory response in the lung after *N. brasiliensis* has been successfully eliminated from the lung and gut and protective immunity has developed. Supporting this view is our finding that IL-10 deficient mice mount stronger and longer-lasting Th2 responses in the lung than wild-type mice infected with *N. brasiliensis*. Furthermore, Grunig *et al.* (36) showed that IL-10 deficient mice also mounted stronger Th2 responses when allergic bronchopulmonary aspergillosis was induced in these animals in comparison to control mice.

In particular Tr cells are very attractive candidates for mediating the observed effects since it has been shown that the development of allergen-induced Th2 responses could be prevented by the application of *in vitro* engineered allergen-specific Tr cell lines secreting IL-10 (37). In addition, Zuany-Amorium *et al.* (38) found that the inhibition of allergen-specific

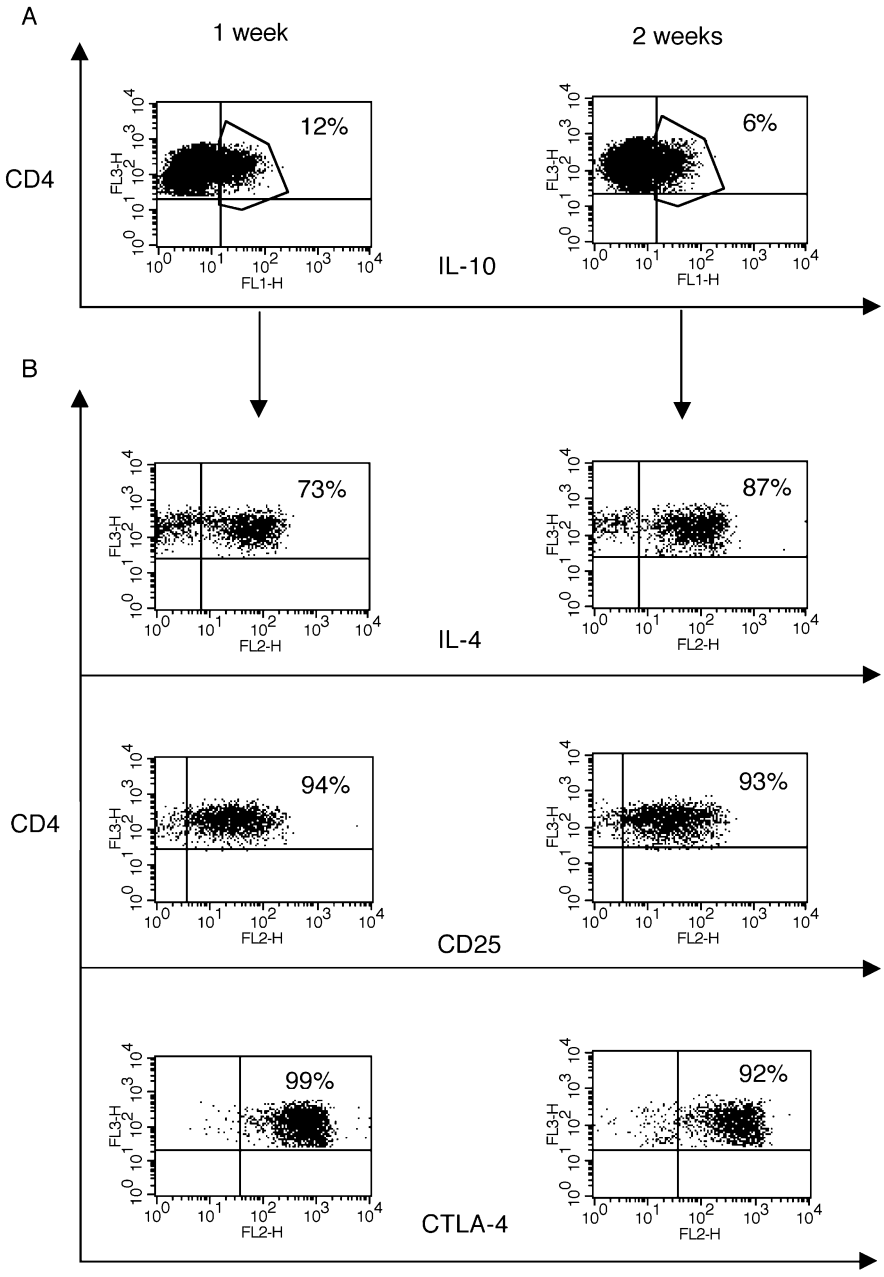

Fig. 7. CD4+ IL-10 producing T cells are present in the BAL fluid of mice infected with *N. brasiliensis*. Mice were infected with *N. brasiliensis*. One week and 2 weeks after infection BALs were performed. BAL cells were stimulated and stained *in vitro* (as described Methods). Shown are cells labeled with antibody and gated on CD4+ T cells present in the BALs 1 and 2 weeks post infection (A) and cells stained with antibody gated on CD4+/IL-10+ cells (B) (the BALs of nine mice/group were pooled). The percentage of CD4+ T cells positively stained for IL-10 production (A) and the percentages of CD4+/IL-10+ cells positively stained for IL-4, CD25 and CTLA-4 (B) are indicated. Positive cell labeling was controlled by using isotype control mAb (data not shown). The 2-week experiment was repeated once with similar results.

Th2 responses after the application of killed *Mycobacterium vaccae* could also be attributed to the generation of Tr cells secreting IL-10. Furthermore, it was also reported that antigen-specific Tr cells secreting IL-10 inhibited the induction of airway hyper-responsiveness in mice (39). However, other cells may also be involved in the observed effect, since Th2 and Tr cells secreting IL-10 were only detected in relatively small amounts. A possible candidate is dendritic cells, since Akbari *et al*. (40) reported that pulmonary dendritic cells secreting IL-10 mediated the suppression of allergen-induced Th2 responses in the lung of mice. The peak of IL-10 production by MLN cells and BAL cells after helminth infection (1–2 weeks) did not correlate with reduced OVA-induced airway eosinophilia observed 4 and 8 weeks after the helminth infection. This suggests that the downregulation of allergen-induced airway eosinophilia and Eotaxin production mediated

by IL-10 may be an event preceeding the i.n. application of OVA. However, this finding also indicates that the effect may not be due to IL-10. It is also possible that other factors besides the lack of IL-10 were responsible for the lack of helminth-infection associated suppression of airway eosinophilia observed in IL-10 deficient mice immunized with OVA. The immune response induced by the helminths and parasite migration and clearance in these animals may be altered in comparison to wild type mice. This question is currently under investigation.

A further aim of our study was to address the question if the helminth infection would influence the generation of allergen-specific B cells secreting IgE or IgG1. Based on previously published results it could be expected that the helminth infection would increase the amounts of B cells secreting allergen-specific IgE or IgG1 by increasing the amounts of IL-4 in these animals (41). On the other hand the report by Wang *et al.* (22) showed that infecting mice with *Strongyloides stercoralis* lead to a reduction of allergen-specific IgE in the BAL fluid. In addition, Bashir *et al.* (42) demonstrated very recently in a model of food allergy that an infection with the enteric helminth *Heligmosomoides polygyrus* results in a decrease of allergen-specific IgE production in the serum. Our results clearly showed that the helminth infection 1, 4 or 8 weeks before OVA i.n. challenge had no effect on the amount of allergen-specific IgE or IgG1 in the serum of the mice. These results clearly indicate that helminth-induced immune response had no influence on the amount of allergen-specific IgE or IgG1 present in the serum. However, similar to the finding of Wang *et al.* (22) we also found a reduction in OVA-specific IgE and IgG1 in the BAL fluid. The discrepancy between our findings in the serum and the report from Bashir *et al.* (42) may be explained by the different helminths used for the studies. Furthermore, it was reported that concurrent Th2 responses in the lung of mice induced by *N. brasiliensis* resulted in the inhibition of aerosol-induced IgE tolerance (induced by the repeated exposure to aerolized OVA) (23). This finding suggests that the experimental protocol used to induce allergen-specific Th2 responses may also impact how the helminth infection influences the development of B cells secreting allergen-specifc IgE.

Numerous epidemiological studies have found that a protective effect of helminth infection on allergic reactivity was associated with high levels of polyclonal IgE in the serum of the patients. Furthermore, it was shown that allergic sensitization of human lung fragments could be inhibited by saturating IgE binding sites (43,44) and that the increase in allergen-induced cutaneous skin reactivity after the treatment of anti-helminthic drugs strongly correlated with a decrease in total IgE levels (5). However, recent publications have challenged this view, where decreased allergen-induced mast cell reactivity in helminth infected persons could not be attributed to increased IgE levels (10,11). Taken together, it is not clear if the production of polyclonal IgE induced by a helminth infection inhibits allergen-mediated mast cell degranulation in humans. For this reason we challenged mice infected with *N. brasiliensis* and immunized with OVA (inducing up to 50-fold increased amounts of IgE in the serum before OVA immunization) or OVA-only immunized mice s.c. with different amounts of OVA and measured cutaneous mast cell reactivity. We found no decrease in allergen-induced mast cell reactivity in the helminth-infected and OVA-immunized mice. These results do not support the 'IgE blocking hypothesis'. However, in mice in contrast to humans, IgG1 crosslinking can also lead to mast cell degranulation (29). For this reason we cannot rule out that polyclonal IgE-mediated suppression of mast cell degranulation could not be detected, since IgG1-mediated crosslinking may be sufficient to induce maximal degranulation of the mast cells in mice.

In conclusion, we have demonstrated that infection with the helminth *N. brasiliensis* suppresses the development of allergen-induced airway eosinophilia without affecting allergen-specific Th2 cell reactivity in the lung. This effect was not observed in IL-10 deficient mice. It is tempting to speculate that the helminth infection induces the secretion of IL-10 in order to down regulate the eosinophilic inflammation of the airways induced by the L3 larvae. The development of allergen-induced airway eosinophilia at the same site and time point is then also inhibited. We obtained similar results in BALB/c mice (data not shown) suggesting that it is a general phenomenon in mice. Taken together, our results support the hypothesis that helminth infections may protect humans from allergic diseases. However, our results also clearly indicate that the time point of infection in relationship to allergen-exposure is critical for the degree of helminth-mediated suppression of allergic inflammation. A further understanding of the mechanism(s) responsible for helminth infection-induced suppression of eosinophilic inflammation in the lung may contribute to clinical intervention of asthmatic disease.

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Abbreviations

BAL	bronchoalveolar lavage
i.n.	intranasal
MESLN	mesenteric lymph nodes
MLN	mediastinal lymph nodes
OVA	ovalbumin
Tr	T regulatory

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