

Proteins secreted by the parasitic nematode *Nippostrongylus brasiliensis* act as adjuvants for Th2 responses

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Infections with parasitic helminths such as *Nippostrongylus brasiliensis* induce dominant type 2 responses from antigen-specific T helper cells. The potency of the Th2 bias can also drive Th2 responses to bystander antigens introduced at the same time as infection. We now report that the Th2-promoting effect of infection can be reproduced with soluble *N. brasiliensis* excretory-secretory proteins (NES) released by adult parasites *in vitro*. Immunization of BALB/c mice with NES results in the production of IL-4 with elevated total serum IgE and specific IgG1 antibodies. NES is also able to stimulate IL-4 and polyclonal IgE production in other mouse strains (C57BL/6, B10.D2, CBA). These features are seen whether NES is administered without adjuvant as soluble protein in phosphate-buffered saline or with complete Freund's adjuvant which normally favors Th1 responses. Thus, NES possesses intrinsic adjuvanticity. Moreover, co-administration of hen egg lysozyme (HEL) with NES in the absence of other adjuvants results in generation of HEL-specific lymphocyte proliferation, IL-4 release and IgG1 antibody responses, documenting that NES can act as an adjuvant for third-party antigens. Proteinase K digestion or heat treatment of NES before immunization abolished the IL-4-stimulating activity, indicating that the factors acting to promote Th2 induction are proteins secreted by the adult parasite.

Key words: Helminth parasite / Immunomodulator / Th1/Th2

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1 Introduction

Activated CD4⁺ Th cells differentiate into distinct populations of effector cells, of which Th1 and Th2 form opposing poles [1, 2]. Multiple interrelated components of the immune system influence the fate of naive Th cells, including exposure to cytokines, APC type, co-stimulation, choice of adjuvant, and the overall strength of TCR ligation [3–5]. Interestingly, many infectious pathogens induce selective differentiation towards one arm of the Th cell spectrum [4, 6]. For example mycobacteria are potent stimulators of Th1 responses, probably due to preferential induction of IFN- γ release by innate immune system cells reactive to bacterial products. In contrast, multicellular helminth parasites are the strongest known agents which drive Th2 responses, but the molecular basis for this effect has yet to be defined.

Nippostrongylus brasiliensis is a rodent nematode parasite which induces strong Th2 type responses in both rats [7], its natural host, and mice [8–12]. A CD4⁺ T cell-dependent IL-4 and/or IL-13 response mediates expulsion of adult parasites from the small intestine, probably via goblet cell proliferation and mucin hypersecretion [13–17]. Infected animals display other features of Th2 cell activation, such as IL-5-dependent eosinophilia [18, 19], mast cell hyperplasia [20] and an IL-4-dependent polyclonal IgE response [21, 22], but these appear to play no role in the expulsion of a primary *N. brasiliensis* infection [13, 23]. Th2 development is so pronounced in the environment of a *N. brasiliensis* infection that responses to coincident bystander antigens are also driven in this direction [24].

Little is known about how *N. brasiliensis* selectively induces Th2 activation or about the parasite antigens recognized by the activated Th2 cells. There may be parallels with the tissue-dwelling trematode *Schistosoma mansoni*, eggs of which switch Th responses into the Th2 pathway, and exposure to which biases cytokine responses to unrelated antigens in the same direction [25–27]. Indeed, the skewing of the immune response

[1 20310]

Abbreviation: HEL: Hen egg lysozyme LNC: Lymph node cells NES: *Nippostrongylus brasiliensis* excretory-secretory proteins

can be so potent that the normally Th1-dominant response to a concurrent infection is inhibited [28] and Th1-mediated autoimmune disease can be ablated [29]. Thus, the Th2-inducing properties of helminth parasites can override an established Th1 bias, and in so doing may exert a beneficial effect on the host. It would be highly desirable to reproduce this effect with defined parasite products, rather than using live infectious organisms.

We show here that the Th2-driving effect can be achieved with a set of soluble proteins secreted from adult *N. brasiliensis*. We demonstrate that a single injection of this soluble *N. brasiliensis* excretory-secretory (NES) antigen induces IL-4 release by peripheral lymph node cells as well as polyclonal IgE production, and that these effects do not require any extraneous adjuvant. Furthermore, the effect is independent of mouse strain, is mediated by a protease- and heat-sensitive component, and is able to stimulate Th2 responses to a soluble bystander antigen administered at the same site of injection.

2 Results

2.1 Immunization with NES results in a Th2 cytokine profile

It is well established that infection with *N. brasiliensis* stimulates an exclusive Th2 cytokine profile [7, 10, 12], and previous work has shown that NES products stimulate IgE [11] as well as strong Th2 recall responses in T cells from infected mice [12]. We therefore investigated the possibility that NES antigens alone could prime murine lymph node cells (LNC) and stimulate a Th2 cytokine profile similar to that observed during infection. Mice were immunized with NES and a control protein, hen egg lysozyme (HEL), emulsified in CFA; for comparison, two other groups of mice were injected with viable or heat-killed infective larvae at an s.c. site. Seven days after infection or immunization, mesenteric or popliteal LNC were rechallenged *in vitro* and the proliferative and cytokine (Fig. 1) responses determined.

As previously reported, *N. brasiliensis* infection stimulated antigen-specific proliferative responses *in vitro* and high levels (400–800 pg/ml) of IL-4. IFN- γ production remained at background levels in cells isolated from infected animals, even in mitogen-stimulated cultures

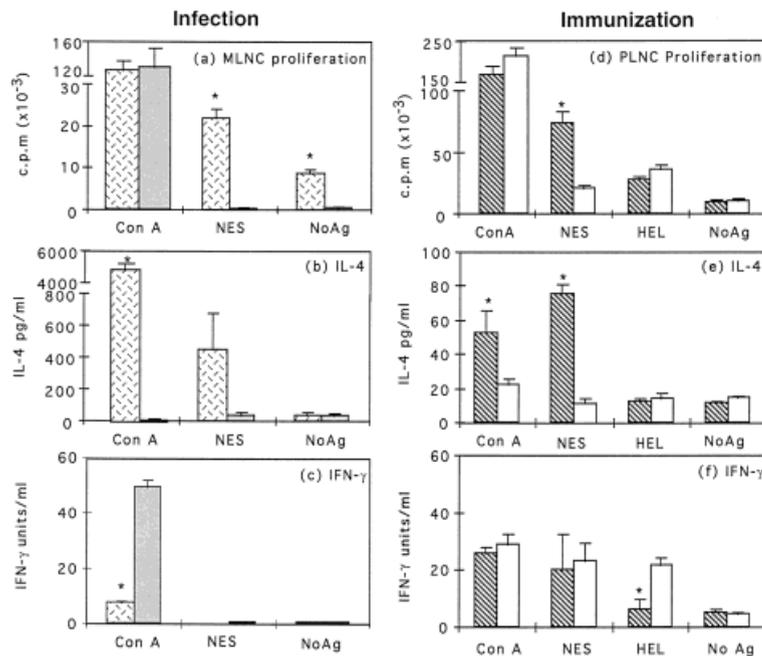


Fig. 1. Th2 responses are induced both by *N. brasiliensis* infection and by immunization with NES. (a–c) Responses of mesenteric LNC (MLNC) isolated from BALB/c (H-2^d) mice 7 days after infection with 500 *N. brasiliensis* infective larvae (stippled bars) or mice injected with 500 heat-killed larvae (grey bars). (d–f) Response of popliteal LNC (PLNC) isolated 7 days after hind footpad immunization with either NES/HEL (hatched bars) or HEL (open bars) in CFA. Mice received 50 μ g per footpad NES and/or 10 μ g per footpad HEL in CFA. Data represent mean \pm S.D. of five individual mice per group. * $p < 0.05$.

(Fig. 1 a–c). Normal production of IFN- γ was preserved in cells from animals receiving killed parasites, but no antigen-specific proliferation or type 2 cytokine production was evident. Mitogen stimulation of cells from infected animals, however, resulted in the greatest amounts of IL-4, indicating that the majority of T cells isolated after infection are Th2 cells.

Although antigen delivered in CFA is considered to predispose towards Th1 differentiation [30, 31], immunization with NES in CFA induced a cytokine profile strikingly similar to that seen after infection (Fig. 1 d–f). IL-4 was the major cytokine produced after antigen challenge *in vitro* although at lower levels (60–80 pg/ml) than those seen after infection. IL-5 and IL-13 responses were also observed (data not shown). In contrast to responses after infection, some IFN- γ was consistently produced in response to NES from cells primed *in vivo* with NES/CFA. Nevertheless, a single immunization with NES drove a response which reproduced the key qualities of the response observed in live infection with a high dose of parasites.

2.2 NES immunization stimulates polyclonal IgE synthesis

Polyclonal IgE synthesis resulting in large increases in total serum IgE is a classical sign of infection with helminths. Increases in total serum IgE are also indicative of Th2 responses due to IL-4-induced switching and the production of factors with allergen-like characteristics. After an initial s.c. immunization with NES in CFA, BALB/c mice were rechallenged after 28 days with NES/IFA in each hind footpad. Mice immunized with HEL in adjuvant served as controls. Levels of serum IgE were increased fourfold at day 28 in NES-immunized mice compared with controls, but the most dramatic effect was observed following challenge, in which NES immunization elicited over 100-fold increases in total serum IgE 7 days after boosting (Fig. 2 a). IgG antibodies to NES (Fig. 2 b) and HEL (Fig. 2 c) were also measured after challenge. NES-specific responses were dominated by IgG1 and IgG3, and mice immunized with HEL alone produced no NES-reactive antibodies (Fig. 2 b). Anti-HEL responses in mice co-immunized with NES showed significant depletion of IgG2a and IgG2b isotypes, while IgG1 levels remained the same as in HEL-only-primed animals (Fig. 2 c). Anti-HEL IgG3 responses were absent in both groups of mice, which may be accounted for by the absence of glycosylated epitopes in HEL.

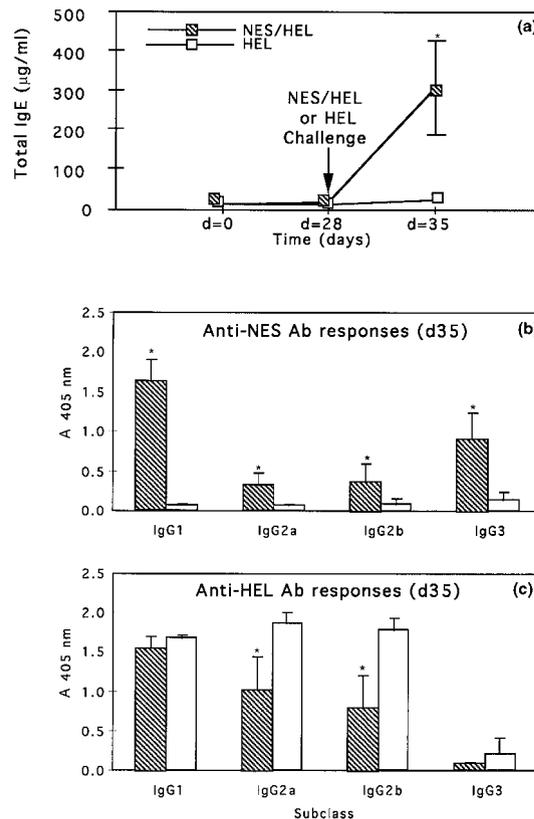


Fig. 2. Polyclonal IgE and IgG isotype responses to NES immunization. (a) Total serum IgE levels in BALB/c mice immunized s.c. with 50 μ g NES and 10 μ g HEL in CFA (hatched symbols) or 10 μ g HEL in CFA (open symbols). After 28 days mice were rechallenged with 50 μ g/footpad NES and 10 μ g/footpad HEL in IFA or 10 μ g/footpad HEL in IFA. Data represent mean \pm S.D. of four individual mice per group. * $p < 0.05$. (b, c) Anti-NES and anti-HEL IgG subclass responses of BALB/c mice described in (a). Data represent the A_{405} values measured at 1/300 serum dilution. * $p < 0.05$.

2.3 NES stimulates IL-4 production and polyclonal IgE synthesis in a number of murine genotypes

Since type 2 cytokine responses are thought to be more readily stimulated in BALB/c mice than in many other genotypes [32], we tested the ability of NES to stimulate type 2 responses in different murine strains. Mice were selected which either shared the BALB/c MHC haplotype (H-2^d) on a disparate genetic background (B10.D2) or were mismatched at both MHC and background loci [CBA/Ca (H-2^k) and C57BL/6 (H-2^b)]. In each strain, NES immunization in CFA resulted in NES-specific IL-4 recall responses from LNC *in vitro* which equalled or exceeded that stimulated by Con A (Fig. 3). Each mouse strain also showed dramatic levels of polyclonal IgE stimulation when given successive NES immunizations as previously

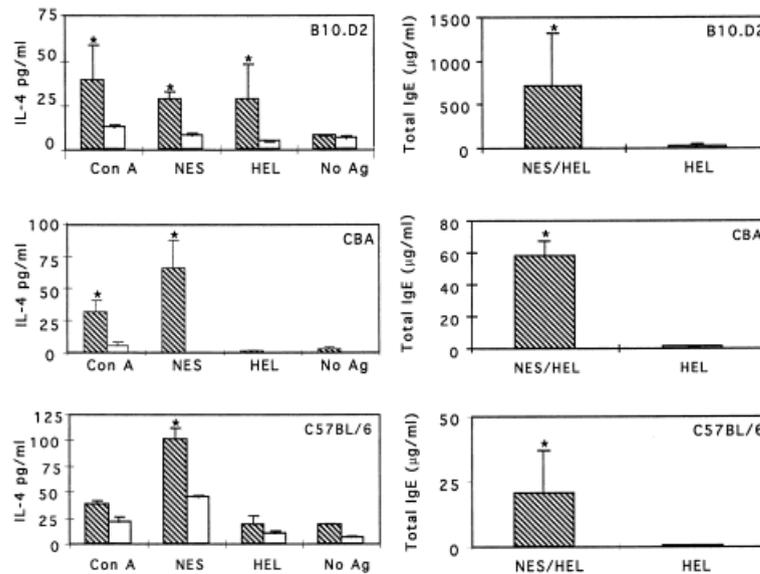


Fig. 3. NES induces Th2 responses in different strains of mice. Left panels: IL-4 responses of popliteal LNC from B10.D2 (H-2^d), CBA/Ca (H-2^b) and C57BL/6 (H-2^b) mice immunized with NES/HEL in CFA (hatched bars), or HEL alone in CFA (open bars) are shown. Cells were isolated 7 days after immunization and challenged *in vitro* with 2 µg/ml Con A, 10 µg/ml NES or culture medium alone. Culture supernatants were removed after 48 h and tested by ELISA. Right panels: Total serum IgE levels in B10.D2, CBA and C57BL/6 mice, immunized s.c. with 50 µg NES/10 µg HEL in CFA (hatched bars) or 10 µg HEL in CFA (open bars). After 28 days mice were rechallenged with 50 µg/footpad NES and 10 µg/footpad HEL in IFA or 10 µg/footpad HEL in IFA. Data represent mean ± S.D. of four individual mice per group. * $p < 0.05$.

described for BALB/c mice (Fig. 2). Although expressing similar levels of IL-4 at day 7 of the primary response, the three strains displayed very different levels of total IgE by day 35 following the challenge immunization. Thus, early IL-4 is not an accurate predictor of the magnitude of mature IgE responses, and other factors such as duration of cytokine production must also be at play.

2.4 CD4⁺ cells are the source of IL-4 when challenged *in vitro*

To demonstrate the cellular source of cytokine production after immunization with NES, cells were separated into CD4⁺ and CD4⁻ fractions. Seven days after immunization popliteal LN were removed from BALB/c mice and separated by magnetic-activated cell sorting (MACS). Cells were then restimulated *in vitro* using irradiated naive splenocytes as APC. Culture supernatants were harvested after 72 h culture. IL-4 secretion in response to NES separated entirely with the CD4⁺ population with only background levels in the CD4⁻ population and in either population of cells isolated from HEL/CFA-immunized animals (Fig. 4). IFN- γ responses to specific antigen were evident in both the CD4⁺ and CD4⁻ populations but these levels were low as observed in previous

experiments with NES. Identical results were obtained in independent experiments with CBA mice (data not shown).

2.5 NES stimulates exclusive Th2 responses and displays self adjuvanticity in the absence of extrinsic adjuvant

In most model antigen systems, the generation of significant and long-lasting immune responses is dependent upon enhancing the immunogenicity of the antigen by delivery with an adjuvant. One adjuvant which is widely used, CFA, contains killed mycobacteria in oil, and generally stimulates Th1 responses [30, 31]. IFA consists of the mineral oil vector alone and is more favorable to Th2 induction [30, 31]. We found that regardless of which adjuvant was used to immunize either BALB/c or B10.D2 mice with NES, LNC generated IL-4 but no IFN- γ in response to NES (data not shown). In contrast, HEL immunization in CFA and IFA followed the reported bias towards type 1 and type 2 responses, respectively.

We then investigated the possibility that NES possessed an intrinsic adjuvant activity, and could stimulate type 2 immune responses in the absence of either CFA or IFA.

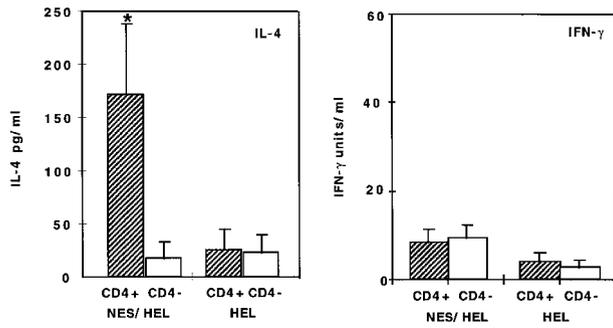


Fig. 4. NES-induced IL-4 production is derived from CD4⁺ T cells. NES-specific IL-4 and IFN-γ production by LNC isolated from BALB/c mice 7 days after immunization with 50 μg NES/10 μg HEL in CFA per footpad or 10 μg HEL in CFA per footpad is shown. Popliteal LNC were separated into CD4⁺ (hatched bars) and CD4⁻ (open bars) populations by MACS and 2 × 10⁵ cells/well challenged with NES *in vitro*. Irradiated splenocytes (10⁵/well) from naive syngeneic mice were used as APC. Culture supernatants were collected after 72 h stimulation and cytokines quantified by ELISA. Mean levels of IL-4 and IFN-γ in cells cultured without antigen were <10 pg/ml IL-4 and 2.98 ± 1.98 U/ml IFN-γ, respectively. The percentage of cells staining positive for CD4 after MACS selection were: CD4⁺-selected 94.25 ± 2.25 %; CD4⁻-depleted 0.84 ± 0.26 %. Data are mean ± S.D. from four individual animals/group. Stimulation of CD4⁺ and CD4⁻ cells isolated from LNC of CBA mice after immunization with 50 μg NES/10 μg HEL in CFA per footpad or 10 μg HEL in CFA per footpad produced identical findings (data not shown). * *p* < 0.05.

BALB/c and C57BL/6 mice were injected in the hind footpads with either NES and HEL, or HEL alone, without any adjuvant. After 7 days popliteal LNC were collected and challenged *in vitro*. In both BALB/c and C57BL/6 mice, a single injection of soluble NES/HEL induced NES-specific proliferation and cytokine production, and HEL-specific proliferative responses in responder BALB/c animals (data not shown). IFN-γ production remained at background levels throughout, and no HEL-specific proliferation was induced in LNC from mice immunized with HEL alone.

Similar results were obtained using BALB/c LNC taken 28 days following NES/HEL priming, in which mice had been boosted with HEL alone at day 21. This regimen induced *in vivo* antigen-specific proliferative and cytokine response both to NES and HEL (Fig. 5a–c). The strong IL-4 recall response to HEL indicated that NES has intrinsic Th2 adjuvant properties. IFN-γ production was not induced and remained at background levels in all groups. Again, no HEL-specific proliferation or cytokine production was induced in LNC from mice immunized with HEL alone.

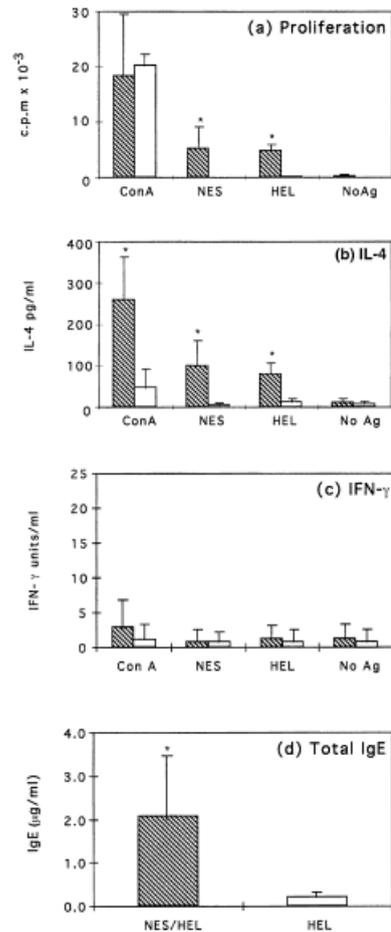


Fig. 5. Soluble NES without extraneous adjuvant induces Th2 responses and acts as an adjuvant for HEL. Responses of BALB/c LNC isolated 28 days after primary footpad immunization with soluble NES/HEL (hatched bars) or HEL alone (open bars) without adjuvant are shown. At 21 days after primary immunization all animals were challenged with HEL alone. All data represent mean ± S.D. of four individual mice per group. (a) Specific proliferation was measured at 60 h after *in vitro* culture. Data are mean ± S.D. of four individual mice/group. (b–c) IL-4 and IFN-γ production determined after 48 h *in vitro* culture. (d) Total IgE levels 28 days after a single immunization of BALB/c mice without adjuvant.

Antibody responses in BALB/c mice showed similar evidence for Th2 activation. All animals immunized with NES/HEL showed elevated total serum IgE levels (Fig. 5d), and specific anti-HEL IgG1 which reached high titers within a week of challenge with HEL alone (Fig. 6a, b); in contrast, no anti-HEL antibodies were detectable after a single injection of soluble HEL, and only low levels after a homologous challenge. Similar results were obtained in inducing NES-specific responses with soluble NES in C57BL/6 animals, but NES was unable to overcome the MHC-determined unresponsiveness of this strain to HEL (data not shown).

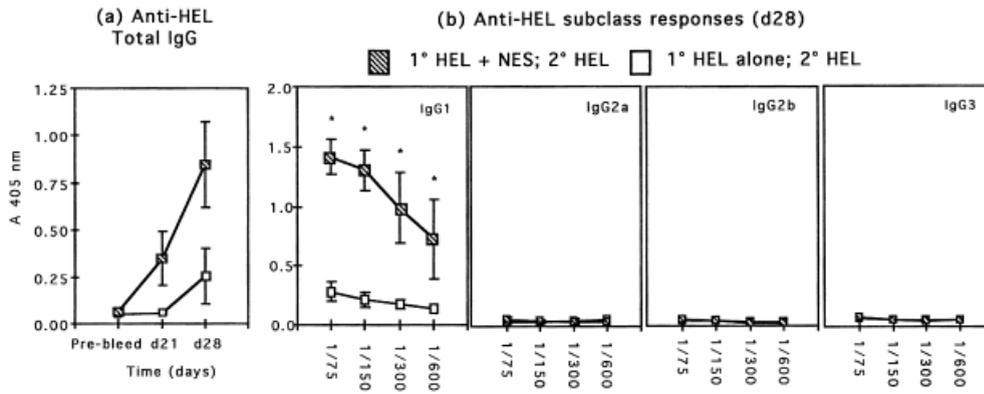


Fig. 6. Soluble NES acts as an adjuvant for HEL responses and induces IgG1 isotypes responses to HEL. (a) Specific anti-HEL IgG responses in BALB/c mice primed with either NES/HEL or HEL alone, and boosted at day 21 with HEL in the absence of NES. Sera were taken at day 0, 21 and 28. Antibodies were tested by ELISA against HEL at a dilution of 1/200. Data are mean ± S.D. of four individual mice/group. **p* < 0.05. (b) Specific anti-HEL serum IgG subclass responses 7 days after HEL challenge of BALB/c mice primed with NES + HEL or HEL alone. Data represent the A₄₀₅ for each dilution point. Data are mean ± S.D. of four individual mice/group. **p* < 0.05.

2.6 IL-4 stimulation by NES requires native protein structure

NES is a complex mixture of molecules, any one or several of which may be responsible for the preferential stimulation of type 2 cytokine responses. To determine whether a proteinaceous antigen is involved, NES sam-

ples were enzymatically digested with proteinase K, or degraded by incubation at 100 °C for 10 min, before admixture with CFA and immunization of BALB/c mice. Untreated NES acted as a positive control. The IL-4 and IFN-γ responses of LNC isolated 7 days after immunization are shown in Fig. 7. Heat treatment abolished the IL-4-stimulating capacity of NES whilst proliferation and

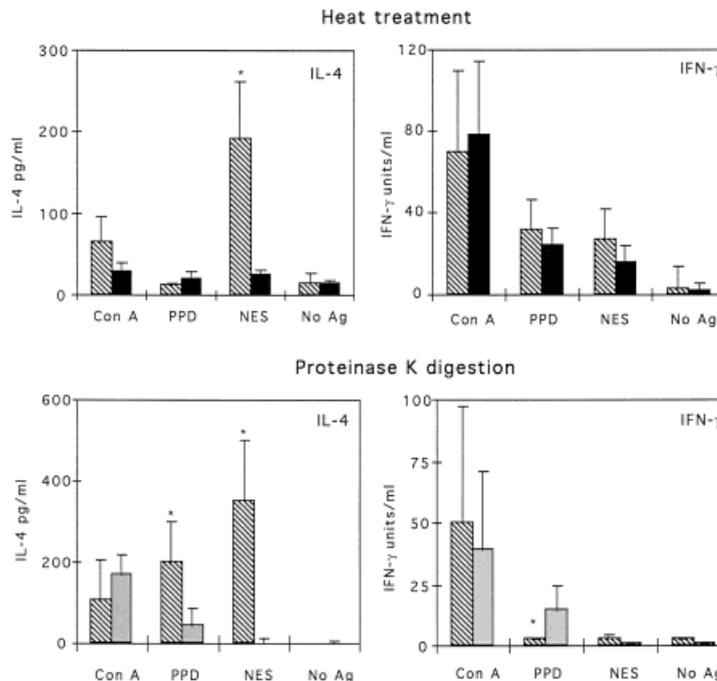


Fig. 7. Heat and protease sensitivity of Th2-inducing activity of NES. IL-4 and IFN-γ produced by popliteal LNC from BALB/c mice immunized with NES (hatched bars) in CFA or with heat-treated (100 °C: 10 min) NES (black bars) in CFA. Alternatively NES was incubated with proteinase K prior to emulsification in CFA and immunization (grey bars). **p* < 0.05.

IFN- γ responses were unaffected. Proteinase K digestion (confirmed by silver staining of a sample on SDS-PAGE) abolished proliferative, IL-4 and IFN- γ NES-specific responses. In one experiment, immunization with intact NES shifted the profile of responses to mycobacterial PPD (a constituent of CFA) from a pure type 1 (IFN- γ) to a dominant type 2 (IL-4). This shift was lost after proteolysis (Fig. 7). Together, these observations indicate that the IL-4-stimulating capacity of NES requires an intact protein component.

3 Discussion

Th cell differentiation is balanced by a series of immune system interactions, particularly at the level of T cell receptors for antigen, cytokines and co-stimulatory molecules [3–5, 33]. Many pathogens appear to deregulate these controls and elicit highly polarized responses, or induce shifts from one Th subset to the other [34, 35]. These extreme responses are of great interest, first as they are likely to represent innate immune system recognition systems (e.g. binding to bacterial LPS [36]; secondly, because they may help identify the ultimate control of T cell subset selection; and thirdly, since novel immune modulators may be discovered from pathogens which will prove invaluable in clinical intervention.

Helminth infections stimulate strong immune responses which are polarized towards the Th2 phenotype [13, 37–40]. We demonstrate here that secreted, soluble proteins from a parasitic nematode can reproduce this effect. The stimulus towards Th2 is robust, being independent of mouse genotype, strongly reflected in cytokine, IgG subclass and IgE profiles, and achieved by soluble components alone without recourse to any extraneous adjuvant. While it is known that presentation of protein antigens in soluble form favors Th2 development [41], NES displays a much more fundamental property in overriding Th1 induction by CFA. Indeed, one of the most significant findings is that NES itself acts as an adjuvant in driving both NES-specific Th2 response and also Th2-dependent antibody responses to co-immunized antigens such as HEL.

In addition to parasite-specific Th2 responses, this bystander effect, in which third-party antigens also become subject to a Th2 bias, is well known following infection [25, 42]. Bystander type 2 responses can also be induced with somatic extracts of helminth parasites such as *Ascaris* [43]. In many ways, helminths act as a mirror image of mycobacteria in driving T cell differentiation towards a selective endpoint. We have observed that co-immunization with NES and CFA can result in the emergence of Th2 responsiveness to PPD, a mycobac-

terial antigen which normally elicits an exclusive Th1 response [34, 35]. A similar finding was reported in mice co-immunized with PPD and somatic extracts of the filarial nematode *Brugia malayi* [44].

In the case of *N. brasiliensis*, there is compelling evidence that the resulting Th2 response results in parasite clearance from the gut [13, 15–17, 23, 45–48]. It is also likely that eosinophils play a role attacking migratory larvae in transit from skin to stomach via the tissues and lungs [49, 50]. However, for most other helminth parasites, both Th1 and Th2 pathways are likely to be required for parasite killing without immunopathological consequences [6, 51, 52]. Since protection against *N. brasiliensis* is so strongly associated with Th2 responses, it seems unlikely that the parasite is secreting substances which stimulate this pathway to its own advantage. Indeed, NES can effectively immunize animals against challenge with live parasites [53], implying that the host immune system recognizes components of the worm's secretions and responds by activating Th2 differentiation.

There are clearly differences between live *N. brasiliensis* infection and immunization with NES, especially with regard to antigen dose and the tempo of continuous antigenic challenge during infection. Some important indications have been drawn from immunological studies of infected mice, beyond the numerous observations of a strong type 2 response. A consistent finding is that infection-generated Th2 cells respond strongly to secreted (NES) antigens [12, 54]. The genesis of the Th2 response in infection is much less well defined, as diverse cell types appear to participate in the production of IL-4. There is an early IL-4 response from $\gamma\delta$ T cells [55], while non-B, non-T cells contribute significant amounts of IL-4 later in infection [56]. Although CD1-restricted NKT cells may also release IL-4, it has been established that the mature Th2 profile develops normally in *N. brasiliensis*-infected $\beta 2$ -microglobulin^{-/-} mice [57], and that the polyclonal IgE response to infection is intact in V α 14 TCR gene-targeted, NKT cell-deficient animals [58]. Moreover, most ligands for NKT and $\gamma\delta$ T cells appear to be non-proteinaceous in nature [59, 60]. Our new data showing that Th2 differentiation is dependent upon a heat-labile, protease-sensitive secreted protein fraction acting through the CD4⁺ T cell population supports a model in which interaction with conventional class II-restricted $\alpha\beta$ TCR T cells is the major feature of the response to NES.

One possibility is that a functional property, such as proteolytic activity [61], of NES proteins explains the Th2 bias [62]. We have separated NES into several fractions by ion exchange chromatography, and found that all

IL-4-inducing activity segregates into a single fraction devoid of protease activity (M. J. Holland, unpublished results). However, other functional non-proteolytic enzymes are known to be secreted, with acetylcholinesterase [63] and platelet-activating factor acetylhydrolase [64] activities; an involvement of such enzymes in T cell modulation is entirely plausible.

An attractive proposition is that NES activates or modulates specific populations of APC, and induces these to drive naive NES-specific T cells towards the Th2 fate. In preliminary experiments, we have observed Th2 stimulation by murine bone marrow-derived dendritic cells pulsed *in vitro* with NES and transferred to naive hosts; similar exposure of dendritic cells to non-parasite antigens failed to provoke Th2 responsiveness (M. J. Holland, unpublished results). We will now be using similar experiments to test the individual fractions of NES, in order to identify both the active parasite molecule(s) and their immediate target cell type within the host immune system.

4 Materials and methods

4.1 Parasites and NES antigen

The *N. brasiliensis* life cycle was maintained in Sprague-Dawley rats (Harlan Olac, Oxfordshire, GB) as previously described [12]. Six days after s.c. injection of 5000 infective stage (L3) larvae animals were killed and adult worms harvested from the gut by dissection. Adult worms were isolated in saline using a Baermann apparatus. Collection of NES followed the protocol described by Healer et al. [61]. After five saline washes worms were preincubated in RPMI 1640 containing 1000 U/ml penicillin and 1 mg/ml streptomycin for 30 min at room temperature followed by five washes in RPMI 1640 before incubation in serum-free medium supplemented with 2 % glucose, 100 U/ml penicillin and 100 µg/ml streptomycin for 7 days at 37 °C. Cultures contained approximately 1000 worms per ml of medium. The medium was replaced at days 1, 3 and 5. Medium from the first 24 h of culture was discarded to rule out any contribution of rat intestinal proteins to the final parasite antigen preparation [61]. Medium was collected on days 3, 5 and 7, filtered through a 0.2-µm filter (Millex, Millipore) and stored at –20 °C. NES antigen was then prepared from 1–1.5 l of parasite culture medium by concentration using an Amicon with a 10-kDa retaining filter. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). NES aliquots were stored at –70 °C until used.

4.2 Animals and immunizations

Six- to eight-week-old female mice were immunized in each hind footpad with either or both 50 µg NES and 10 µg HEL.

Initial dose-response experiments determined that significant IL-4 production was induced by doses of 5–50 µg NES per footpad. A dose of 50 µg/footpad was selected for all subsequent experiments and is equivalent to the amount of NES antigen released from approximately 125 adult worms. Where stated antigens were emulsified in CFA (Sigma). BALB/c, C57BL/6, CBA/Ca and B10.D2 mice were obtained from Edinburgh University. Mice were killed 7 days post-immunization and popliteal LNC isolated. When sera were required, animals were pre-bled on the day of immunization, and at 21 or 28 days after immunization and 7 days (day 35) after an additional secondary immunization. Four animals were used in each experimental and control group and the data are presented as the mean ± S.D. of individual mice.

4.3 Proliferation assays

LNC were cultured in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mM glutamine and 10 % FCS. Cells were cultured at 2×10^6 /ml in 200-µl quadruplicate cultures containing various antigens (10 µg/ml), mitogen (2 µg/ml) or controls (medium only). After 60 h in culture 100 µl of culture supernatant was removed for cytokine assays. Proliferation of remaining cells was assessed by the addition of 1 µCi [³H] thymidine for the last 18 h of culture. Cells were then harvested using an automated cell harvester and the activity incorporated estimated by scintillation counting. Data from proliferation assays are expressed as mean cpm ± S.D. for each individual in each group of mice. When CD4⁺ LNC were required, CD4⁺ cells were positively selected by mini-MACSTM (Miltenyi Biotech, Camberley, Surrey, GB). After staining with PE-conjugated anti-CD4, aliquots of each cell fraction were removed and FACS analysis was performed to verify the efficacy of the separation procedure and the data analyzed using CellQuest software (Becton-Dickinson, Cowley, Oxford, GB). The separated CD4⁺ or CD4[–] popliteal LNC (2×10^5 /well) were challenged with 10 µg/ml NES *in vitro*. Irradiated splenocytes (10^5 /well) from naive syngenic mice were used as APC. Supernatants were collected for cytokine estimation as detailed above.

4.4 Cytokine assays

IFN-γ and IL-4 concentrations in supernatants were determined by capture ELISA. IFN-γ capture/detection antibody pairs have been described previously [65] and are based on R46A2/XMG1.2 (PharMingen, Becton Dickinson, Ltd, Oxford, GB). IL-4 was detected using an ELISA capture assay developed using the antibody 11B.11 (gift of National Cancer Institute, Frederick, MD) and detected using biotinylated anti-IL-4 (BVD6-24G2; PharMingen, Becton Dickinson Ltd, Oxford, GB). Cytokine levels were determined from a standard curve constructed in DMEM-10 using recombinant murine cytokines (Sigma, Poole, GB) for each assay. All

cytokine capture assays used an extravidin-alkaline phosphatase (Sigma UK, Poole, GB) amplification system and were developed using the SigmaFast system as substrate. Once developed, A_{405} was measured using an Anthos 2001 microtiter plate reader (Anthos Labtec Instruments, Salzburg, Austria) and the data manipulated for curve fitting and interpolation using Deltasoft (Denley Instruments, Billingham, GB) software.

4.5 Antibody assays

Total IgE was determined using a capture ELISA using the antibodies R35–72 (2 µg/ml) and biotinylated R35–92 (2 µg/ml). Bound R35–92 was detected using extravidin-horseradish peroxidase (Sigma, Poole, GB) and the reaction developed using the substrate ABTS (KPL Biotechnology, Gaithersburg, MD). The reaction was stopped after 30 min by the addition of an equal volume of 1% SDS and A_{405} determined as described previously. The final concentration of IgE was calculated from a standard curve constructed using a murine monoclonal IgE (Clone IgE-3) supplied by PharMingen (Becton Dickinson Ltd, Oxford, GB). Specific anti-NES or anti-HEL IgG and IgG subclasses were determined by ELISA using horseradish peroxidase-conjugated anti-IgG and subclass antibodies as described previously [65] supplied by Southern Biotechnology Associates Inc. (Birmingham, AL).

4.6 Statistical analyses

Differences in mean values of data sets between experimental groups were compared using Student's unpaired *t*-test. *p* values < 0.05 were considered statistically significant and are indicated by * in the figures.

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