

Research Article

Secretory products from infective forms of *Nippostrongylus brasiliensis* induce a rapid allergic airway inflammatory response

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Summary Allergic asthma is responsible for widespread morbidity and mortality and its incidence has increased dramatically in industrialized countries over the past two decades. Here, we describe a new murine model of allergic asthma utilizing a novel allergen with intrinsic enzymatic activity similar to that reported for many environmental allergens. The allergen, NES, is excreted and secreted from the nematode *Nippostrongylus brasiliensis*, and can readily be isolated from *in vitro* parasite cultures. When NES is administered intranasally to presensitized mice, allergic airway disease develops, including airway hyper-responsiveness, airway eosinophilia, IgE antibody production and Th2 cytokine production. This disease is characteristic of atopic asthma and can be induced within 11 days, thus providing a platform for the rapid analysis of allergic disease and high throughput testing of immunomodulatory factors.

Key words: allergen, asthma, nematode, *Nippostrongylus*, Th2.

Introduction

The incidence of asthma has increased dramatically in industrialized countries over the past two decades, and accordingly, numerous experimental investigations have focused on the factors responsible for both its development and its pathogenesis. Many of the key discoveries that have revealed the immunological mechanisms responsible for atopic asthma have come from studies utilizing murine models of allergic airway inflammation. These models are characterized by the activation of antigen-specific Th2 cells that secrete IL-4, IL-5 and IL-13.^{1,2} The action of these cytokines results in the maturation and migration of eosinophils from the bone marrow to the airways,^{3,4} B cell isotype switching to IgE,⁵ mucus production and airway hyper-responsiveness.⁶

Numerous experimental protocols are implemented to induce asthma-like responses, the majority of which involve the sensitization of animals to innocuous proteins such as ovalbumin (OVA), followed by multiple i.n. challenges to induce an asthma-like response.^{7,8} These models typically involve sensitization by i.p. injection of antigen adsorbed in alum adjuvant; however, in some cases, alum adjuvant is not used as an antigen depot, with the consequent requirement for numerous injections of antigen to sensitize mice.⁷ A significant disadvantage of such models is that much manipulation of mice is required over an extended period of time, and the proteins used are not natural environmental allergens likely to possess the common features responsible for inducing atopic asthma. Protocols that use environmental allergens such as the common house dust mite allergen, Der p1, and cockroach extracts can be difficult to work with experimentally. These proteins are

allergens which induce T-cell activation and can also act directly upon bronchial epithelial cells and inflammatory cells such as eosinophils, basophils and T cells^{9,10} to specifically induce Th2 immune responses. However, these allergens are difficult to isolate in sufficient quantities for *in vivo* experiments, and recombinant expression and refolding can result in loss of intrinsic protease activity, a feature that is thought to be key to a protein's ability to stimulate atopic responses.

Another common model used to study Th2 immune responses is infection with *Nippostrongylus brasiliensis*. Infection with *N. brasiliensis* induces a Th2 immune response characterized by the presence of CD4⁺ Th2 cells and eosinophils, mucus cell hyperplasia and IgE production by B cells.^{11–13} In contrast to typical allergic airway inflammation models, *N. brasiliensis* larvae infect via the skin and migrate to the lung via the vasculature. Although *N. brasiliensis* eventually emigrates from the lung, a Th2 immune response characteristic of allergic airway inflammation is sustained at the initial site for an additional 14 days after the larvae have migrated, probably due to the secretion and deposition of allergens.^{13,14} The larvae mature to the L4 stage and leave the lung, possibly via active migration, or by being coughed up and swallowed, leading to their localization in the jejunum.¹⁵ *N. brasiliensis* of either the infective L3, migrating L4 or gut localized L5 adult stages can readily be cultured *in vitro*, enabling the allergens secreted by them to be isolated from culture supernatant, and providing an ideal source of allergens for use in experimental investigations.

In this study, we found that the airways of mice can be sensitized to produce profound allergic responses when challenged with the secreted products of the infective forms of *N. brasiliensis* (NES). This lung inflammatory response has many of the hallmark pathological features of atopic asthma and has the significant advantage of inducing this response within a much shorter time frame than other models available to date. In addition, the isolated allergens used here more

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closely resemble common asthma-inducing allergens than do those from conventional models, which use innocuous antigens such as OVA. The atopic asthma model described here allows characterization of the immunological pathways responsible for allergic responses and provides a platform for the rapid investigation of factors that may modulate the development of asthma.

Materials and methods

Mice

C57BL/6 mice used in these experiments were bred by the Biomedical Research Unit, Wellington School of Medicine, University of Otago, Wellington, New Zealand. Six- to 10-week-old mice were used in all of the experiments. All experimental procedures described in this study were approved by the Wellington School of Medicine Animal Ethics Committee and carried out in accordance with the guidelines of the University of Otago, New Zealand.

Preparation of NES allergens

L3 infective *N. brasiliensis* larvae were isolated from faecal cultures, washed five times in sterile PBS then a further five times in an antibiotic cocktail (RPMI medium with 1:20 penicillin and streptomycin and 1:100 gentamycin [Gibco-BRL, Grand Island, NY, USA]). After the final wash, larvae were incubated for 60 min at room temperature in the antibiotic cocktail. Larvae were then incubated for 48 h at 37°C in RPMI 1640 (RPMI medium with 1:100 penicillin and streptomycin and 1:100 gentamycin) supplemented with 1% glucose. The supernatant was concentrated in Vivaspinn 20 centrifugation concentrators (Vivascience Sartorius Group, Hannover, Germany) and the amount of protein NES was determined by absorbance at 280 nm. NES was filter sterilized and frozen in aliquots at -20°C until required.

NES and OVA inoculation

For the two-prime model, mice were primed by two i.p. injections of OVA (Sigma, St Louis, MO, USA) or NES in 200 µL of 2% Al(OH)₃ alum adjuvant (Serva Electrophoresis, Heidelberg, Germany) at the indicated concentrations. A booster inoculation was given 2 weeks after initial priming. Seven days after the booster inoculation, mice were anaesthetized using xylazine and ketamine (Phoenix, Auckland, New Zealand) and OVA or NES (at the indicated concentrations) in 50 µL of PBS was administered by i.n. inoculation. Four days following the intranasal challenge, mice were killed and tissues were taken for analysis. For the one-prime model, mice were primed with one i.p. injection of OVA (Sigma) or NES in 200 µL of 2% Al(OH)₃ alum adjuvant (Serva), followed by a i.n. inoculation 7 days later. At the indicated time points following i.n. challenge, mice were killed and tissues were taken for analysis.

Quantification of airway eosinophilia

Mice were killed by i.p. injection of a lethal dose of anaesthetic. The trachea was cannulated and bronchoalveolar lavage (BAL) was performed by flushing the lungs and airways three times with 1 mL Iscove's modified Dulbecco's medium (IMDM) containing 5% FCS. The BAL cells were counted and 1×10^5 cells were spun onto glass slides. Slides were stained with Diff-Quik (Dade Behring, Newark, DE, USA) according to the manufacturer's instructions. The percentage of macrophages, lymphocytes, eosinophils and neutrophils in a minimum of 200 total cells was determined microscopically using standard histological criteria.

Histological analysis

Lungs were fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin wax. Sections were cut and stained with haematoxylin-eosin, or combined alcian blue and periodic acid-Schiff, using standard histological protocols.

Enzymatic digestion of lung tissue

Lungs were cut into small pieces using a scalpel blade under sterile conditions and incubated in 5 mL complete IMDM (Gibco-BRL) containing 2.4 mg/mL collagenase type II (Gibco-BRL) and 0.1% DNase 1 (Sigma) for 30 min at 37°C. Free floating cells were collected in 50 mL tubes, placed on ice, and remaining tissue was broken down by passage through an 18 gauge needle. The cell suspension was incubated for a further 30 min in 5 mL new enzyme cocktail mix (fresh collagenase and DNase). The resulting cell suspension was pooled with the lung cells collected earlier. Lung cells were washed twice in IMDM, then underlaid with 70% Percoll (Amersham Bioscience, Uppsala, Sweden). Cells were centrifuged at 800 g for 20 min with a slow deceleration to ensure cell layers were not disturbed. Lymphocytes were removed from the interface between the Percoll and IMDM and used for further analysis.

Cell culture

Mediastinal lymph nodes (MLN) were taken and made into a single cell suspension by gentle teasing through sterile gauze. Isolated cells, at a concentration of 1×10^6 cells/mL, were cultured in 24-well plates in the presence of dendritic cells (DC) and 100 µg/mL NES allergens for either 6 or 48 h. All cultures used IMDM supplemented with 5% FCS, 2 mmol/L glutamine, 1% penicillin-streptomycin and 5×10^{-5} mol/L 2-mercaptoethanol (all from Gibco-BRL).

Intracellular cytokine staining and FACS analysis

Approximately 5×10^5 cells from BAL samples were stimulated with NES-pulsed DC for 6 h at 37°C in IMDM. For the final 2 h, Brefeldin A (10 µg/mL; Sigma) was added to the cultures to retain cytokines in the cytoplasm. Thereafter, cells were washed with PBS/0.1% BSA and incubated with anti-CD32/CD16 mAb for 30 min at 4°C to block Fc binding. After another washing step, cells were stained with allophycocyanin (APC)-labelled anti-CD4 mAb (BD Biosciences Pharmingen, San Diego, CA, USA) for 15 min at 4°C. Subsequently, cells were washed with PBS/0.1% BSA, then again in PBS, and then they were fixed with 2% paraformaldehyde for 20 min at room temperature. Fixed cells were then incubated in permeabilization buffer (0.5% saponin in PBS/1% BSA) containing FITC-labelled anti-IFN-γ and phycoerythrin (PE)-labelled anti-IL-4 mAb (BD Pharmingen) for 30 min at room temperature. Cells were washed twice in permeabilization buffer and then resuspended in PBS/1% BSA and analysed by flow cytometry (FACSCalibur; Becton Dickinson, Mount View, CA, USA) and Flowjo software (Tree Star, Ashland, OR, USA).

Cytokine assays

A sandwich ELISA using TRFK5 capture and TRFK4-biotin conjugated detecting antibodies was used to measure IL-5 cytokine production. Between each of the following steps, plates were washed five times in PBS. Polyvinyl chloride 96-well plates were coated overnight at 4°C with capture antibody, followed by blocking with 2% BSA in PBS for 60 min at room temperature. Appropriate dilutions of samples and murine IL-5 internal standard were added

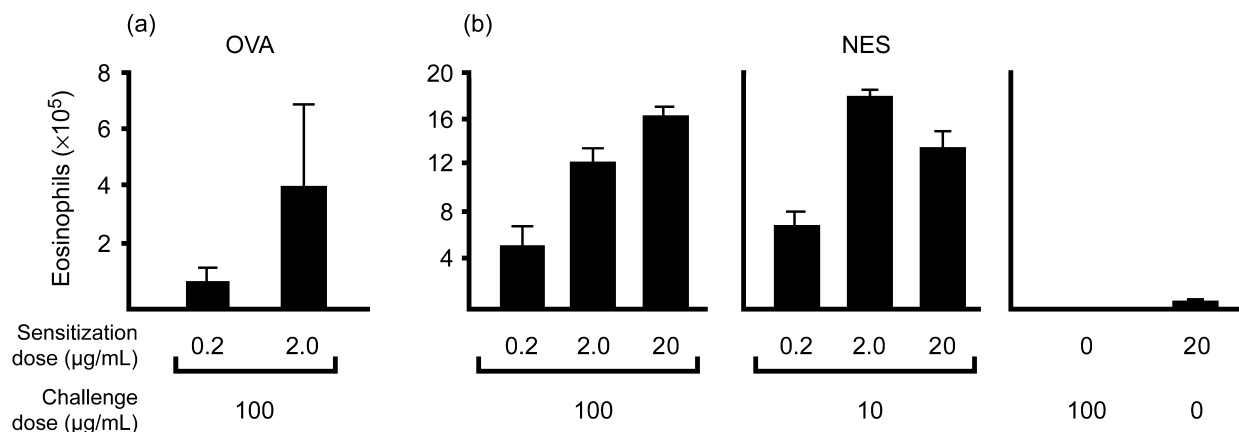


Figure 1 *Nippostrongylus brasiliensis* secretory products induce airway eosinophilia in a dose-dependent manner. Mice were sensitized on days one and 14 by i.p. injection of either (a) ovalbumin (OVA) protein or (b) NES, then challenged with the same allergen 7 days later via the i.n. route. Four days following i.n. challenge, bronchoalveolar lavage (BAL) was performed, total cells were counted and the number of eosinophils determined as described in the Materials and methods. These data are from a representative experiment using three to four mice per group, and represent the mean total number of eosinophils for each group $\pm$ standard deviation.

and incubated for 2 h at room temperature. Peroxidase-labelled streptavidin was then added and incubated for 1 h at room temperature. The reaction was developed using 2,2' azino-di(3-ethylbenzthiazoline sulphonic acid) (ABTS; Sigma). A 2 mmol/L sodium azide solution was used to stop the reaction, and plates were read at 414 nm using the Benchmark microplate reader (Bio-Rad, Hercules, CA, USA).

Antibody ELISA

Total IgE levels in serum were determined by ELISA. IgE levels were assayed using the anti-IgE mAb 6HD5 as the capture antibody and R1E4-biotin conjugate as the detector antibody. Peroxidase-labelled streptavidin was then added and reactions were developed. The same protocol for ELISA was carried out as for cytokine assays.

Measurement of airway responsiveness

On day four post i.n. challenge with NES, mice were placed in individual unrestrained whole body plethysmograph chambers (Buxco Electronics, Petersfield, UK). Airway responsiveness was assessed in mice by inducing airflow obstruction with aerosolized methylcholine-chloride (MetCh; Aldrich Chemie, Steinheim, Germany) following the manufacturer's instructions (Buxco Electronics) and as described previously.^{16,17} Briefly, this procedure estimates total pulmonary airflow in the upper and lower respiratory tracts. The chamber pressure was used as a measure of the difference between thoracic expansion (or contraction) and air volume removed from (or added to) the chamber during inspiration (or expiration). Pulmonary airflow obstruction was assessed by measuring PenH using BioSystem XA software (Buxco Electronics). Measurements of MetCh responsiveness were obtained by exposing mice for 3 min to incremental doses of aerosolized MetCh and monitoring the breathing pattern for 5 min after initiation of aerosol dose.

Statistics

Statistical significance was analysed by the Student's *t*-test. Unless otherwise indicated, data represent the mean $\pm$ standard deviation. Data with *P*-values < 0.05 were considered to be statistically significant.

Results

Profound airway eosinophilia results from the sensitization and challenge of mice with NES

Nippostrongylus brasiliensis derived allergens are known to promote Th2 immune responses;¹⁸ however, they have not been evaluated as potential allergens for producing the symptoms of atopic asthma. We directly compared the ability of NES allergens and OVA protein to induce an airway eosinophilia. A common OVA immunization and challenge protocol used to induce an airway eosinophilia involves i.p. injections of OVA adsorbed in alum adjuvant on days one and 14, followed by an i.n. challenge of OVA protein in PBS 7 days later.¹⁹ Four days following the i.n. challenge, which has previously been established to be the peak of eosinophil infiltration (data not shown), lung lavage was performed (BAL) and the total number of eosinophils in the airways enumerated. This protocol was implemented with titrated doses of both OVA and NES. As previously established, when OVA-sensitized mice were challenged with OVA i.n., an airway eosinophilia developed (Fig. 1a). Similarly, when mice were sensitized and challenged with NES, an airway eosinophilia was induced; however, using the same protein concentration the response induced following NES challenge was threefold higher than that of OVA (Fig. 1b). The NES-induced airway eosinophilia increased in a dose-dependent manner when increasing concentrations of NES were used for the sensitization part of the protocol. Interestingly, increasing the concentration of NES used for i.n. challenge of mice had no apparent effect on the induction of an airway eosinophilia (Fig. 1b), nor did it influence the percentage of eosinophils in the cellular infiltrate, which remained between 70 and 80% (data not shown). Mice that were sensitized with alum alone and challenged with NES, or sensitized with the highest concentration of NES but challenged with PBS, did not develop an airway eosinophilia (Fig. 1b). Taken together, these data show that NES allergens are capable of inducing a profound allergic airway eosinophilia and that NES allergens provide a stronger model allergen when compared to OVA protein.

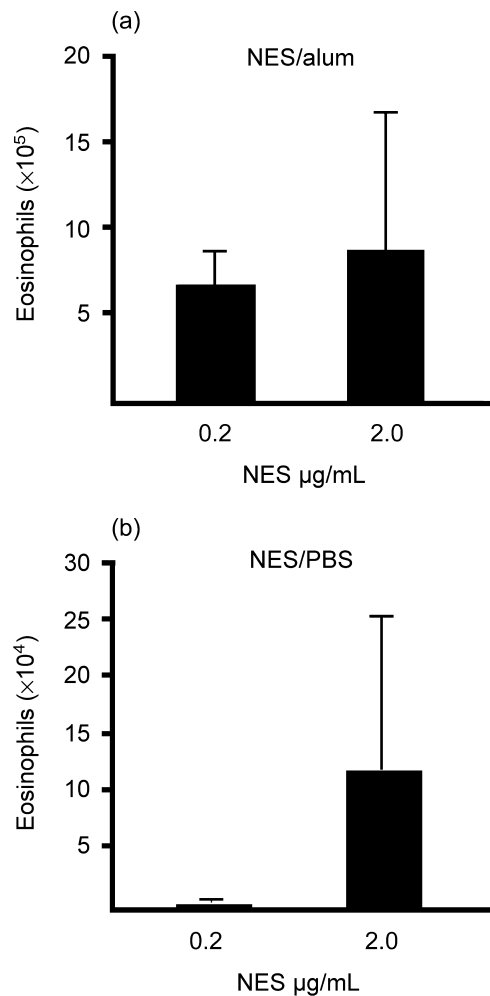


Figure 2 *Nippostrongylus brasiliensis* secretory products can induce an airway eosinophilia following a single i.p. sensitization and i.n. challenge. (a) Mice were sensitized i.p. with the indicated concentration of NES adsorbed in alum adjuvant on day zero, and 7 days later were challenged with 10 μg NES i.n. Four days following challenge, bronchoalveolar lavage (BAL) was performed and the total number of airway eosinophils quantified. (b) Mice were sensitized i.p. by the indicated concentration of NES in PBS on day zero, and 7 days later were challenged with 10 μg NES i.n. Four days following challenge, BAL was performed and the total number of eosinophils quantified. These data are from a representative experiment using three to four mice per group, and represent the mean total number of eosinophils for each group $\pm$ standard deviation.

A single immunization with NES is sufficient to sensitize mice for a subsequent airway eosinophilia

We reasoned that because the NES response shown in Figure 1b was dramatically stronger than the OVA response, a single i.p. immunization may suffice for the optimum expansion of NES-specific cells. Therefore, we immunized mice i.p. with NES adsorbed in alum adjuvant on day one, then challenged mice i.n. on day seven and enumerated the total number of eosinophils that had migrated into the airways 4 days later. A large infiltration of eosinophils occurred (Fig. 2a), which was comparable in size to that observed with

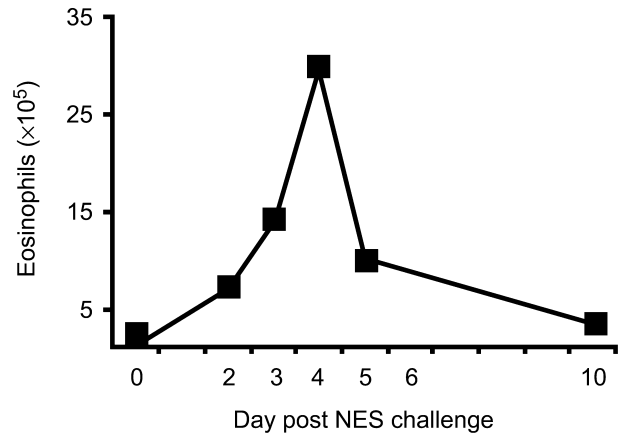


Figure 3 *Nippostrongylus brasiliensis* secretory product-induced airway eosinophilia peaks at day four post i.n. challenge. Mice were sensitized i.p. with 2 μg NES adsorbed in alum adjuvant on day zero, and 7 days later were challenged with 10 μg NES i.n. At the indicated time points following challenge, bronchoalveolar lavage (BAL) was performed and the total number of eosinophils quantified. These data are from a representative experiment using pooled samples from three to four mice per time point, and represent mean total number of eosinophils per mouse.

two immunizations of NES (Fig. 1b), and accordingly three-fold greater than that induced with two immunizations of OVA (Fig. 1a). We next investigated whether alum adjuvant was required during the sensitization phase. As shown in Figure 2b, a single immunization with NES in the absence of alum adjuvant was sufficient to sensitize mice to respond to an i.n. challenge with NES with airway eosinophilia; however, this response was weak and variable. It is likely that NES is cleared too quickly from the peritoneum to allow adequate antigen uptake and consequent optimal activation of NES-specific cells in the draining lymphoid tissue. It is possible that this may be overcome by multiple injections of NES alone; however, such a protocol was not pursued because one immunization with alum induced a rapid and robust response. Accordingly, the remainder of this manuscript focuses upon the allergic airway response induced using the one immunization NES/alum protocol.

Infiltration of eosinophils into the airways peaks at day four post NES challenge

While it has previously been established in our laboratory that the infiltration of eosinophils into the airways peaks at day four post OVA challenge (N. Harris, unpubl. data, 1995), we considered it important to determine whether this timing applied to the optimized NES model described in the previous section. Accordingly, mice were sensitized to NES in alum adjuvant on day one and challenged with NES in PBS 7 days later. On the indicated days, BAL of the airways was performed and the total number of eosinophils determined (Fig. 3). In agreement with prior observations using the two immunization OVA model, the peak eosinophil infiltrate measured in the airways occurred at day four post i.n. challenge, and returned to baseline levels by day 10 post challenge. These data show that whilst the sensitization of

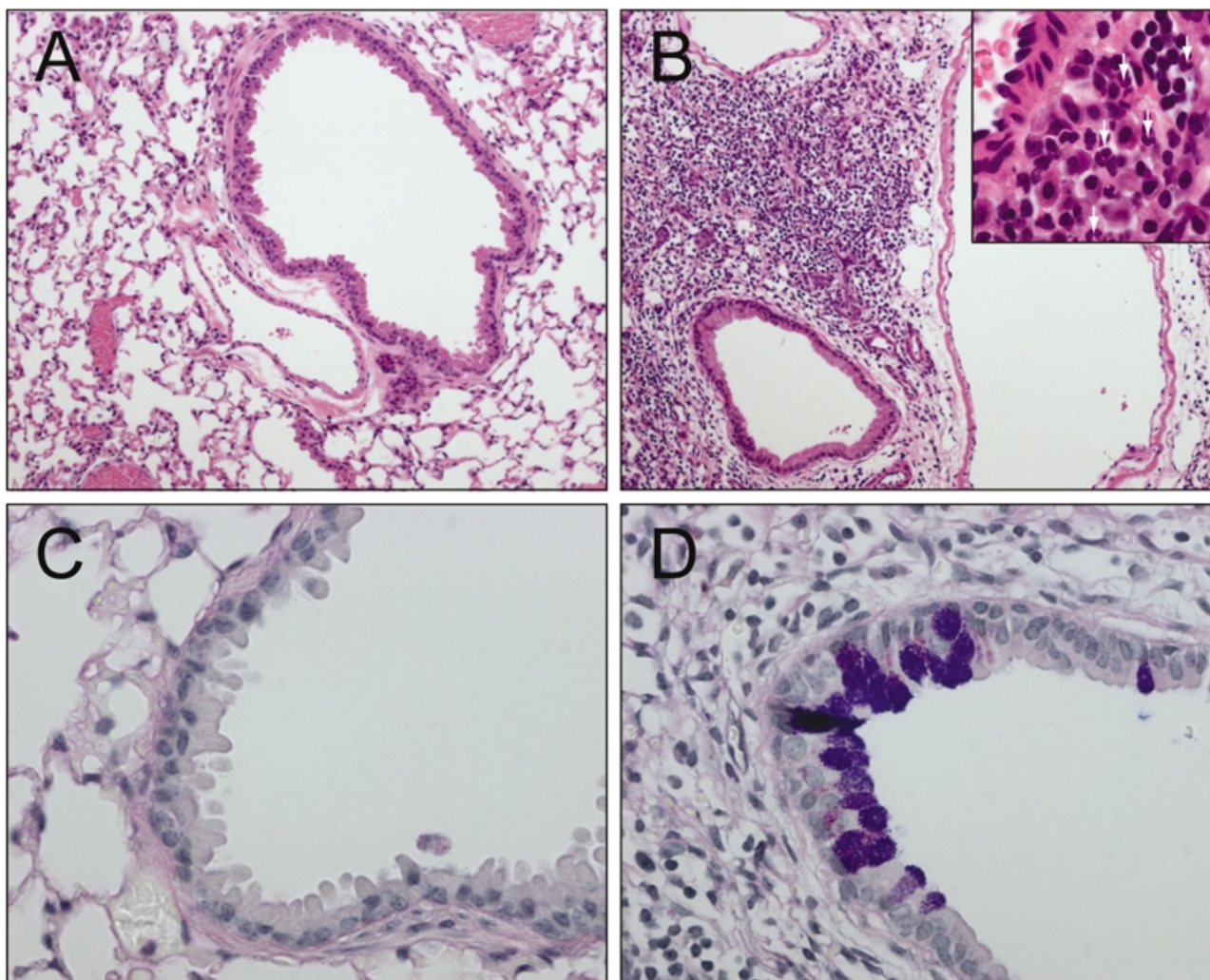


Figure 4 Immunization and challenge with *Nippostrongylus brasiliensis* secretory products results in the acute infiltration of inflammatory cells into the lung parenchyma and mucus cell hyperplasia. Mice were sensitized i.p. with 2 μ g NES adsorbed in alum adjuvant on day zero, and 7 days later were challenged with (A,C) PBS alone or (B,D) 10 μ g NES i.n. Four days following challenge, mice were killed and their lungs removed for histological analysis. Microphotographs show haematoxylin–eosin staining (A,B) and combined alcian blue and periodic acid–Schiff staining (C,D) with a 40 $\times$ objective. The arrows in (B) indicate infiltrating eosinophils. These data are from a representative experiment using three to four mice per group.

mice to NES occurred more rapidly than that against proteins such as OVA, the timing of the allergic inflammation response in the airways following i.n. challenge with NES was identical to that observed with an OVA protein challenge.

NES immunization and airway challenge results in the acute infiltration of inflammatory cells into the airways and lung parenchyma

We examined the lungs of NES sensitized and challenged mice by histology to investigate whether histological features of atopic asthma were evident in this model. As expected, mice that had been sensitized with NES adsorbed in alum and subsequently challenged i.n. with PBS alone had no inflammatory cell infiltrate (Fig. 4A). In comparison, mice that had been sensitized and challenged with NES displayed intense peribronchular and perivascular inflammatory infiltrates

which consisted predominantly of eosinophils, macrophages and lymphocytes (Fig. 4B). Furthermore, whilst no mucus cell hyperplasia or mucin production was evident in PBS-treated lungs (Fig. 4C), pronounced mucus cell hyperplasia and mucin production was evident in NES-treated lungs (Fig. 4D). These features are consistent with the induction of strong allergic airway inflammation.

NES immunization and airway challenge results in increased levels of serum IgE

The antibody isotype IgE is a major effector arm of allergic responses. IgE binds to high affinity receptors on mast cells, and upon cross-linking with specific antigen induces the rapid degranulation of these cells and release of inflammatory products that mediate many of the symptoms of atopic asthma. Measurement of the production of IgE is thus a key readout of

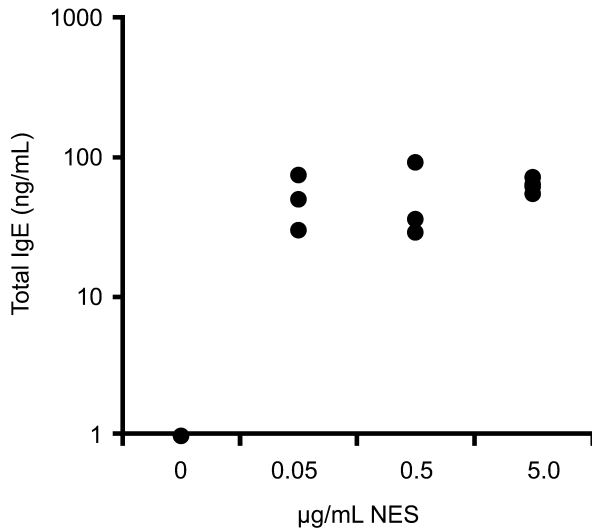


Figure 5 *Nippostrongylus brasiliensis* secretory products induce IgE production in the serum. Mice were sensitized i.p. with the indicated concentration of NES adsorbed in alum adjuvant on day zero, and 7 days later were challenged with 10 µg NES i.n. Four days following challenge, blood was taken and total IgE in serum measured by ELISA. These data are from a representative experiment using three mice per NES concentration.

the intensity of an allergic response. We sought to determine whether immunization and challenge with NES induced production of serum IgE antibodies. We found that i.p. immunization with 0.05–5 µg NES followed by i.n. challenge of 10 µg NES in PBS was sufficient to induce the production of IgE as determined by ELISA of serum samples (Fig. 5).

Ex vivo restimulation of NES-specific CD4⁺ T cells results in the production of IL-4 and IL-5

A further important measurement of allergic responses is the production of the cytokines IL-4 and IL-5 by CD4⁺ T cells. IL-4 is the prototypic Th2 cytokine and is responsible for Th2 cell differentiation and IgE isotype switching. IL-5 is a key factor required for the maturation and proliferation of eosinophils, which as described earlier, is a key immunological mechanism associated with the symptoms of atopic asthma. To determine whether IL-4 and IL-5 production could be measured following NES immunization and challenge, cells from BAL fluid were re-stimulated *ex vivo* in the presence of DC and NES. Intracellular cytokine staining was performed after 6 h stimulation of BAL cells, and IL-4- and IFN-γ-producing CD4⁺ T cells were detected by flow cytometry. NES-activated CD4⁺ T cells were found to produce IL-4, but not IFN-γ (Fig. 6a), consistent with a highly polarized Th2 phenotype. Next, lymphocytes were isolated from lung tissue and the draining MLN of naive mice and previously NES-challenged mice. After 48 h of stimulation with NES, culture supernatants were assessed for IL-5 production by ELISA. As expected from the lung eosinophilia evident in Figure 4B, high levels of IL-5 were detected in lung samples from NES-challenged mice (Fig. 6b). Moderate levels of IL-5 were detected in the MLN and no NES-induced IL-5 was found in

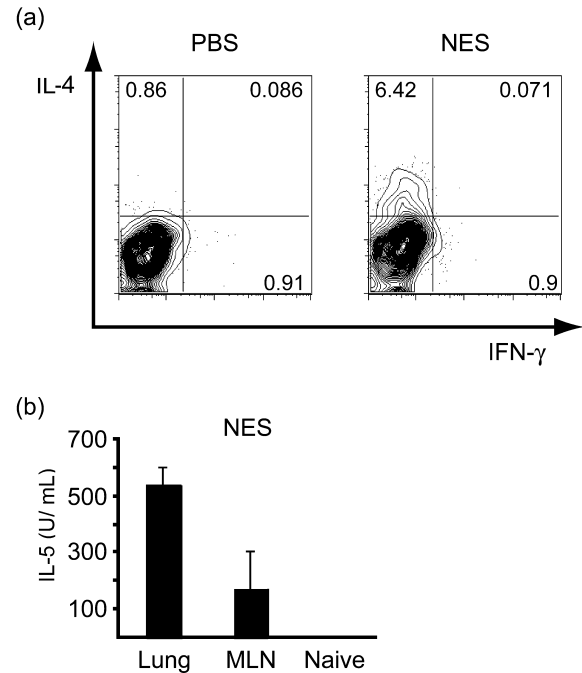


Figure 6 CD4⁺ T cells specific for *Nippostrongylus brasiliensis* secretory products produce IL-4 and IL-5, but not IFN-γ. Mice were sensitized i.p. with 2 µg NES adsorbed in alum adjuvant on day zero, and 7 days later were challenged with 10 µg NES i.n. Four days following challenge, bronchoalveolar lavage (BAL) was performed and the lung and draining mediastinal lymph node (MLN) removed. (a) BAL cells were stimulated for 6 h in the presence of dendritic cells (DC) and NES. Brefeldin A was added during the final 2 h of culture and IL-4- and IFN-γ-producing cells were detected by intracellular staining and flow cytometry. Numbers represent percentage of CD4⁺ T cells staining positive for either IL-4 or IFN-γ. (b) Lymphocytes isolated from NES-challenged lung tissue and the draining MLN, or naive lung tissue, were stimulated with NES for 48 h and culture supernatant measured for IL-5 by ELISA. These data are from a representative experiment using three to four mice per group.

the lungs of naive mice (Fig. 6b). Taken together, these two techniques confirm the polarized Th2 nature of the NES-induced response and provide useful tools for the analysis of this antigen-specific response in future investigations.

NES sensitization and challenge induces airway hyper-responsiveness

The hallmark feature of asthma is airway hyper-responsiveness. Accordingly, we examined whether one immunization with NES followed by an i.n. challenge 7 days later induced airway hyper-responsiveness, alongside the Th2-mediated inflammation described above. As shown in Figure 7, 4 days after NES challenge of previously sensitized mice, a marked increase in airway sensitivity to MetCh challenge was evident as compared to non-immunized controls. These data provide further evidence that NES sensitization and challenge of mice results in disease development and pathogenesis consistent with atopic asthma.

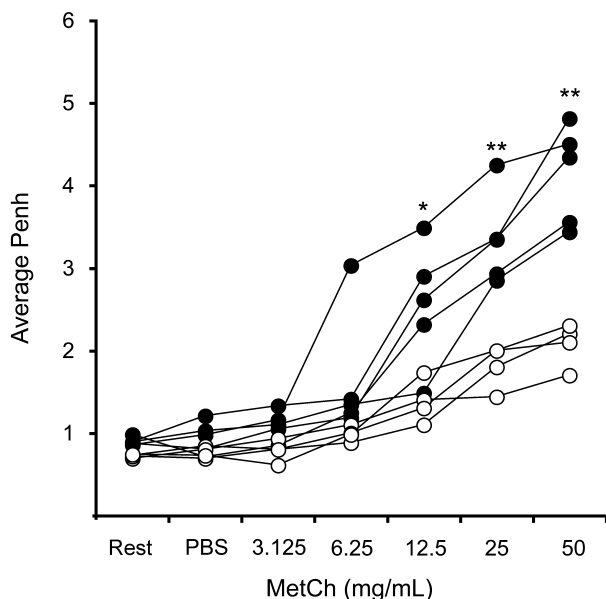


Figure 7 Sensitization and challenge of mice with *Nippostrongylus brasiliensis* secretory products induces airway hyper-responsiveness. Mice were sensitized i.p. with 2 µg NES adsorbed in alum adjuvant on day zero, and 7 days later were challenged with either 10 µg NES (●) or PBS (○) i.n. Four days following challenge, airway hyper-responsiveness was determined to increasing doses of aerosolized MetCh in a whole body unrestrained plethysmograph. These data are from a representative experiment using four to five mice per group. Statistical significance between the NES and PBS groups was determined by the Student's *t*-test (** $P < 0.005$, * $P < 0.05$).

Discussion

In this study, we have described the potential of secreted products from the infective forms of *N. brasiliensis* to produce many of the responses associated with atopic asthma, including airway eosinophilia, Th2 cytokine production, IgE antibody production and most importantly, airway hyper-responsiveness. By using this model, atopic asthma-like pathogenesis can be examined within 11 days, and involves only two manipulations of mice, in comparison to many currently established models, which can take more than 21 days and require repeated immunization and challenges. The ease and cost-effectiveness of NES isolation makes it an ideal source of allergens and represents perhaps the most natural source of allergens for which the mammalian immune system has evolved to deal with.

The Th2-inducing properties of NES have previously been described, and are likely to contribute to the robust allergic airway inflammation induced following immunization and challenge. Holland and colleagues have shown that NES isolated from adult parasites *in vitro* can stimulate IL-4 production and serum IgE.¹⁸ The Th2-inducing properties were abolished following heat treatment or proteinase K digestion, indicating that the response is driven by protein(s) in NES.¹⁸ The Th2-inducing ability of NES has also been used as an adjuvant to induce OVA-²⁰ and hen egg lysozyme-¹⁸specific IL-4 production by T cells. In addition, NES has

been described to suppress IFN- γ production by rat lymphocytes whilst not effecting IL-4 or IL-10 production.²¹ The model described in the current manuscript concurs with such observations where we showed that following *ex vivo* activation, NES-specific CD4⁺ T cells produced IL-4 and IL-5 but not IFN- γ .

The immunomodulatory influences of parasite-derived factors are well established. Excretory/secretory products from *N. brasiliensis* have recently been shown to inhibit neutrophil recruitment following LPS instillation *in vivo*.²² A cytokine homologue of macrophage migration inhibitory factor²³ is secreted by *Brugia malayi*, which alone is sufficient to recruit eosinophils,²⁴ and a wide range of helminth proteins have been shown to inhibit serine proteinases, which play critical roles in host defence.²⁵ In line with this, many common allergens exhibit protease activity, which may influence the induction of Th2 immune responses in allergic individuals. Studies of Der p1 have shown that its proteolytic activity on human T cells and DC increases IL-4 production while decreasing IFN- γ and IL-12,^{26,27} and enhances IgE production by B cells.²⁸ Furthermore, human eosinophils have been shown to be directly activated by cysteine proteases derived from house dust mite allergen.²⁹ Nematodes, including *N. brasiliensis*, produce proteases including acetylcholinesterases and lactate dehydrogenases.³⁰ A study that compared protease activity of Der p1 and secreted allergens from *Necator americanus* showed that both were able to induce IL-4, IL-5 and IL-13, but not IFN- γ cytokine production from basophils. Similarly, the immunomodulating activity of these allergens was demonstrated to be through proteases, because protease inhibitors abrogated cytokine expression.⁹

Interestingly, the BALB/c strain of mice is typically used in models of atopic asthma as it is postulated that these mice induce stronger allergic responses. However, in this study, we used C57BL/6 mice and still found a strong allergic response was induced. Of note, we have also performed this model in BALB/c mice and found comparable induction of the allergic response (data not shown).

Taken together, the data in this manuscript details the development and characterization of a novel model of atopic asthma. We have taken advantage of the Th2-inducing properties of allergens to induce a rapid allergic response that displays the characteristic parameters of atopic asthma, including airway eosinophilia, IgE antibody isotype switching, Th2 cytokines and airway hyper-responsiveness. In addition to the short duration of this protocol, the allergens used are more likely to model the physiologically relevant allergens responsible for atopy than the commonly used protein antigens such as OVA. NES allergens can also readily be isolated in their enzymatically active form for experimental use. We conclude that NES allergens are highly effective at inducing Th2 immune responses and the model described represents a useful tool for the rapid and physiologically relevant induction of atopic asthma in mice.

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