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This information is current as of March 6, 2021.

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J Immunol 2004; 173:2699-2704; ;
doi: 10.4049/jimmunol.173.4.2699
<http://www.jimmunol.org/content/173/4/2699>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



A Secreted Protein from the Human Hookworm *Necator americanus* Binds Selectively to NK Cells and Induces IFN- γ Production

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Parasitic helminths induce chronic infections in their hosts although, with most human helminthiases, protective immunity gradually develops with age or exposure of the host. One exception is infection with the human hookworm, *Necator americanus*, where virtually no protection ensues over time. Such observations suggest these parasites have developed unique mechanisms to evade host immunity, leading us to investigate the role of the excretory/secretory (ES) products of adult *N. americanus* in manipulating host immune responses. Specifically, we found that a protein(s) from ES products of adult *N. americanus* bound selectively to mouse and human NK cells. Moreover, incubation of purified NK cells with *N. americanus* ES products stimulated the production of augmented (4- to 30-fold) levels of IFN- γ . This augmentation was dependent on the presence of both IL-2 and IL-12 and was endotoxin-independent. This is the first report of a pathogen protein that binds exclusively to NK cells and the first report of a nematode-derived product that induces abundant levels of cytokines from NK cells. Such an interaction could provide a means of cross-regulating deleterious Th2 immune responses in the host, thereby contributing to the long-term survival of *N. americanus*. *The Journal of Immunology*, 2004, 173: 2699–2704.

More than 740 million people throughout the world's tropical and subtropical regions are infected with the human hookworm, *Necator americanus*, and to a lesser extent, *Ancylostoma duodenale* (1). Despite their global importance and the chronicity of infections, little is known about precisely how these parasites interact with their hosts, negotiate host anatomy to arrive in the gut, and survive the complex immune responses generated against them. *N. americanus* infections are typically characterized by Ab responses, dominated by IgG4 and IgE. Although such findings suggest that infected individuals develop protective immunity over the course of infection (2), there is little evidence that these vigorous responses are protective (reviewed in Ref. 3). In fact, a strong positive correlation between intensity of infection and age has been demonstrated in human populations in China (4) and Brazil (5), suggesting that hookworms are not affected by the humoral and cellular responses they induce, but rather subvert the immune response toward a phenotype that promotes their long-term survival. Taken together, these findings suggest that, unlike most other helminthiases, human hookworm infections are not associated with the development of acquired immunity.

Descriptions of cytokine profiles from human PBMCs stimulated with *Necator* adult worm Ag have been reported only recently. These studies demonstrated a mixed anti- and proinflammatory response, characterized by IL-5, IL-10, as well as IFN- γ and IL-12 (6, 7). Of particular interest to the maintenance of chronic infections, is the presence of high levels of IL-10, a cytokine typically associated with immunoregulation of effector responses (8). However, given that elevated levels of IL-10 are found both in helminth infections where acquired immunity develops, such as schistosomiasis (9, 10), as well as helminth infections where immunity fails to develop, such as necatoriasis, it seems unlikely that IL-10 is the main mechanism for the persistent immune evasion observed with hookworms. An alternative mechanism of immune suppression is the cross-regulation of protective responses, in this case, Th2 responses. Many nematodes, including hookworms, secrete a myriad of products with the potential for immunomodulation. In the case of several different nematodes, including *Brugia malayi*, *Nippostrongylus brasiliensis* and *Toxocara canis*, injection of excretory/secretory (ES)³ products alone is sufficient to induce immune responses similar to those observed during infection with the live parasites in laboratory animals (11). These responses included the suppression of cellular proliferation, suggesting a capacity for immune suppression/down-modulation by ES products. In the current studies, we propose that a protein(s) present within hookworm ES products might contribute to the observed down-regulation of host immune responses by selectively skewing the host's immune response away from a protective Th2 phenotype.

In this study, we show that an ES product from adult *N. americanus* binds exclusively to murine and human NK cells. Moreover, exposure of NK cells to ES products results in production and

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Received for publication September 4, 2003. Accepted for publication June 15, 2004.

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³ Abbreviations used in this paper: ES, excretory/secretory; bioES, biotinylated ES product; EU, endotoxin unit; PMB, polymyxin B; NKBP, NK binding protein.

secretion of augmented levels of IFN- γ by the cells. This is the first report of a pathogen protein that binds exclusively to NK cells, and the first report of a nematode-derived product that induces abundant levels of cytokines from NK cells. We hypothesize that the local production of proinflammatory Th1 cytokines, such as IFN- γ , interferes with the effector function of protective Th2 responses, contributing to the longevity of hookworm survival in infected people.

Materials and Methods

Collection and labeling of hookworm ES products

N. americanus was maintained in hamsters (12). The dog hookworm, *Ancylostoma caninum*, was maintained in experimental beagles (13). Adult worms were removed from the intestines of euthanized animals, washed in PBS, then cultured overnight in RPMI 1640, 100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, and 0.25 μ g/ml amphotericin B (all reagents from Sigma-Aldrich, St. Louis, MO). ES products were concentrated using microconcentrators (Millipore, Bedford, MA) with a 10 kDa cut-off membrane. ES products were electrophoresed on Tris-glycine 4–20% polyacrylamide gels (Gradipore, Hawthorne, NY) and stained with Coomassie brilliant blue or a Silver Stain Plus kit (Bio-Rad, Hercules, CA). Aliquots of ES products were biotinylated by amine coupling using NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. Biotinylated ES products (bioES) were then dialyzed against 1000 volumes of PBS overnight at 4°C. A mixture of protease inhibitors (AEBSF, bestatin, pepstatin A, E-64, and phosphoramidon; Invitrogen Life Technologies, Carlsbad, CA) was then added to bioES which was subsequently stored at 4°C until needed.

Endotoxin test

ES products (diluted to a concentration at which they were used in tissue culture) were tested for the presence of endotoxin using a semiquantitative *Limulus* Amebocyte Lysate (LAL) assay (E-Toxate kit; Sigma-Aldrich, as well as a quantitative chromogenic LAL assay (BioWhittaker, Walkersville, MD). A maximum of 0.5 endotoxin units (EU)/ml endotoxin was detected in the various batches of ES products used. These assays were also used to test the levels of endotoxin within preparations of LPS (Sigma-Aldrich). Dilutions of 1 and 10 ng/ml LPS were found to contain 10 and 100 EU/ml endotoxin, respectively.

Cell isolations

Murine spleen cells and peripheral blood cells were obtained from male C57BL/6 mice. All mice were used at 6–8 wk of age and were purchased from the National Cancer Institute (Frederick, MD). Human peripheral blood cells were obtained from healthy volunteers. For all studies, mononuclear cells were enriched from mouse spleen, mouse blood, or human blood by centrifugation over Lymphocyte Separation Medium (ICN Bio-medicals, Costa Mesa, CA). The recovered cells were washed once in PBS and then used immediately (for FACS studies) or were further processed to obtain purified populations of NK cells. Mouse NK cells were isolated using anti-NK (DX5) microbeads followed by positive selection on a MACS LS column using a VarioMACS magnet (Miltenyi Biotec, Auburn, CA). Purity of the recovered cells was routinely 85–90% NK1.1⁺ by FACS analysis. Human NK cells were isolated using an NK Cell Isolation Kit (Miltenyi Biotec) in which non-NK cells (T, B, and myeloid cells) are depleted using a mixture of hapten-conjugated Abs against CD3, CD14, CD19, CD36, and anti-IgE, followed by incubation with anti-hapten microbeads and retention on a MACS LS column. Purity of the recovered effluent cells was routinely 95–99% CD56⁺/CD16⁺ by FACS analysis. For some experiments, the bound cells were recovered from the MACS column by flushing and used as a source of non-NK cells (see Fig. 4C). All of the described studies using human and animal cells were conducted under Institutional Review Board and Institutional Animal Care and Use Committee approval.

Binding of ES products to cells and FACS analysis

Populations of mouse or human mononuclear cells were resuspended at 5×10^6 in 1 ml PBS containing 1.0 μ g of bioES from *N. americanus* or *A. caninum* and incubated for 1 h on a rocking platform at 37°C. Control groups of cells were incubated either with 1.0 μ g of unbiotinylated ES products or PBS alone. The cells were then washed twice with PBS containing 5% FCS and stained on ice with FITC-conjugated streptavidin (for the detection of bioES) plus one of the following Abs (purchased from BD Biosciences, San Jose, CA or Caltag Laboratories, Burlingame, CA). For

studies using mouse cells: PE-labeled anti-CD3, anti-CD4, anti-CD8 α , anti-B220, anti-FcR (clone 2.4.G-2), or anti-MHC class II. In some experiments, PerCP-labeled anti-NK1.1 was also included. For studies using human cells: PE-labeled anti-CD20, anti-HLA-DR, anti-CD16/56, or tri-color-labeled anti-CD4. PerCP-labeled anti-CD3 was included in the anti-CD16/56 staining to exclude any CD56⁺CD3⁺ cells during analysis. Stained cells were then fixed in 1% paraformaldehyde and analyzed by FACS.

Measuring cytokine production induced by binding with ES products

NK cells purified from mouse spleen or human blood were set up in 48-well tissue culture plates at 1×10^6 cells per ml in Click's medium (Sigma-Aldrich) containing 5% FCS and various combinations of recombinant cytokines and *N. americanus* ES products. For mouse cells, recombinant murine IL-2 was used at a final concentration of 25 U/ml and recombinant murine IL-12 was used at 5 ng/ml. For human cells, recombinant human IL-2 was used at 100 U/ml and recombinant human IL-12 was used at 10 ng/ml. Other than for the dose response study (Fig. 4B), ES products were added at 1 μ g per well. In some experiments, polymyxin B (PMB; Calbiochem, San Diego, CA) was included in cultures at a final concentration of 0.1 μ g/ml. This dose of PMB was confirmed to inhibit IFN- γ production by non-NK cells stimulated either with 1 or 10 ng/ml LPS (see Fig. 4C). All cultures were incubated for 24 h at 37°C and supernatants were removed and tested for the presence of mouse or human cytokines. Commercial kits were used to quantify the presence of each cytokine: IFN- γ and TNF- α (Pierce, Woburn, MA), and IL-4, IL-5, and IL-10 (R&D Systems, Minneapolis, MN). In some experiments, intracellular IFN- γ was measured in human NK cells after incubation with ES products. Purified NK cells were mixed 1/1 with total PBMC and set up as above except 1 μ l of GolgiPlug solution (BD Biosciences) was added to the cultures after the first 2 h of incubation. The following day, the cells were stained with a combination of PE-labeled anti-CD16/CD56 and PerCP-labeled anti-CD3 and then fixed/permeabilized using a BD FACS Lysing and Permeabilization kit (BD Biosciences). Cells were then stained with FITC-labeled anti-IFN- γ or Ig isotype Ab and analyzed by FACS.

Results

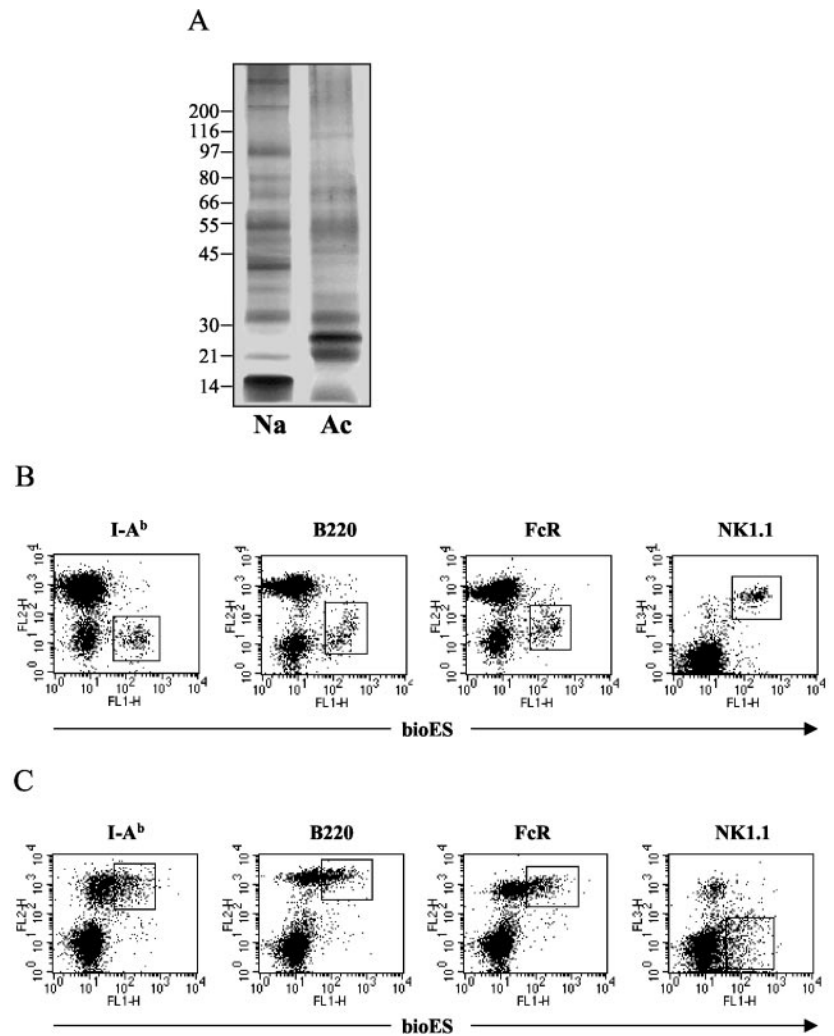
ES products from *N. americanus* and *A. caninum* bind to distinct populations of mouse leukocytes

Many nematodes, including hookworms, excrete/secret multiple macromolecules with the potential for immunomodulation. However, the protein profiles of ES products differ significantly, even between closely related species. Despite their morphological similarities (14), and genetic similarities (Ref. 15; A. Loukas, unpublished observations from expressed sequence tag projects—see www.nematode.net), the protein profiles of adult *N. americanus* and *A. caninum* were notably distinct under denaturing and reducing conditions (Fig. 1A). Both parasites secreted a similar number of proteins (as determined by silver-stained SDS-PAGE gels), but the major proteins secreted by *N. americanus* did not correspond to the major proteins secreted by *A. caninum*.

ES products from *N. americanus* and *A. caninum* bind to distinct populations of mouse leukocytes

A major goal of our studies was to establish how ES products from *N. americanus* hookworms, compared with other hookworm species, might interact with mammalian leukocytes. Thus, our first studies were designed to determine whether any ES product(s) from *N. americanus* might bind directly to mouse or human leukocytes. The data shown in Fig. 1B depicts the findings using bioES from adult *N. americanus* and murine spleen cells. Strikingly, the binding of bioES was completely restricted to a population of cells that were I-A^b negative, but expressed intermediate levels of B220 and FcR, a phenotype suggestive of NK cells (16, 17). Indeed, using an Ab specific for mouse NK1.1⁺ cells, we next confirmed that *N. americanus* ES products bound exclusively to NK cells (Fig. 1B). This population represented 4–5% of total spleen mononuclear cells, with >85% binding bioES. Of importance, this population of NK1.1⁺ cells was found to be CD3⁻ and

FIGURE 1. ES products from different hookworm species are distinct and bind to different subsets of leukocytes. ES products were collected from adult *N. americanus* and *A. caninum* hookworms and aliquots from each were biotinylated to generate bioES. A, Silver stain of a 4–20% gradient SDS-PAGE gel showing separated proteins from *N. americanus* (Na) vs *A. caninum* (Ac) ES products. Murine spleen cells were incubated with bioES from *N. americanus* (B) or *A. caninum* (C) followed by staining with FITC-streptavidin and various PE- or PerCP-conjugated anti-leukocyte Abs. FACS dot plots show the presence of bioES (FL1 on X-axis) on cells costained for I-A^b, B220, FcR, or NK1.1. All dot plots are gated on live lymphoid cells (we previously established that bioES did not bind to nonlymphoid cells). Boxed regions highlight the presence of FL1⁺ cells.



CD4⁻ (data not shown), excluding the contribution of NK1.1 T cells (18). Interestingly, when we examined the binding of biotinylated ES products from *A. caninum* to mouse leukocytes, we observed no binding to NK cells. Instead, *A. caninum* ES products preferentially bound to cells that were MHC class II bright, B220 bright, and FcR bright, but NK1.1 negative (Fig. 1C). Taken together, these data suggest that a protein(s) within ES products from *N. americanus* hookworms selectively binds to NK cells and that such a binding might be unique to that species of hookworm.

ES products from N. americanus also bind selectively to human NK cells

Because humans are the permissive, definitive host for *N. americanus*, we next tested whether ES products from this parasite would also bind to human NK cells. As shown in Fig. 2A, *N. americanus* bioES was observed to bind exclusively to leukocytes from human peripheral blood that stained negative for HLA-DR, CD20, and CD4, but stained positive for the NK cell markers, CD16/56. Of note is that the intensity of binding of bioES on human NK cells was lower than observed on mouse spleen cells (see Fig. 1B). However, a side-by-side comparison of the binding profiles of *N. americanus* bioES on cells from mouse spleen, mouse peripheral blood, and human peripheral blood showed considerable variation in the level of binding between NK cells from different tissues of the same species (mouse), as well as from different species (Fig. 2B). This could reflect the presence of different

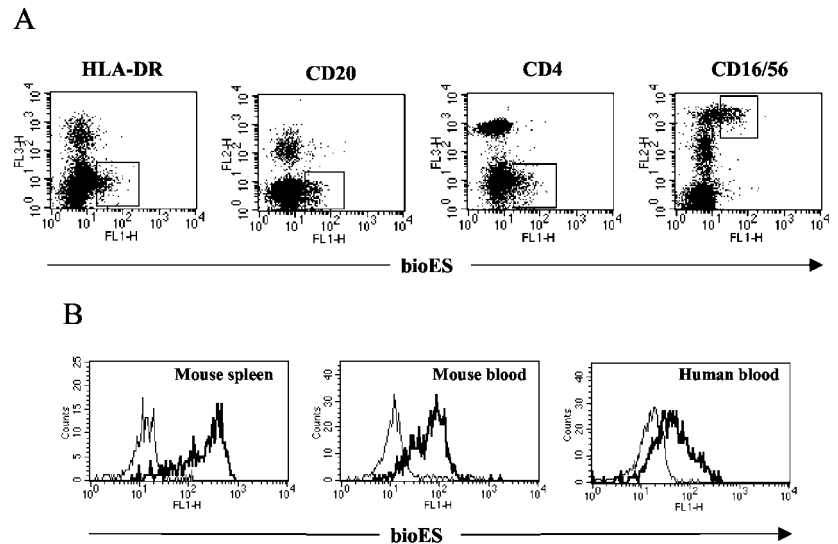
subsets and/or maturational forms of NK cells present within each of these tissues (19).

N. americanus ES products induce IFN- γ production by NK cells

Given the capacity of NK cells to produce abundant levels of cytokines following interaction with innate components of many different types of pathogens, we next examined whether ES products from *N. americanus* might similarly induce cytokine secretion, notably IFN- γ , by NK cells. As shown in Fig. 3, while mouse NK cells incubated with ES products alone failed to secrete any IFN- γ , the addition of ES products to NK cells cultured in the presence of IL-2/IL-12 induced a 4-fold increase in IFN- γ production. The effect was even more striking using human NK cells, where the addition of ES products to cells cultured with IL-2/IL-12 induced a >30-fold augmentation in IFN- γ secretion (Fig. 4A). The observed effect was dose dependent, as demonstrated by adding variable doses of ES products to human NK cells (Fig. 4B). During the course of these studies we also looked for the presence of IL-4, IL-5, IL-10, and TNF- α in the NK cell cultures. For each of these cytokines, the level was below the limit of detection of the ELISA (IL-4 and TNF- α were <15 pg/ml, IL-5 was <12 pg/ml, and IL-10 was <31 pg/ml), suggesting a selective augmentation in IFN- γ production by ES products.

To rule out the possibility that the augmentation in IFN- γ production induced by ES products might be due to the presence of

FIGURE 2. *N. americanus* ES products bind to human NK cells. PBMCs were isolated from human blood, mouse blood or mouse spleen and incubated with bioES from *N. americanus* followed by staining with FITC-streptavidin and various anti-leukocyte Abs. A, FACS dot plots show the presence of bioES (FL1 on X-axis) on human PBMCs costained for HLA-DR, CD20, CD4, or CD16/56 (with CD3⁺ cells excluded). B, FACS histograms show a comparison of bioES binding after gating on NK1.1⁺ cells from mouse spleen and mouse blood, and gating on CD16/CD56⁺ (CD3⁻) cells from human blood. All plots are gated on live lymphoid cells (we previously established that bioES did not bind to nonlymphoid cells). Fine lines are cells incubated with unbiotinylated ES products; bold lines are cells incubated with bioES.



contaminating LPS within preparations, we added PMB, a drug inhibitor of endotoxin activity, to cultures of NK cells. As shown in Fig. 4C, the presence of PMB had no impact on the capacity of ES products to augment IFN- γ secretion by NK cells. Importantly, as part of our experimental setup, we demonstrated that the same concentration of PMB was able to inhibit the induction of IFN- γ secretion by non-NK leukocytes stimulated with doses of LPS containing >10-fold higher levels of endotoxin than detected in ES products (Fig. 4C). Taken together, these findings confirm that the effects of ES products on IFN- γ production by NK cells are not endotoxin-mediated. In addition, we have found that pretreatment of ES products with proteases (either trypsin or pronase) completely abrogated their capacity to bind to and augment IFN- γ production by NK cells (data not shown), suggesting the effects of ES products are mediated by a protein(s).

Further confirmation that the observed augmentation in IFN- γ was due solely to an increase in NK cell-derived secretion was obtained by staining for the presence of intracellular IFN- γ in a mixed population of human PBMCs cultured in IL-2/IL-12 with or without addition of ES products. Figure 4D shows that NK cells (CD16/CD56⁺, with CD3⁺ cells excluded) stained positive for intracellular IFN- γ in the presence of IL-2/IL-12 and that the frequency of IFN- γ -producing cells was increased by the addition of

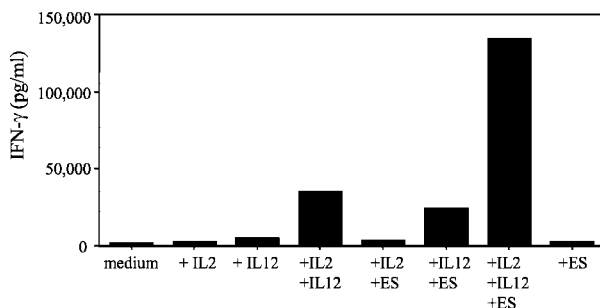


FIGURE 3. *N. americanus* ES products induce IFN- γ production by mouse NK cells. NK cells were purified from mouse spleen and set up in culture in the presence of various combinations of recombinant cytokines and *N. americanus* ES products. After 24 h of culture, levels of IFN- γ were measured by ELISA. Data show the concentration of IFN- γ detected in supernatants of mouse spleen NK cells cultured with combinations of IL-2, IL-12, and ES products.

ES products. These findings demonstrate that the effects of ES products on cytokine secretion are NK cell-specific.

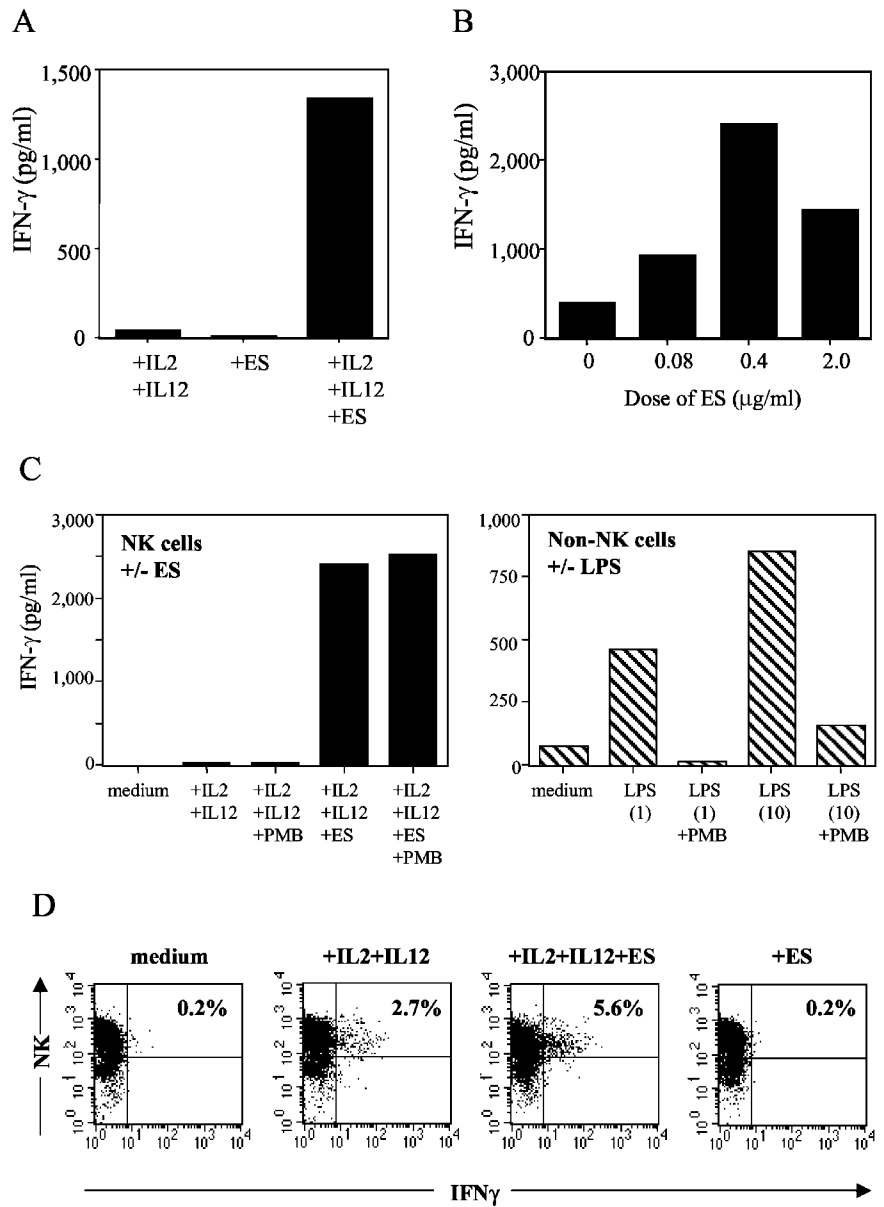
Discussion

Unlike many other parasitic worm infections, there is little evidence that human hookworm infections are associated with the development of acquired immunity. Protection against gastrointestinal nematodes is usually mediated by Th2 responses, where IL-4 plays a prominent role (20). Although human hookworm infections exhibit some of the hallmark features of Th2 responses (IgE and local and systemic eosinophilia), these immune responses clearly fail to protect the host (3). The reason for the observed failure of Th2 cells to mount effective anti-hookworm responses remains unknown.

The findings in the current studies demonstrate the capacity of a protein(s) from *N. americanus* ES products to induce NK cells to augment their secretion of IFN- γ . A similar finding has been reported for protozoan parasites, including the erythrocytic stage of *Plasmodium falciparum* (21) and *Leishmania major* (22, 23). In both studies, the presence of IL-12 or IL-12-producing adherent cells was shown to be critical for the observed augmentation in IFN- γ secretion by NK cells. These findings fit well with our current observations using hookworm products and further emphasize the essential role of IL-12 in NK cell responses to many types of pathogens (24, 25). Interestingly, the *Plasmodium* studies also showed a marked synergistic effect between IL-18 and infected erythrocytes/IL-2/IL-12 for optimal NK cell activation (21). No such synergy was observed between IL-18 and Necator ES products (S. L. Constant, unpublished data), suggesting the mechanism(s) of NK cell activation might differ between hookworm and *Plasmodium* products. It should also be noted that, unlike our current Necator findings, neither of the *Plasmodium*- or *Leishmania*-derived molecules was shown to directly bind to NK cells. Thus, hookworms may have a unique mechanism of interaction with NK cells.

Although NK cells are known to be instrumental in innate immune responses against intracellular pathogens, including viruses, bacteria and protozoa, a role for NK cells in helminth infections is not obvious. To date, there are few reports of a possible contribution of NK cells during worm infections. For example, mice infected with the filarial nematode, *Litomosoides sigmodontis*, undergo an expansion of NK cells with reduced expression of the

FIGURE 4. *N. americanus* ES products induce IFN- γ production by human NK cells. NK cells from human blood were set up in culture in the presence of recombinant cytokines and *N. americanus* ES products. In some groups, PMB was included. After 24 h of culture, levels of IFN- γ were measured by ELISA or intracellular cytokine staining. **A**, Concentration of IFN- γ detected in supernatants of human blood NK cells cultured with IL-2 and IL-12, with or without ES products. **B**, Concentration of IFN- γ detected in supernatants of human blood NK cells cultured with IL-2 and IL-12 plus different doses of ES products. **C**, Concentration of IFN- γ detected in supernatants of human blood NK cells cultured with IL-2, IL-12, and ES products, with/without addition of PMB. To confirm the efficacy of PMB to block endotoxin activity, non-NK cells were stimulated with 1 or 10 ng/ml LPS, with/without addition of PMB. **D**, Intracellular IFN- γ detected in human blood NK cells mixed with total PBMCs and cultured with combinations of IL-2, IL-12, and ES products. FACS dot plots show IFN- γ expression in cells costained for CD16/CD56 with CD3⁺ cells excluded.



Ly49 family of inhibitory receptors (26). Moreover, infections of mice lacking NK cells have demonstrated that host NK cells serve an essential (albeit undefined) role in the growth of the filarial parasite, *Brugia malayi* (27). In contrast, several other studies, in which NK cells were depleted by anti-NK1.1 treatment (thus depleting both NK cells and NKT cells), showed no significant impact on host immune responses to *Trichuris muris* (28) and *Schistosoma mansoni* (29). Similarly, studies using a rat model of *Fasciola hepatica* infection concluded that NK cells do not play a significant role in the course of infection (30).

The most important contribution of NK cells during immune responses to pathogen infections is usually the production of type I cytokines and the induction of Ab-dependent cell-mediated cytotoxicity. Although neither of these mechanisms is typically associated with protection against helminth infections, one way in which type I cytokines (specifically IFN- γ) might benefit nematode survival is via its ability to limit the effectiveness of Th2 effector cells. Thus, hookworms may have evolved the capacity to stimulate the secretion of IFN- γ as a means of cross-regulating destructive Th2 responses. This would most likely be effective at a localized site, for example at the site of adult worm attachment

in mucosal tissues, where ES products would be present at high concentrations. Although such a hypothesis cannot be tested directly, due to the lack of a suitable animal model for *Necator* infections, support for a correlation between IFN- γ production and ES products in vivo is provided by studies comparing cytokine levels in infected mothers (where adult worms were present) and their neonates (no adult worms present). Although IL-5 and IL-10 were equally elevated in both mothers and neonates, IFN- γ (and IL-12) levels were significantly higher in the mothers only (6), suggesting a correlation between type I cytokines and the presence of live parasites. Furthermore, the injection of ES products alone from various nematodes is sufficient to induce potent immune responses in laboratory animals, including IFN- γ production (11).

Of interest also are the current observations that *A. caninum* ES products failed to bind NK cells (Fig. 1C) and induce IFN- γ secretion (our unpublished observations). Unlike *N. americanus* that successfully evades the immune responses of its human host, *A. caninum* is readily cleared by its canine host (31). Instead, *A. caninum* ES products bound to all subsets of leukocytes expressing high levels of FcR. Much of the observed binding of *A. caninum* ES products can probably be attributed to binding of the secreted

glycoprotein, neutrophil inhibitory factor, to its integrin receptor CD11b/CD18 (32). To date, a neutrophil inhibitory factor homologue has not been reported from *Necator* and the corresponding cDNA has not been identified from several thousand expressed sequence tags deposited in public databases (A. Loukas, unpublished data). Thus, ES products of *N. americanus* may contain specific proteins, not present in other species of hookworm, that contribute to the long-term survival of this parasite in its host. Of interest is that we have examined the binding properties of several different recombinant proteins from adult and larval hookworms and none of these were found to bind NK cells. Instead, some of these bound either to monocytes or to platelets (S. L. Constant and A. Loukas, unpublished data), further confirming the selective capacity of *N. americanus* ES products to bind NK cells. Characterization of the composition of hookworm and other nematode ES products has demonstrated the presence of many different types of proteins including proteases, protease inhibitors, C-type lectins, anti-oxidants, pathogenesis-related proteins, and anti-inflammatory proteins (3). Studies are currently underway to characterize and eventually clone the *N. americanus* NK binding protein (NKBP). Identifying the proteins in NKBP, as well as the receptor for NKBP on NK cells, will shed light on this potentially unique strategy developed by hookworms to avoid immune destruction. In addition, it will enable us to establish the potential utility of NKBP as a proinflammatory adjuvant.

Acknowledgments

We thank Reshad Dobardzic and Azra Dobardzic for maintenance and handling of the hamsters infected with *N. americanus* hookworms.

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