

Helminth-derived Products Inhibit the Development of Allergic Responses in Mice

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Rationale: Epidemiological studies suggest that infections with helminths protect from the development of asthma. Supporting this view is our published finding that infection with *Nippostrongylus brasiliensis* decreased ovalbumin-induced Th2 responses in the lung of mice.

Objectives: To evaluate if *N. brasiliensis* excretory–secretory products also prevent the development of asthma.

Methods: Mice were immunized with ovalbumin/alum intraperitoneally in the absence or presence of helminthic products and then challenged intranasally with ovalbumin. Six days later, we analyzed if the mice developed Th2 responses in the lung.

Main Results: The application of the helminthic products together with ovalbumin/alum during the sensitization period totally inhibited the development of eosinophilia and goblet cell metaplasia in the airways and also strongly reduced the development of airway hyperreactivity. Allergen-specific IgG1 and IgE serum levels were also strongly reduced. These findings correlated with decreased levels of IL-4 and IL-5 in the airways in product-treated animals. The suppressive effects on the development of allergic responses were independent of the presence of Toll-like receptors 2 and 4, IFN- γ , and most important, IL-10. Interestingly, suppression was still observed when the helminthic products were heated or treated with proteinase K. Paradoxically, we found that strong helminth product-specific Th2 responses were induced in parallel with the inhibition of ovalbumin-specific responses.

Conclusion: Our results suggest that helminths suppress the development of asthma by secreting substances that modulate allergic responses without affecting the generation of helminth-specific Th2 immunity. The identification of these products may lead to the design of novel therapeutic intervention strategies for the treatment of asthma.

Keywords: allergy; helminth; *Nippostrongylus brasiliensis*; products; inhibition

Allergic immune responses to common environmental antigens lead to clinical disorders such as allergic asthma, hay fever, eczema, and allergic rhinitis and are associated with Th2 cells producing IL-4, IL-5, IL-9, and IL-13. The secretion of these cytokines induces the production of allergen-specific IgE by B cells, the development of eosinophilia, smooth muscle contrac-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Epidemiological studies and animal work support the hypothesis that infections with helminths protect from the development of allergic responses.

What This Study Adds to the Field

Helminths may suppress the development of asthma by producing substances that interfere selectively with the induction of allergen- but not helminth-specific Th2 responses.

tion, and mucus secretion. IgE cross-linking induces the degranulation of eosinophils and mast cells, which are two critical factors leading to the development of allergic disorders (1–3).

Although the role of heritability in the development of these diseases is unquestionable, it can not explain the marked increase in the incidence of atopic disorders during the last few decades (4–8). This observation has been attributed, in part, to the steady decline of infectious diseases in the developed world, a phenomenon called the “hygiene hypothesis” (7). It suggests that the exposure to infectious agents in the early childhood prevents the development of allergen-specific Th2 cells because they establish Th1-based immunity (8). To view the rise in allergic diseases only as the result of lacking Th1 responses appears to be too simplistic, because recent epidemiological studies have indicated a possible protective effect of helminth infections, in particular, schistosomes or hookworms (9, 10), on the development of atopy. Experiments performed in animals also found suppressive effects of infections with helminths on the development of allergic diseases. Wang and colleagues (11) showed that infecting mice with *Strongyloides stercoralis* led to a reduction of allergen-specific IgE in the bronchoalveolar lavage (BAL) fluid. In addition, Bashir and colleagues (12) demonstrated 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. We have also found that an infection with the helminths *Nippostrongylus brasiliensis* suppressed allergic features in mice (13). The mechanism of how helminths suppress the development of allergic responses is unclear but appears to be at least in part associated with the induction of T-regulatory (Tr) and not Th1 responses (8, 14).

In the present study, we investigated if products derived from helminths could also suppress the development of allergic responses. For this purpose, we applied *N. brasiliensis* excretory–secretory products (NES) together with ovalbumin (OVA)/alum

(Received in original form January 13, 2006; accepted in final form November 22, 2006)

Supported by the Bundesministerium für Bildung und Forschung, the Bayerisches Staatsministerium für Wissenschaft, Forschung, und Kunst, and the Bayerische Forschungsförderung-Forimmun.

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Am J Respir Crit Care Med Vol 175, pp 336–344, 2007

Originally Published in Press as DOI: 10.1164/rccm.200601-0540C on November 22, 2006
Internet address: www.atsjournals.org

intraperitoneally to mice and analyzed if OVA-specific Th2 responses were generated in the presence of the helminth-derived products. We found that NES strongly inhibited the development of OVA-specific Th2 responses (published as abstracts in References 15 and 16) and that the effect seems to be mediated by nonprotein components of NES. Our results encourage the evaluation of helminth-derived products as novel therapies aimed at preventing allergic diseases.

METHODS

Mice

Female C57BL/6, Balb/c, and C3H/HeJ mice (carrying a spontaneous mutation in the Toll-like receptor 4 gene (TLR-4 knockout [KO]) were purchased from Charles River (Sulzfeld, Germany). IFN- γ -deficient mice were generously provided by O. Liesenfeld (Berlin, Germany). IL-10-deficient mice were a gift of W. Müller (Cologne, Germany). TLR-2-deficient mice (with seven back-crosses on C57BL/6) were bred at the Research Center Borstel Germany (originally obtained from Tularik, Inc., San Francisco, CA). Mice transgenic for the OVA₃₂₃₋₃₂₉-specific $\alpha\beta$ T cell receptor (TCR) (OT-2) were kindly provided by A. Schimpl (Würzburg, Germany). All animals except the TLR-4 KO mice were on the C57BL/6 genetic background. At the onset of the experiments, animals were between 6 and 8 weeks of age. All experiments were performed according to the guidelines of the local and government authorities.

Immunization Protocols

C57BL/6 mice and the different KO strains were sensitized intraperitoneally with a mixture of 2 μ g OVA (Sigma Chemical Co., St. Louis, MO) in 200 μ l alum (Serva, Heidelberg, Germany) (Days 0 and 14) in the presence or absence of 50 μ g of NES (alum-precipitated together with OVA). Where indicated, less NES was also used. On Day 24, the mice were then anesthetized by an intraperitoneal injection of ketamine/xylazine (Sigma Chemical Co.) and challenged intranasally with 50 μ l phosphate-buffered saline (PBS) containing 100 μ g OVA or 50 μ g of NES in PBS. Analyses were performed 6 days later (Day 30) unless otherwise indicated. In one experiment, we applied 50 μ g NES+alum intraperitoneally, 6 days after the mice were treated with OVA/alum (Days 6 and 20).

Mice were also immunized subcutaneously in the footpads with 25 μ g of NES in PBS or PBS only on Days 0 and 7. On Day 11, CD4⁺ T cells from the draining lymph nodes (LN) producing IL-4 or IFN- γ were detected as described previously (17).

Balb/c mice were sensitized by intraperitoneal injections of 20 μ g OVA (Serva) in 200 μ l PBS/alum (or PBS/alum only) on Days 1, 14, and 21, with or without 50 μ g of NES precipitated together with OVA/alum, and challenged (Days 26 and 27) by inhalation with a 1% OVA (or PBS) solution for 20 minutes and analyzed on Day 28.

BAL

BAL was performed as previously described (17).

N. brasiliensis Parasites and Preparation of NES

Seven days after subcutaneous injection of 3,000 infective-stage (L3) larvae (13), animals were killed, and adult worms were harvested from the gut by dissection. NES was prepared as previously described (18). Protein concentration was determined by Bradford assay (Bio-Rad Laboratories GmbH, Munich, Germany). NES aliquots were stored at -20°C. Endotoxin levels in our NES preparations were less than 1 μ g/ml (determined by Limulus assay, Bio Whittaker, Walkersville, MD). LPS-depleted NES (endotoxin levels < 0.01 μ g/ml) was also prepared by using the Detoxi-Gel AffinityPak prepacked columns (Pierce, Rockford, IL) following the manufacturer's instructions.

Digestion of NES with Proteinase K

Proteinase K agarose beads (Sigma Chemical Co.) were suspended at 1 mg/ml in distilled water for 1 hour at 2 to 8°C and washed three times with ice-cold 20 mM Tris HCl at pH 7.2. The pellet was then mixed with 1 ml of the NES preparation (4,000 μ g protein/ml) at a 20% vol/vol

ratio. The mixture was incubated at 50°C under agitation, replacing the beads at 2, 24, and 48 hours. After 72 hours, the proteinase beads were removed by centrifugation for 1 minute at 13,000 rpm and the protein digestion of the bead-free supernatant was then confirmed by Bradford assay (Bio-Rad Laboratories GmbH) and by silver staining of a polyacrylamide gel (Silver Stain Plus kit; Bio-Rad Laboratories GmbH).

Detection of Immunoglobulins and Cytokines

OVA-specific immunoglobulins in the serum and cytokine levels in the BAL fluid and culture supernatants were determined by ELISA as described elsewhere (13, 17). Where indicated, ELISA for NES-specific immunoglobulins was also performed. In this particular case, microtiter plates were coated overnight at 4°C with NES (10 μ g/ml), and the NES-specific antibody titers were detected in the serum by using biotinylated monoclonal antibodies (mAbs) against mouse IgG1 (A85-1), IgG2a (R19-15), or IgE (R35-118). BAL fluid was concentrated threefold using Millipore's Amicon Ultra-15 centrifugal filter devices with a 5-kD retaining filter (Millipore GmbH, Schwalbach, Germany). CD4⁺ T cells from BAL producing IL-5 were detected by using two-color fluorescence-activated cell sorter analysis as described elsewhere (13, 17). All antibodies were purchased from Becton Dickinson (Becton Dickinson GmbH, Heidelberg, Germany).

Histological Analysis

Histological analysis was performed as previously described (17). The intensity of peribronchial and perivascular inflammation as well as the degree of goblet cell metaplasia were scored by two independent observers (0 = no inflammation or goblet cell metaplasia, 1 = slight inflammation or goblet cell metaplasia, 2 = strong inflammation or goblet cell metaplasia, 3 = very strong inflammation or goblet cell metaplasia).

Measurement of Airway Hyperreactivity

Airway hyperreactivity (AHR) was assessed by methacholine-induced airflow obstruction, using a whole-body plethysmograph (in C57BL/6 mice) determining enhanced pause (Penh) values (model PLT UNR MS; Emka Technologies, Paris, France) as previously described (17) or using a head-out system (in Balb/c mice) measuring midexpiratory airflow (EF₅₀) as previously described (19).

Proliferation Assay

Lymph node cells (a mixture of mediastinal and mesenteric lymph nodes: 4 $\times$ 10⁵/well) from OT-2 TCR transgenic mice were cultured in 96-well flat-bottom plates (Nunc, Wiesbaden, Germany) for 48 hours either in medium, in the presence of OVA (40 μ g/ml; Sigma), or with an mAb to CD3 (145-2C11, 25 μ g/ml; Becton Dickinson) together with 200 U/ml recombinant human IL-2 (Novartis, Basel, Switzerland). Where indicated, NES (10 μ g/ml, 5 μ g/ml, 1 μ g/ml) was added 1 hour before the initiation of the culture. DNA synthesis was measured using [³H]thymidine incorporation.

Treatment of Mice with Anti-IL-10 Receptor Antibodies

Anti-IL-10 receptor (anti-IL-10R) mAbs were generously provided by Dr. M. Lutz (Erlangen, Germany). C57BL/6 mice were treated with anti-IL-10R mAbs (320 μ g/mouse intraperitoneally) on Days -1, 6, 13, and 20. As controls, groups of mice were treated with the isotype-matched mAbs (also 320 μ g/mouse intraperitoneally). Simultaneously, the protocol to induce OVA-specific Th2 responses using either OVA/alum or OVA+NES/alum was initiated as described above.

Statistical Analysis

Statistical differences between two different groups were evaluated using Student's *t* test. One-way analysis of variance together with the Tukey test for multiple comparisons was used to establish differences between three or more groups. A *p* value of less than 0.05 was considered significant.

RESULTS

Application of NES with OVA/Alum Inhibits the Development of Allergen-induced Airway Inflammation and Hyperreactivity

We evaluated the capacity of NES, an excretory-secretory product derived from adult *N. brasiliensis* (18), to suppress

OVA-specific allergic responses in mice. NES was applied together with OVA/alum intraperitoneally during the OVA sensitization period, and subsequently an OVA intranasal challenge was performed (Figure 1A). The development of OVA-specific Th2 allergic responses was evaluated 6 days later. As shown in Figure 1B, the application OVA+NES/alum was able to profoundly reduce the eosinophilia and the inflammatory response in the airways in comparison to the application of only OVA/alum (200-fold reduction in the airway eosinophilia compared with OVA only-treated mice). NES also significantly reduced the OVA-specific IgG1 and IgE serum levels, although the production of OVA-specific IgG2a was not affected (Figure 1C). Because the production of IgG2a in mice is stimulated by IFN- γ , these results suggest that the mechanism underlying the down-regulatory effects of NES on OVA-specific Th2 responses is not related to the induction of OVA-specific Th1 responses *in vivo*. This view is supported by the unaltered levels of IFN- γ found in the BAL fluid of the NES-treated animals (Figure 1D). Levels of IL-10 detected in the BAL fluid were also not altered by the application of NES (Figure 1D). In contrast to untreated naive animals, IL-4, IL-5, RANTES (regulated upon activation, normal T-cell expressed and secreted), monocyte chemoattractant protein-1 (MCP-1), and eotaxin could be detected in the BAL of NES-treated mice; however, with the exception of eotaxin, the levels were significantly reduced in comparison to OVA-only-treated animals (Figures 1D and E). The production of eotaxin was also reduced in the NES-treated mice, albeit not significantly (Figure 1E).

Mice treated with NES also showed a strong reduction in perivascular and peribronchial inflammation (mean scores: PBS, 0; OVA/alum, 2; OVA+NES/alum, 0; Figures 2A–2C) and no goblet cell metaplasia in the airways (mean scores: PBS, 0; OVA/alum, 3; OVA+NES/alum, 0; Figures 2D–2F) in comparison to the OVA/alum-only immunized mice. Furthermore, Figure 3A shows that the AHR induced by the application of high doses of methacholine was also significantly decreased in NES-treated animals. This observation was associated with a reduction in the number of eosinophils in the airways (Figure 3B). We also repeated this experiment in Balb/c mice using a similar experimental approach (see METHODS) and found that AHR determined by measuring the midexpiratory airflow (EF₅₀) using head-out body plethysmography technology was also significantly reduced in the NES-treated mice (OVA control, 30.8 ± 6.3 ; PBS

control, $46.5 \pm 5.8^*$; OVA/NES, $73.8 \pm 12.3^{**}$ ED₅₀ methacholine [mg/ml]; mean values $\pm$ SEM of 6–8 mice/group; $*p < 0.05$, $**p < 0.01$ compared with the OVA group). Total eosinophil numbers in the BAL and OVA-specific IgG1 and IgE serum levels were also significantly reduced in the NES-treated animals (data not shown). Taken together, our results clearly show that the application of NES together with OVA strongly inhibits the development of systemic and local allergen-specific Th2 responses. Interestingly, we found that when mice immunized and challenged with OVA received NES+alum intraperitoneally 6 days after the application of OVA/alum, they no longer showed a reduced development of airway eosinophilia (OVA, 2.02 ± 0.64 ; OVA+NES < 0.01 ; NES after OVA, 1.7 ± 0.55 eosinophils/ml BAL $\times 10^6$ mean of 7 to 8 mice/group $\pm$ SEM) or reduced amounts of OVA-specific IgE or IgG1 in the serum (data not shown).

We further determined that amounts of NES as low as $0.05 \mu\text{g}/\text{mouse}$ still had the ability to decrease the OVA-induced allergic responses, demonstrating that this helminth-derived component is very efficient in this model (eosinophils/ml BAL $\times 10^3$ in mice treated with OVA/alum, 399.7 ± 90.97 ; OVA+NES [$50 \mu\text{g}/\text{mouse}$]/alum, $0.33 \pm 0.21^*$; OVA+NES [$0.05 \mu\text{g}/\text{mouse}$]/alum, $33.67 \pm 15.26^*$; mean of 6 to 7 mice/group $\pm$ SEM; $*p < 0.05$).

NES Does Not Interfere with the Proliferation of OVA-specific Transgenic T Cells *In Vitro*

Previous studies with NES have shown the presence of enzymes with protease activity in this product (20). Therefore, one possible explanation for the previously shown results is that the proteolytic activity of NES may degrade the OVA protein to a level where OVA peptides can no longer be presented by antigen presenting cells after the application of OVA+NES. To investigate if NES has this effect, OVA lymph node cell suspensions from OT-2 OVA-specific $\alpha\beta$ TCR transgenic mice (95% of the CD4⁺ T cells express this TCR) were stimulated with OVA in the presence or absence of different concentrations of NES. OVA was incubated together with NES 1 hour before the cells were added. T-cell proliferation was determined by using [³H]thymidine incorporation. Table 1 shows that the OVA-induced proliferation of OVA-specific TCR transgenic cells was not affected by the presence of different amounts of NES.

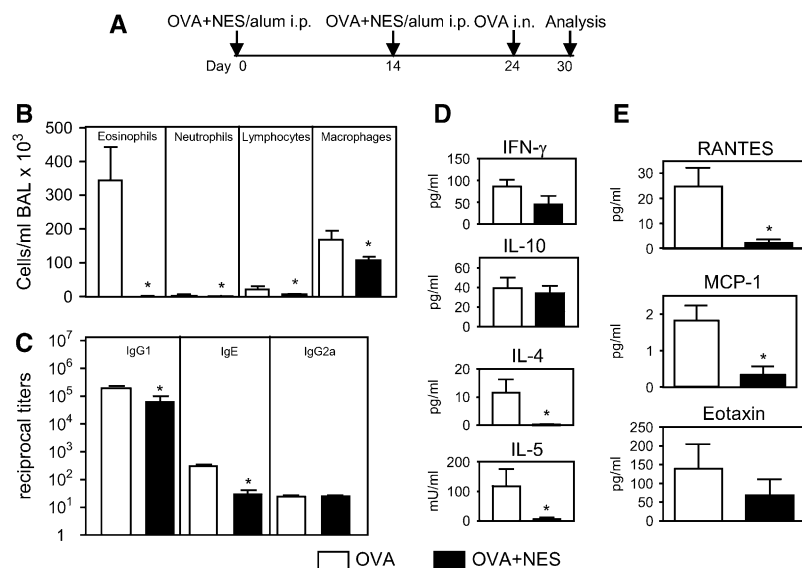


Figure 1. *Nippostrongylus brasiliensis* excretory-secretory products (NES) suppresses ovalbumin (OVA)-specific allergic Th2 responses. (A) Mice were vaccinated with OVA+NES/alum during the OVA-sensitization period and then the induction of OVA-specific Th2 responses was analyzed 6 days after the OVA intranasal challenge. Depicted are the absolute number of the different cell types present in the (B) bronchoalveolar lavage (BAL), (C) the reciprocal titers of OVA-specific serum antibodies, and (D) the cytokine and (E) chemokine levels present in the BAL fluid. Mean values of six mice/group $\pm$ SEM are shown. The experiment was repeated four times with similar results. $*p < 0.05$ compared with the values obtained from mice sensitized only with OVA.

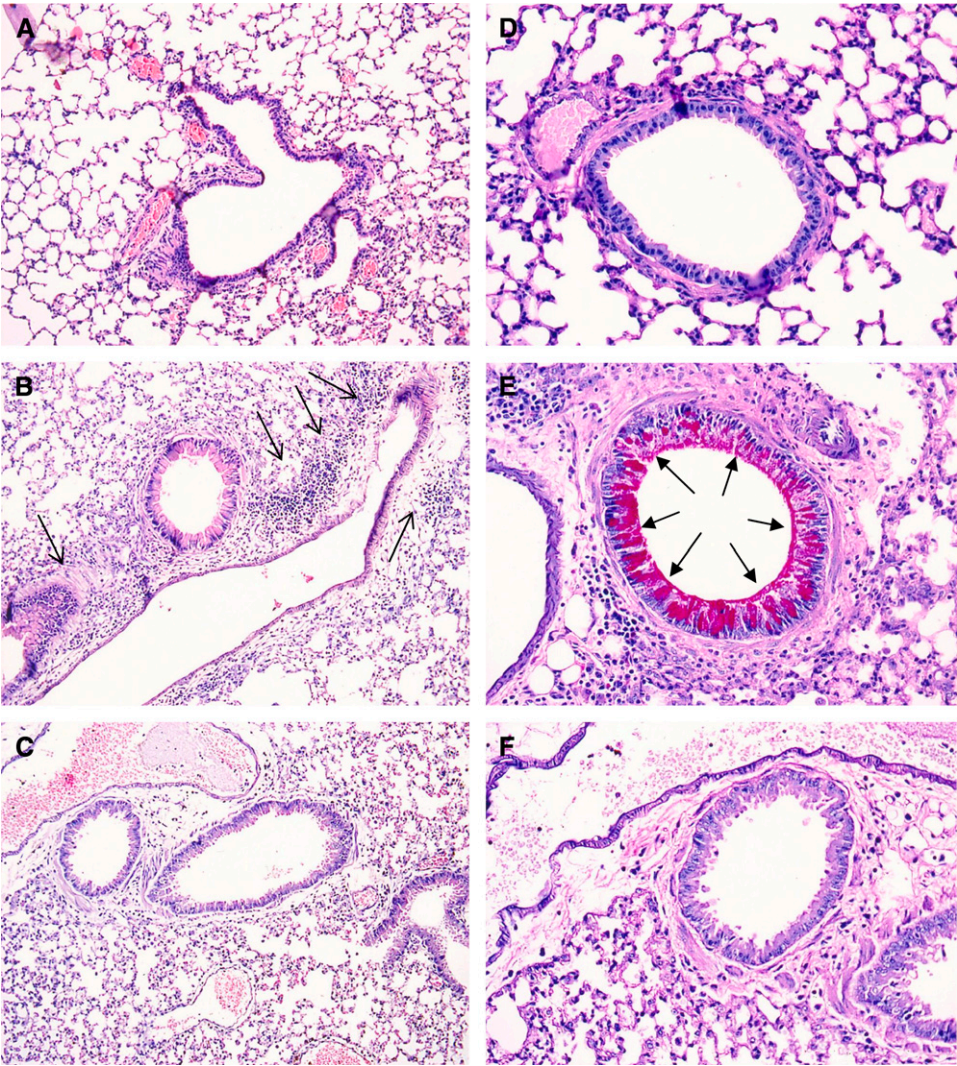


Figure 2. Airway inflammation and goblet cell metaplasia in OVA-sensitized/challenged mice are also decreased after the application of NES. Mice were vaccinated as described in Figure 1. Shown are the lung sections subjected to (A–C) hematoxylin and eosin or (D–F) periodic acid–Schiff staining from (A, D) phosphate-buffered saline–, (B, E) OVA–, and (C, F) OVA+NES-immunized mice. Representative examples of six mice/group are shown (A–C, 20×, and D–F, 200× magnification). Arrows indicate areas with peribronchial and perivascular inflammation (B) or goblet cell metaplasia (E).

NES-induced Inhibition of Allergen-specific Responses is Proteinase K and Heat Stable

In an attempt to elucidate the components of NES responsible for its ability to down-modulate OVA-specific allergic responses,

NES was treated with proteinase K beads. The proteinase K–digested NES was applied to the mice together with OVA/alum, and the induction of allergic responses was evaluated after the OVA challenge using the protocol described in Figure 1A.

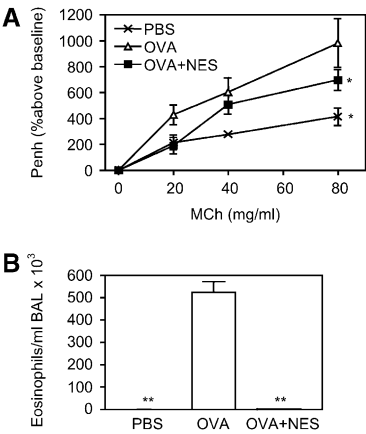


Figure 3. NES decreases airway hyperreactivity (AHR) after repetitive OVA intranasal challenges. Mice were injected intraperitoneally with OVA/alum or OVA+NES/alum at Days 0 and 14. They were then subjected to OVA intranasal challenges on Days 24, 25, 26, and 31, 32, and 33, and analyses were performed at Day 34. The AHR in response to increasing concentration of methacholine (MCh; A) and the absolute number of eosinophils in the BAL (B) are illustrated. Mean

values of six mice/group ± SEM are shown. *p < 0.05 compared with the values obtained from mice sensitized only with OVA; **p < 0.01.

TABLE 1. NIPPOSTRONGYLUS BRASILIENSIS EXCRETORY–SECRETORY PRODUCTS DO NOT INTERFERE WITH THE EFFICIENT PROLIFERATION OF OVALBUMIN-SPECIFIC TRANSGENIC T CELLS

	Medium	OVA	Anti-CD3/IL-2
Medium	4,000 ± 365	164,008 ± 8,354	69,036 ± 960
NES 10	4,051 ± 491	176,356 ± 16,298	58,243 ± 10,574
NES 5	3,711 ± 326	144,021 ± 20,160	44,516 ± 3,497
NES 1	4,020 ± 695	160,870 ± 4,120	36,952 ± 2,791

Definition of abbreviations: NES = *Nippostrongylus brasiliensis* excretory–secretory products; OVA = ovalbumin. Lymph node cells from OT-2 (H-2^b) T cell receptor transgenic mice (recognizing the OVA_{323–339} epitope) were cultured for 48 h either in medium, OVA (40 µg/ml), or with a monoclonal antibody to CD3 (25 µg/ml) together with recombinant IL-2 (200 U/ml). Where indicated, NES was added to the cultures (10, 5, or 1 µg/ml). To measure DNA synthesis, cells were pulsed with 0.25 µCi/ml [³H]thymidine overnight and harvested. [³H]Thymidine incorporation was determined using liquid scintillation counting in a β-plate. Results are expressed as counts per minute, means ± SD of triplicate cultures.

The complete digestion of all protein components of NES was monitored by a silver stain of a polyacrylamide gel (data not shown). Proteinase K–digested NES retained the ability to suppress OVA-induced airway eosinophilia (Figure 4A). However, this inhibitory effect was not as pronounced as the effect of nondigested NES (see Figure 1), suggesting that NES contained proteins that contributed to the decreased OVA-specific allergic responses *in vivo*. It is also possible that a nonprotein ligand is attached to a protein backbone, reducing the affinity/availability of the ligand after proteinase treatment. Proteinase K–digested NES also significantly suppressed the production of OVA-specific IgG1 in the serum (Figure 4B). We additionally performed experiments using NES that had been previously heated at 100°C for 20 minutes to denature all the protein components of NES. Remarkably, heat-treated NES was still able to significantly decrease airway eosinophilia and serum OVA-specific IgG1 levels in OVA-sensitized/challenged mice (Figure 4). Taken together, these experiments indicate that nonprotein components of NES are mainly responsible for its suppressive effects on allergic immune responses in this model.

The Ability of NES to Down-modulate OVA-induced Th2 Responses Is Independent of TLR-2, TLR-4, or IFN- γ

Next, we also assessed the antiallergic effect of NES in the absence of TLR-2 and TLR-4 (21) as described in Figure 1A. Figure 5A shows that neither the absence of TLR-2 nor TLR-4 affected the suppressive capacity of NES on airway eosinophilia. Also, TLR-2–deficient and TLR-4 KO mice vaccinated with NES showed reduced levels of OVA-specific IgG1 and IgE in the serum in comparison to mice injected only with OVA/alum (Figure 5B). These data show that TLR-2 as well as TLR-4 are not necessary for the antiallergic effects of NES and also suggest that our observed effects are not due to the small contamination of endotoxin still present in our NES preparations. Nevertheless, to rule out this possibility, we compared our LPS-contaminated NES with LPS-depleted NES on the ability to suppress OVA-induced allergic responses. We found that these two preparations of NES had the same inhibitory effects on airway eosinophilia (eosinophils/ml BAL $\times 10^3$ in mice treated with OVA/alum, 601.00 ± 208.20 ; OVA+NES [$< 0.01 \mu\text{g/ml}$ LPS]/alum, $4.00 \pm 1.48^*$; OVA+NES [$< 0.01 \mu\text{g/ml}$ LPS]/alum, $5.71 \pm 1.55^*$; mean of 7 mice/group $\pm$ SEM; $*p < 0.05$) and on the serum levels of OVA-specific IgG1 and IgE (data not shown).

We also investigated if IFN- γ was involved in the suppressive effect of NES. For this purpose, IFN- γ –deficient and control mice were treated as described in Figure 1A. The absence of IFN- γ did not affect the suppressive effect of NES on the development of airway eosinophilia (eosinophils/ml BAL $\times 10^3$ in IFN- γ –deficient mice treated with OVA/alum, 141.1 ± 32.26 ; OVA+NES/alum, $6.87 \pm 4.37^*$; mean of 7–8 mice/group $\pm$ SEM

from two separate experiments; $*p \leq 0.05$). IFN- γ –deficient NES-treated mice also exhibited reduced levels of serum OVA-specific IgG1 and IgE in comparison to mice injected only with OVA/alum (data not shown).

Suppressive Effect of NES on the Development of OVA-specific Th2 Allergic Responses Is Not Mediated by IL-10

Recent findings suggest that NES may be mediating its suppressive effect through an IL-10–dependent mechanism (13, 22–24). To test this hypothesis, we evaluated whether NES was still able to suppress OVA-induced allergic responses in the absence of IL-10 or IL-10–mediated signaling. Monoclonal blocking antibodies against the IL-10 receptor (IL-10R) were applied intraperitoneally and, in parallel, mice were treated as described in Figure 1A. Mice injected intraperitoneally with an isotype-matched antibody were used as controls. Surprisingly, mice vaccinated with NES could significantly suppress airway eosinophilia and OVA-specific IgG1 and IgE in the serum after blocking IL-10–mediated signaling by using IL-10R mAbs (Figure 6A). These results were confirmed in IL-10–deficient mice (Figure 6B).

NES Suppresses OVA-specific Th2 Responses and Simultaneously Induces NES-specific Th2 Responses

Previous studies have shown that NES induces strong Th2-type immune responses in mice (18, 25). These observations are in contrast to our findings in which NES clearly leads to the inhibition of Th2 responses. To test if our NES preparation may be different from the previously used NES, we analyzed if our NES is also able to induce Th2 responses. For this purpose, mice were immunized with OVA+NES/alum, as described before, and were then either challenged with OVA or with NES intranasally. Surprisingly, although the application of NES+OVA/alum suppressed the development of airway eosinophilia after the application of OVA intranasally, the application of NES+OVA/alum and then intranasal NES induced a strong airway eosinophilia (Figure 7A). The induction of airway eosinophilia correlated with an increase in the percentage and absolute numbers of IL-5–producing CD4⁺ cells present in the BAL of the NES+OVA/alum immunized and NES intranasally challenged animals (Figures 7B and 7C). The production of NES-specific antibodies in the serum was also evaluated. Figure 7D shows that the application of NES intraperitoneally and then intranasally significantly enhanced the levels of NES-specific IgG1 and IgE in the serum. Taken together, these results clearly show that NES induces Th2 responses against its own components and simultaneously inhibits the development of OVA-induced Th2 responses. We further confirmed this observation by applying NES in the footpads of mice and evaluating the frequency of IL-4– and IFN- γ –producing CD4⁺ T cells from the draining LN after restimulation

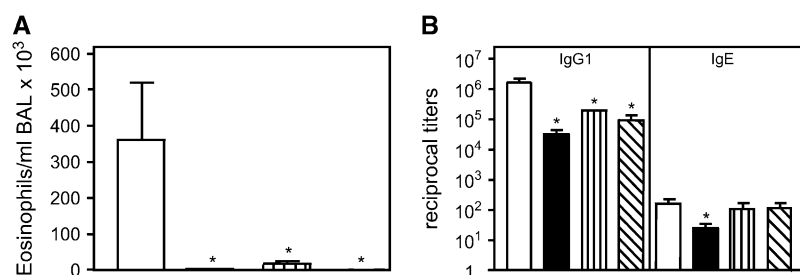


Figure 4. The antiallergic effect of NES is mainly mediated by nonprotein components. Mice vaccinated with either NES, proteinase K–digested (PK) NES, or heat-treated (ht) NES were subjected to the OVA-sensitization protocol as described in Figure 1A. Depicted are the absolute number of eosinophils in the BAL (A) and the OVA-specific IgG1 and IgE serum titers (B). Mean values of five to six mice/group $\pm$ SEM are shown. The experiment was repeated once with similar results. Open bars, OVA; solid bars, OVA+NES; vertical-striped bars, OVA+PK NES; hatched bars, OVA+ht NES. $*p < 0.05$ compared with the values obtained from mice sensitized only with OVA.

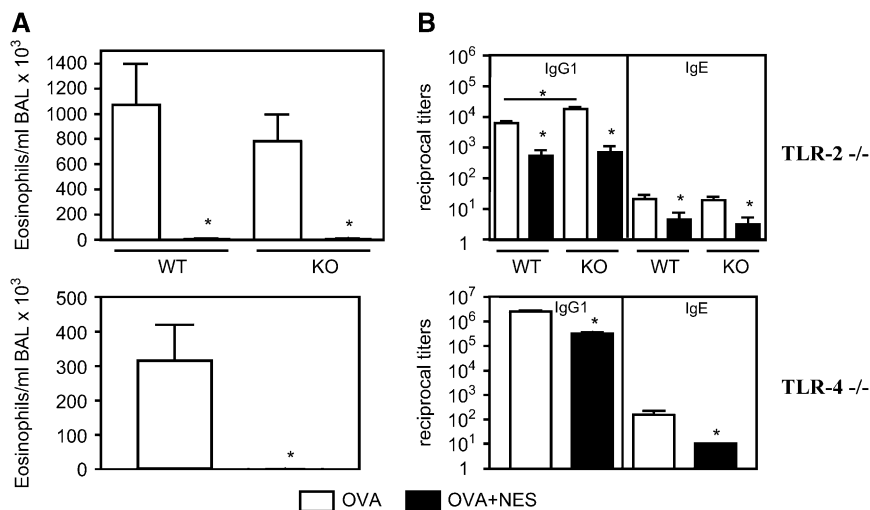


Figure 5. Toll-like receptor (TLR)-2 and TLR-4 are not necessary for the antiallergic effects of NES. TLR-2-deficient mice (knockout [KO]) and C57BL/6 wild-type controls (WT) or TLR-4-deficient mice (C3H/HeJ mice) were vaccinated with OVA+NES/alum using the same protocol described in Figure 1A. Shown are the absolute number of eosinophils in the BAL (A) and the OVA-specific IgG1 and IgE serum titers (B). Mean values of four to six mice/group $\pm$ SEM are shown. * $p < 0.05$ compared with the values obtained from mice sensitized only with OVA.

in vitro. Intracellular staining revealed that the percentage of IL-4-producing CD4⁺ T cells increased from 0.2% (PBS-treated mice) to 2% in the NES-treated mice. Total numbers of CD4⁺IL-4⁺ cells/LN also greatly increased (data not shown). No changes were observed in the percentages and absolute numbers of IFN- γ -producing CD4⁺ T cells between PBS- and NES-treated mice (data not shown). Taken together, these results clearly demonstrate the Th2-promoting activity of NES and its simultaneous ability to inhibit OVA-induced allergic responses.

DISCUSSION

Recent publications suggest that infections with helminths can protect from the development of allergic disorders both in animals and in humans (8). Furthermore, products derived from *Ascaris suum* have also been shown to have antiallergic effects (26), suggesting that helminth infection-induced suppression of allergen-specific Th2 responses is mediated by products released by the parasites. In our current study, we investigated if *N. brasiliensis* produces substances that also have antiallergic effects. NES was highly effective in suppressing eosinophilia, mucus production, and inflammatory responses in the airways as well as AHR when applied together with OVA/alum during

the OVA-sensitization period. The finding that only low levels of OVA-specific IgE and IgG1 could be detected in the NES/OVA-treated mice suggests that the presence of NES interfered with the development of OVA-specific Th2 responses during the allergen-sensitization phase, resulting in the strongly reduced airway inflammation after the intranasal application of OVA. Nevertheless, the detected OVA-specific IgG1 and IgE may be responsible for the enhanced Penh measurement in the NES-treated mice in comparison to the PBS-only-treated group. Interestingly, NES did not reduce the development of allergen-specific Th2 responses, when applied 6 days after the intraperitoneal sensitization with OVA/alum, suggesting that the presence of NES during allergen-specific Th2 cell development is necessary to have an suppressive effect.

How can NES suppress the development of OVA-specific Th2 responses? There are several possibilities. First, it was previously reported that NES contains enzymes with protease activity (20). The proteolytic activity of NES may degrade the OVA protein to a level where OVA peptides can no longer be presented by antigen presenting cells after the application of OVA+NES. We found that the OVA-induced proliferation of OVA-specific TCR transgenic cells was not affected by the presence of different amounts of NES, suggesting that NES does

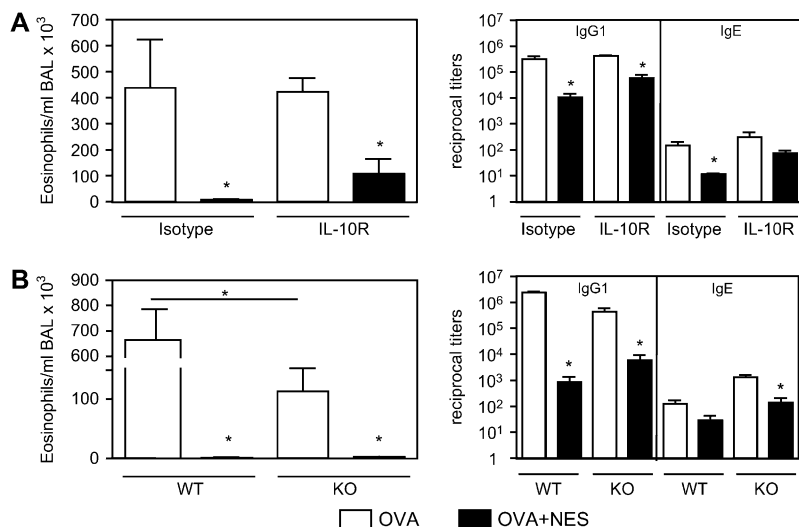


Figure 6. IL-10 is not required for the suppressive effect of NES on the development of OVA-induced allergic responses. The involvement of IL-10 on the NES-mediated reduction of OVA-specific allergic responses was evaluated either by blocking IL-10-mediated signaling by treating mice with IL-10 receptor (IL-10R) monoclonal antibodies (mAbs) ("IL-10R" in the figure) or isotype-matched control mAbs ("Isotype" in the figure), as described in METHODS (3–6 mice/group) (A), or in the absence of IL-10 by using IL-10-deficient mice (WT, wild-type strain; KO, IL-10-deficient mice; 6–7 mice/group) (B) using the protocol described in Figure 1A. Shown are the absolute numbers of eosinophils in the BAL and the OVA-specific IgG1 and IgE serum titers. Mean values of four to six mice/group $\pm$ SEM are shown. * $p \leq 0.05$ compared with the values obtained from mice sensitized only with OVA.

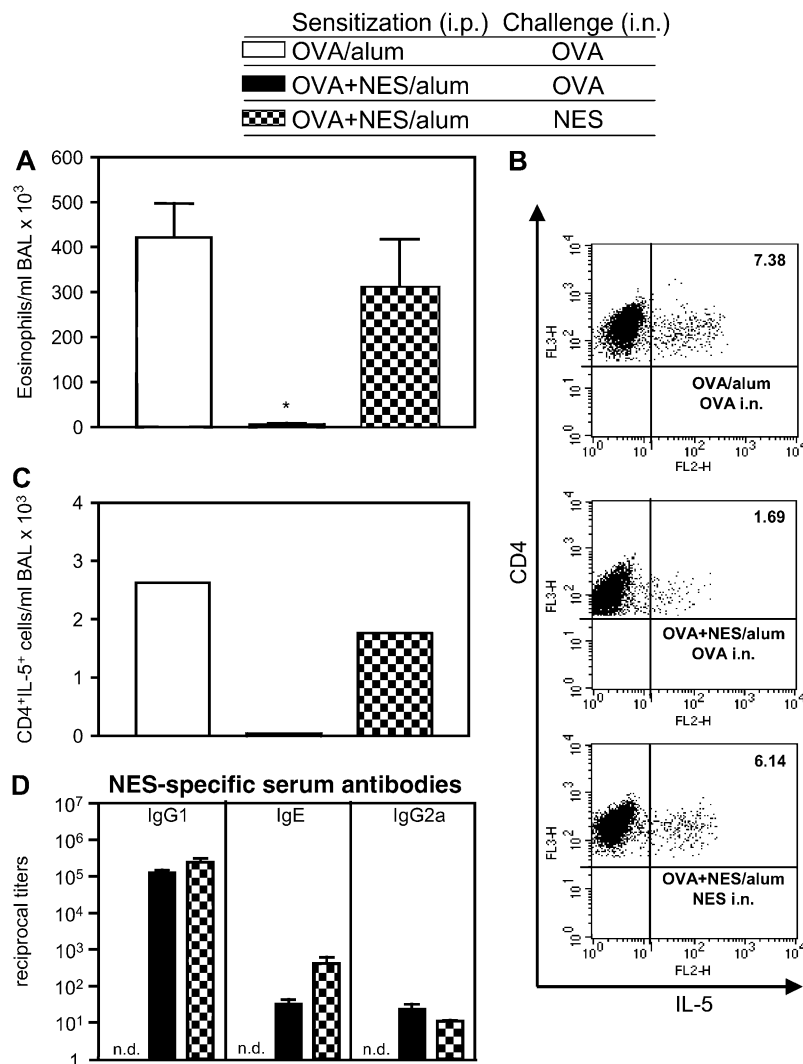


Figure 7. NES inhibits the development of OVA-specific allergic responses but simultaneously induces the development NES-specific Th2 responses. Mice were vaccinated using the protocol described in Figure 1A. Where indicated, NES was applied during the intranasal (i.n.) challenge instead of OVA. Shown are the absolute number of eosinophils in the BAL (A), the percentage (B) and absolute number (C) of IL-5-producing CD4⁺ T cells in pooled cells from the BAL, and the reciprocal titers of NES-specific serum antibodies (D). Mean values of five to six mice/group $\pm$ SEM are shown. The experiment was repeated once with similar results. i.p. = intraperitoneal; n.d. = not detected. * $p < 0.05$ compared with the values obtained from mice sensitized and challenged only with OVA.

not generally interfere with the efficient presentation of OVA-derived peptides. However, Boitelle and colleagues (27) recently reported, using an adaptive transfer model, that helminth products did not influence the initial primary response to OVA but interfered with the efficient expansion of OVA-specific CD4⁺ T cells *in vivo*. Currently, we cannot totally rule out this possibility.

A second explanation of our finding is that we applied an excess amount of NES in comparison to OVA (a 25:1 ratio). We also tested if the excess amounts of NES may be responsible for the inhibition of OVA-specific Th2 responses. Amounts of NES as low as 0.05 μ g added together with OVA/alum still had the ability to strongly decrease OVA-induced airway eosinophilia, strongly indicating that the inhibitory effect of NES is not simply due to the presence of an excess amount of helminth-derived protein during allergen sensitization. Supporting this view was our finding that boiled NES or proteinase K-digested NES also retained their inhibitory effects on allergen-specific Th2 responses. Furthermore, Balb/c mice, in which 20 μ g of OVA were applied together with 50 μ g of NES, also showed the strong reduction in AHR, airway eosinophilia, and OVA-specific IgG1 and IgE serum levels. Taken together, these results indicate that NES contains nonprotein products that selectively inhibit the development of allergen-specific Th2 responses.

Among the candidates for the antiallergic activity of NES, several lipoconjugates could be considered. Among others, lipo-

peptides, such as the synthetic lipopeptide LP40, which was already demonstrated to inhibit allergic responses in mice by triggering TLR-2 (28), may also be present in our NES preparations. Another important helminth-derived product to consider is the lysophosphatidylserine family of molecules with acyl chains. These lipid species not found in mammals were reported to be present in adult worms of the helminth *Schistosoma mansoni* (23) and have the ability to activate dendritic cells through TLR-2-inducing Tr cells. However, the ability of NES to decrease OVA-induced allergic responses in our model was independent of either TLR-2 or TLR-4 signaling, suggesting that NES may harbor pathogen-associated molecular patterns interacting with other receptors.

On the basis of previous reports that suggest that infections of humans or animals with helminths induce the generation of IL-10-producing Tr cells, which interferes with Th2 cell effector mechanisms (13, 22–24), we postulated that the suppressive effect of NES on OVA-induced allergy may be mediated by IL-10. Surprisingly, we found that NES-mediated inhibition of OVA-specific Th2 responses was still observed in IL-10-deficient mice and in mice treated with anti-IL-10R mAbs. These data strongly argue that the NES effect was also not mediated by IL-10.

A further candidate for our observed effects is transforming growth factor (TGF)- β . This cytokine has been shown to reduce inflammatory responses *in vitro* and *in vivo* and is also implicated

in the induction of oral tolerance (29). Furthermore, homologs of TGF- β or the TGF- β receptor family are expressed by some helminth parasites (30, 31). We are currently investigating the role of this cytokine on the ability of NES to suppress OVA-induced Th2 responses.

It is also possible that the generation of CD4⁺CD25⁺ Tr cells is stimulated by NES, inducing the IL-10-independent suppression of OVA-induced Th2 responses. Indeed, infection with the helminth *S. mansoni* was reported to induce the generation of CD4⁺CD25⁺ Tr cells in mice, which can inhibit immune responses partially through an IL-10-independent mechanism (32). Supporting this view is the current report from Wilson and colleagues (33). They found that an infection with *Heligmosomoides polygyrus* suppressed airway inflammation in OVA and house dust mite allergen Der p 1-sensitized mice and provided evidence that the suppression was mediated by CD4⁺CD25⁺Foxp3⁺ Tr cells and that the effect was IL-10 independent.

A further cell that possibly mediates our observed effects is the alternatively activated macrophage (AAM). AAMs develop after helminth infection (34), are markedly cytostatic, preventing the proliferation of nonlymphoid as well as T cells, and act through a contact-dependent mechanism (35, 36). They have also been shown to reduce the proliferation of a Th2 cell clone *in vitro* (37). AAMs may be induced during the intraperitoneal application of NES, which, in turn, would suppress OVA-specific Th2 responses, thereby explaining our results.

An intriguing finding of the present study is the ability of NES to inhibit Th2-mediated OVA-specific allergic responses but simultaneously induce the development of NES-specific Th2 responses. Currently, we have no explanation for our observation, but it is tempting to speculate that the immune system can distinguish between signals from helminths (NES) and signals from allergens (OVA) when both stimuli are present at the same time and site, resulting in the development of a response against the pathogen but not the allergen. Our observation may also help explain the apparent paradoxical finding that humans suffering from helminth infections (associated with strongly increased local and systemic Th2 responses) often show less allergic reactions (8, 14).

Taken together, our results clearly show that NES can inhibit the development of allergen-specific Th2 responses and that this effect is independent of TLR-2, TLR-4, IFN- γ , and IL-10. Furthermore, the effect was still observed when NES was heated or treated with proteinase K, suggesting that nonproteins were mediating most of the observed effects. The use of helminth-derived products may also help in the design of novel therapeutic intervention strategies for the treatment of allergic disorders.

Conflict of Interest Statement: C.M.T.-V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.W.-K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.J.E. is employed by Boehringer-Ingelheim (BI). The study, however, was not sponsored by BI.

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