Hookworms Evade Host Immunity by Secreting a Deoxyribonuclease to Degrade Neutrophil Extracellular Traps

Highlights

- Neutrophils swarm around hookworm larvae as they penetrate the skin
- Neutrophils generate extracellular traps (NETs) in response to hookworm larvae
- NETs can directly impair larval viability
- Hookworm larvae degrade NETs by secreting a deoxyribonuclease (DNase II)

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In Brief

Bouchery et al., demonstrate that neutrophils participate in defense against helminth by secreting neutrophil extracellular traps that are toxic to the parasite larvae. In response, the parasite has evolved an evasion strategy based on the secretion of a DNase II that degrades the DNA backbones of the traps.
Hookworms Evade Host Immunity by Secreting a Deoxyribonuclease to Degrade Neutrophil Extracellular Traps

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https://doi.org/10.1016/j.chom.2020.01.011

SUMMARY

Hookworms cause a major neglected tropical disease, occurring after larvae penetrate the host skin. Neutrophils are phagocytes that kill large pathogens by releasing neutrophil extracellular traps (NETs), but whether they target hookworms during skin infection is unknown. Using a murine hookworm, Nippostrongylus brasiliensis, we observed neutrophils being rapidly recruited and deploying NETs around skin-penetrating larvae. Neutrophils depletion or NET inhibition altered larvae behavior and enhanced the number of adult worms following murine infection. Nevertheless, larvae were able to mitigate the effect of NETs by secreting a deoxyribonuclease (Nb-DNase II) to degrade the DNA backbone. Critically, neutrophils were able to kill larvae in vitro, which was enhanced by neutralizing Nb-DNase II. Homologs of Nb-DNase II are present in other nematodes, including the human hookworm, Necator americanus, which also evaded NETs in vitro. These findings highlight the importance of neutrophils in hookworm infection and a potential conserved mechanism of immune evasion.

INTRODUCTION

Hookworms, including Necator americanus (Na) and Ancylostoma duodenale, are highly successful nematode parasites that represent an evolutionarily ancient disease; evidence of infection has been found in human fossils dating between 4,000 and 7,000 years old (Araujo et al., 1988). Modern sanitation methods have largely eradicated these parasites from developed regions. However, approximately 700 million people living in impoverished conditions remain infected, and many of them suffer from morbidity resulting from the anemia caused by nematode feeding on host blood (Hotez, 2008). While the host immune response to hookworm infection is robust, it fails to elicit protection, and individuals tend to exhibit heavier worm burdens with age (Hotez et al., 2016). No protective vaccines currently exist, and their successful development will require an improved understanding of both the host immune response and nematode biology (Allen and Maizels, 2011; Anthony et al., 2007; Hotez, 2008). Neutrophils are highly abundant granulocytes that rapidly enter sites of infection, inflammation, or damage. They have long been known to contribute to pathogen resistance through multiple mechanisms including phagocytosis, production of reactive oxygen species, and the release of granules containing toxic mediators. More recently, activated neutrophils were observed to release extracellular nucleic acids decorated with histones and granular proteins, termed neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). Since their discovery, a large body of research has demonstrated that neutrophils actively form NETs (a process known as NETosis) in response to an array of pathogens—including bacteria, fungi, viruses, and protozoa (Yipp and Kubes, 2013). Evidence that NETs can provide defense against pathogens has also been demonstrated for E. coli (McDonald et al., 2012), Staphylococcus (Yipp et al., 2012), Candida albicans (Byrd et al., 2013; Urban et al., 2006), and HIV (Saitoh et al., 2012). Interestingly, NETs have been shown to be deployed selectively against pathogens that are too large to be killed intracellularly, such as fungal hyphae, and play a critical role in their clearance (Branzk et al., 2014). Several groups have now reported formation of NETs around other large pathogens, including...
Figure 1. Myeloid Cells Form Swarms around Skin Penetrating Hookworm Larvae

(A–E) Mice were injected with the indicated labeled mAb(s) intravenously (i.v.) 1 h before intradermal (i.d.) injection of 250 CFSE-labeled Nb L3 into the belly skin. At 1 and 6 h following infection mice were sacrificed, the infected skin removed, and imaging performed as described in Figure S1A. Data were collected from 2 independent experiments (n = 3–5 mice/time point) and representative images are shown. Scale bar, 100 μm. (A) Mice received 10 μg APC-labeled anti-Gr1 mAb

(legend continued on next page)
**RESULTS**

**Hookworm Infection of the Skin Elicits Rapid Neutrophil Swarming**

Myeloid cell recruitment from the blood to extravascular sites of tissue damage is a hallmark of the early innate immune response. To assess the possible recruitment of myeloid cells into the skin following Nb infection, we injected mice with allopurinol, an anti-Ly6G/Ly6C (Gr1) mAb (monoclonal antibody), followed by infection with carboxyfluorescein succinimidyl ester (CSFE)-labeled Nb delivered via intradermal injection. Myeloid cell recruitment and larvae were then visualized using confocal imaging of excised tissue (Figure S1A). Myeloid cells rapidly accumulated at the site of infection and formed swarms around the larvae by 6 h post-infection (Figure 1A). To determine the relative contribution of monocytes or neutrophils to the swarms, we utilized labeled antibodies specific for neutrophils (anti-Ly6G) or monocytes (anti-Ly6C), and then determined the proportion of each cell type to larvae using a custom-made script as detailed in Figures S2 and S1B. Both neutrophils and monocytes began to accumulate around larvae by 1 h post-infection (Figures 1B and 1C). At 6 h post-infection, swarms of neutrophils were observed in close proximity to larvae, while monocytes were only present at the outer edges of the swarm (Figures 1D and 1E). At 1 h post-infection, larvae are still within their outer sheath (Figure S1C), but by 6 h, larvae were motile and had begun to exsheath (Figure S1D). By 13 h, the majority of larvae had left the skin and mainly empty sheaths remained (Figure S1E). Of note, the few larvae that did remain at this time point were consistently surrounded by large swarms of neutrophils (Figure S1E).

Although experimental infection with Nb is typically performed by injection of infective larvae, natural infections occur via skin penetration following exposure of the host to larvae present in the soil. We therefore addressed whether myeloid cell recruitment to larvae also occurred during a natural infection. For this purpose, we administered CSFE-labeled Nb via topical application to the belly skin of mice previously injected with APC-labeled anti-Gr1 antibody. Mice were subjected to repeated topical applications of larvae until a final dose of 100 L3 was reached, then sacrificed 1 h later. By this time, the majority of larvae penetrating the skin were surrounded by Gr1+ cells (Figure S1F), indicating that myeloid cell recruitment around larvae also occurs in response to natural infection. However, because of the low efficiency of the skin penetration by Nb (approximately 8% of total larvae applied) and the need for repeated topical applications, we returned to the use of intradermal injections for all subsequent experiments.
Myeloid cell recruitment around skin larvae could occur in response to the presence of larvae or as a consequence of tissue damage resulting from larval migration. We predicted that neutrophils could respond directly to larvae, because in vivo injection of killed larvae elicited extensive neutrophil swarming, while sham injections did not (Figures S1G and S1H). To test this hypothesis, we co-cultured neutrophils or monocytes together with larvae in vitro. After 24 h, both neutrophils and monocytes adhered to larvae, with the addition of serum increasing adherence in a manner that was partly dependent on complement (Figures 1F and 1G). Of note, the monocyte-mediated attack was less dramatic than the neutrophil attack (Figure 1G). The addition of serum also resulted in larger numbers of adhering neutrophils compared with monocytes, mimicking our in vivo observations (Figures 1F and 1G). Interestingly, the attraction of neutrophils toward larvae occurred in a chemotactic manner as demonstrated using a transwell system (Figure 1H), however, this process was serum independent.

**Hookworm Larvae Adapt Their Development after Sensing Neutrophils**

To determine whether the myeloid cells that surrounded skin penetrating larvae impacted on their survival, we depleted both neutrophils and monocytes using anti-Gr1 mAb, or only neutrophils using anti-Ly6G mAb, then determined the number of larvae that were able to exit the skin and migrate to the lung. The efficiency of cell depletion was confirmed by visualizing cellular DNA present in cultures containing either live or dead larvae, the addition of serum resulted in a reduction in extracellular DNA (Figure 3A). Despite its ability to enhance neutrophil adherence to larvae, the addition of serum resulted in a reduction in extracellular DNA present in cultures containing either live or dead larvae, similar to what has been recently reported for LPS-induced NETs (Figure 3A; Neubert et al., 2018). To validate that the extracellular DNA originated from NETs, we stained for citrullinated histones (H3) and the granule protein myeloperoxidase (MPO), which are specific NET markers. The cultures with dead larvae contained MPO* and H3* neutrophils and exhibited clear evidence of extracellular staining for the same proteins, indicating that NETosis had occurred, with NETs still visible (Figure 3B). Neutrophils in the cultures containing live larvae did not exhibit extracellular staining for these proteins, confirming that NETs were not present. Of note, however, these neutrophils did exhibit positive intracellular staining for both proteins, suggesting that NETs may have formed but were subsequently degraded (Figure 3B).

The observation that NETosis occurs in response to both live and dead larvae, but that NETs could only be detected in cultures of dead larvae, suggested that live larvae may secrete a DNase capable of degrading the DNA backbone of NETs. Infective larvae respond to the 37°C temperature of their hosts and the presence of serum components by actively secreting enzymes that function to facilitate migration, promote feeding, and modulate host immune responses (Datu et al., 2008; Hadford and Schad, 1990; Weinstein and Jones, 1956). To determine whether the excretory secretory products of Nb infective larvae (NES) also contain enzyme(s) with DNase activity, we tested the ability of NES to degrade NETs formed in response to Candida albicans hyphae. Neutrophils were activated by C. albicans hyphae and NET formation was assessed dynamically using time-lapse microscopy. In the absence of NES, NETs were visible from the time of formation for at least 6–11
Figure 2. Hookworm Larvae Sense Neutrophils and Adapt Their Development to Their Presence

(A) Mice were treated with 250 μg anti-Gr1 mAb or 500 μg of anti-Ly6G mAb by intraperitoneal (i.p.) injection and day −1, 0, and 1. On day 0, mice were infected with 250 antibiotic-treated Nb L3 by i.d. injection and skin and lungs removed 24 and 48 h later. Data are pooled from 2 independent experiments (n = 8–10/group) and analyzed by ANOVA.

(B and C) Mice were treated with anti-Gr1 mAb and anti-Ly6G mAbs, as for (A), and infected with 250 doubled-labeled L3 (CFSE for external labeling, yellow orange (YO) carboxylate microspheres for internal labeling) in the belly skin. The dispersion of larvae was determined in the skin at 6 h following infection by mounting excised skin in a chamber containing fluorobrite medium at 37°C under 5% CO2 stimulation. The skin was imaged from the inside (subcutaneous tissue) with a confocal microscope. Scale bar, 400 μm. Images are representative of 2 independent experiments (n = 3 mice/time point).

(C) The percentage of sheathed versus exsheathed larvae was enumerated and data analyzed by ANOVA. Bonferroni multiple comparisons show significant changes for isotype control versus Gr1-mAb-treated groups (p = 0.0006) and isotype control versus Ly6G-mAb-treated groups (p = 0.0113).

(D) Nb L3 were cultured in the presence or absence of neutrophils and the percentage of sheathed versus exsheathed L3 determined after 24 h using a brightfield microscope. Data are representative of 4–5 independent counts of 100 L3 and analyzed by a t test (p = 0.034).

(E) Mice were treated with depleting mAb and infected with L3, as for (A), and the number of worms in the intestine determined 5 days later. Data are pooled from 2 independent experiments (n = 8–10 mice/group) and analyzed by ANOVA.
Figure 3. Neutrophils Release NETs in Response to Hookworms

(A) Human peripheral neutrophils were co-cultured with 100 live or dead (boiled) Nb L3 for 1 h in the presence of Sytox blue, with or without naive mouse serum. Sytox-positive events were imaged by confocal microscopy at 37°C under 5% CO2 stimulation, with the pinhole nearly closed. NETs were visualized using a “fire” filter in ImageJ and the Sytox intensity shown (black = low intensity, white = high intensity). The area of Sytox-positive event was calculated using a home-made pipeline (see STAR Methods) in ImageJ. Data are shown from one experiment (duplicate wells/condition) and are representative of 3 independent experiments using different donors. Data are analyzed by ANOVA.

(B) Human peripheral neutrophils were co-cultured with 100 live or dead (boiled) Nb L3 for 1 h then fixed and stained with mAbs against MPO (red) or citrullinated histone (H3) (green) then counterstained with DAPI (blue). Images were acquired using a confocal microscope. Representative images are shown from 1 experiment employing 2 independent donors (duplicate wells/condition).

(C) Human peripheral blood neutrophils were stimulated with C. albicans hyphae to induce NET formation in the presence of increasing concentrations of NES extract. NET lifetime was measured by live cell microscopy and is presented as the time it takes for the Sytox signal to disappear in each condition. Data are pooled from 2 independent experiments (n = 2 donors). The negative association between the NES dose and NET lifetime was analyzed by Spearman’s rank correlation (Spearman’s Rho = −0.678, p < 0.001).

(D) Plasmid DNA was incubated with increasing concentrations of NES, or DNase I, in the presence or absence of G-actin, for 15 or 45 min as indicated. DNA degradation was assessed by agarose electrophoresis.
Remarkably, the presence of NES did not impact the formation of NETs (Figure S4A)—however, it did impact in a dose-dependent manner the half-life of NETs, indicating that NES promotes NET degradation (Figure 3C). NES also resulted in the degradation of plasmid DNA, in a time- and dose-dependent manner, confirming the presence of DNase activity (Figure 3D). Interestingly, G-actin, a known inhibitor of DNase I, did not inhibit the DNA-degrading activity of NES, suggesting that the NES DNase is structurally different to DNase I (Figure 3D). A recent study reported that Staphylococcus aureus subverts host immunity by secreting nucleotidases that degrade NETs, leading to the release of factors toxic for surrounding monocytes (Papayannopoulos, 2014; Thammavongsa et al., 2013). We therefore assessed whether NES-mediated degradation of NETs also impacted on monocyte viability, however, we were unable to find any evidence of toxicity in this setting (Figure S4B).

To determine whether degradation of NETs by live larvae also occurs in vivo, we subjected mice previously injected with Sytox to intradermal injection of live or dead larvae and determined the presence of NETs within the skin. At 3 h post-infection, a small amount of extracellular DNA surrounded both live and dead larvae (Figure 4A). However, by 6 h, extracellular DNA could only be found in close contact to dead larvae and was virtually absent from the skin containing live larvae, despite the presence of neutrophils in both settings (Figure 4A). These observations suggested that larvae can secrete a DNase capable of degrading the DNA backbone of NETs in vivo. In support of this hypothesis, the presence of extracellular DNA around dead larvae could be prevented by co-injection of dead larvae together with NES (Figure 4B). This effect was similar to that observed by pre-treatment of mice with a recombinant DNase I (Figure 4B), a protocol widely used to facilitate NET degradation.

The observed ability of Nb infective larvae to degrade NETs suggested an evolutionary advantage of this process, perhaps as a means to prevent larval damage inflicted by NETs. To address this, we treated mice with a recombinant DNase I intraperitoneally at repeated intervals during the first 12 h following infection. Similar to what was observed after neutrophil depletion, less larvae were found in the lungs of treated mice at 48 h post-infection (Figure 4C), and an increased number of adults were recovered from the intestine at day 5–6 following infection (Figure 4D). We confirmed the role of NETs in altering parasite viability using protein arginine deiminase 4 (PAD4)-deficient mice and by treating mice with a neutrophil elastase (NE) inhibitor (Yanagihara et al., 2007) to prevent neutrophils from undergoing NETosis. In both cases, a reduced number of larvae were observed in the lung at 48 h post-infection (Figure 4E).

**Hookworm Larvae Secrete a DNase II to Escape Larval Killing by NETs**

We next set out to identify the protein(s) harboring DNase activity within NES. As the DNase activity in NES was not inhibited by G-actin, we searched for a DNase II motif in the sequences of identified proteins secreted by the L3 stage of Nb (Sotillo et al., 2014) and identified one protein, m.13872 (hereafter called Nb-DNase II; Data S1; Figure 5A). A blast search of NCBI non-redundant protein sequences indicated that Nb-DNase II is highly conserved within clade VI nematodes including Ancylostoma ceylanicum, N. americanus (Na, human hookworms), Haemonchus contortus and Trichuris muris (sheep roundworms), and Heligmosomoides polygyrus bakeri (murine hookworm) (Figure 5A). Interestingly, Nb-DNase II is more highly expressed in the L3 than the adult worms, suggesting an evasion strategy tailored for the transition of early-stage infection to parasitism (Sotillo et al., 2014).

We next produced a recombinant form of Nb-DNase II in E. coli and tested the ability of the recombinant protein (Nb-DNase II) to degrade NETs formed in vitro by neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA). The addition of rNb-DNase II resulted in degradation of NETs in a manner similar to that observed for NES (Figure 5A). We also generated an anti-serum against rNb-DNase II and demonstrated that the addition of this anti-serum to PMA-stimulated neutrophils could reverse NES-mediated degradation of NETs, while naive serum could not (Figure S5A). The ability of the anti-serum to block NET degradation by NES allowed us to assess the impact of intact NETs on larval viability. To this end, we added Sytox (a dye to which the cuticle of live larvae is impermeable) to larval neutrophil co-cultures, with or without anti-serum, and counted the proportion of larvae that took up the dye. The presence of neutrophils alone led to an increased proportion of Sytox+ larvae, indicating that these cells could directly kill the parasite (Figure 5C). Of note, addition of the anti-serum to block the ability of larvae to secrete DNase II resulted in increased larval killing, providing direct evidence that NETs function to impair parasite survival (Figure 5C).

Given that we had identified several putative homologs of Nb DNase II in the Na proteome (Figure 5A), we next aligned the sequence of those putative homologs and found that the two catalytic sites specific of DNase-II were highly conserved, with XP_013307665 presenting the greatest identity (63.39%) (Figure S5B).

These data indicate that Na larvae may also secrete a DNase II capable of degrading NETs. To address this experimentally, we co-cultured live or dead Na larvae together with neutrophils in vitro and assessed the presence of NETs using Sytox staining. NETs were observed more often in association with dead larvae as compared with live larvae, indicating that live larvae are able to degrade NETs (Figure 5D). We next confirmed that the observed extracellular DNA structures were true NETs by co-staining Sytox-positive DNA using mAbs directed against MPO and histone H3 citrullination (Figure 5E). Taken together, these data provide evidence that both human and murine hookworm larvae can evade NET-mediated killing through the secretion of a DNase II.

**DISCUSSION**

The evolution of strategies to overcome trapping by NETs has previously been reported for bacteria, viruses, protozoa, and fungi—the most common of these strategies being the production of a DNase I that targets the backbone structure of the NETs (Derré-Bobillot et al., 2013; Guimarães-Costa et al., 2014; Seper et al., 2013; Sumby et al., 2005; Thammavongsa et al., 2013). The current study demonstrates that NETs directly contribute to the killing of hookworm larvae in vitro and limit parasite viability in vivo. We also identified the secretion DNase...
Figure 4. Live Hookworm Larvae, or Their Secreted Products, Can Degrade NETs *In Vivo*

(A and B) Mice were given an i.v. injection of 5 μg APC-labeled anti-Ly6G mAb and 100 μL of 50 μM Sytox-green 1 h before i.d. injection of 250 Nb L3 into the belly skin and infected skin removed 3 or 6 h later and imaged as described in Figure 1A. Images show neutrophils (red) and extracellular DNA (green) and are representative of 2 independent experiments (n = 3 mice/group). Scale bar, 100 μm. (A) Mice received live or dead (boiled) Nb L3 and the skin imaged 3 or 6 h later. (B) Mice received dead (boiled) Nb L3, with or without 100 μg of NES, or were additionally given an i.p. injection of 1,000 U of DNase I, and the skin imaged 6 h later.

(C and D) Mice were infected with 250 antibiotic-treated Nb L3 by i.d. injection and additionally treated with 1,000 U of DNase I by i.p. injection at 0, 4, and 8 h post-infection. Worm numbers in the lung at 48 h (C) or gut at 6 days (D). Data are pooled from 2 independent experiments (n = 8–10 mice/group) and analyzed by ANOVA.

*Wild-type mice were treated with NE-inhibitor by i.p. injection and day 0, 1, and 1. On day 0, wild-type and PAD-4-knockout (KO) mice were infected with 250 antibiotic-treated Nb L3 by i.d. injection and the number of larvae in the lung determined at 24 and 48 h. Data are pooled from 2 independent experiments (n = 6–8 mice/group) and analyzed by ANOVA.*
Figure 5. Hookworms Share a Conserved DNase II that Allow Their Evasion from NET-Induced Killing

(A) Phylogenetic relationships of DNase II based on Bayesian inference (BI). The posterior probability supporting each clade is indicated (bootstrap: small circle 0.6, big circle 1).

(B) Human peripheral neutrophils were stimulated with 100 nM PMA to induce NET formation in the presence of 10 μg/mL of NES. NETs were visualized and their area quantified as described in Figure 3A. More than 1,000 events were recorded for each condition.

(C) 100 Nb L3 were co-cultured with 1 million human peripheral neutrophils for 24 h in presence of antisera raised against Nb-DNase II (αNb-DNase II, 1:50) or naive serum (1:50). Larvae cultured in the absence of neutrophils were included as a baseline control for viability (Nb only). After overnight co-culture, Sytox green was added (1:100) to the wells and viability of the larvae was assessed using fluorescent microscopy. Larvae were considered dead, or to have impaired viability, when staining positive for the dye. Data are pooled from 3 independent experiments with 1 donor and are representative of 2 additional experiments using a second donor. Data are analyzed by ANOVA.

(D) Human peripheral neutrophils were co-cultured with 100 live (pre-activated overnight at 37°C) or dead (boiled) Na L3 for 3 h in presence of Sytox green (1:1,000) and human serum. The formation of NETs around larvae was identified using a fluorescent microscope. Data are presented as the percentage of larvae covered with NETs and pooled from 3 experiments (n = 2–3 wells/condition) and analyzed by t test.

(E) Larvae cultured as in (D) were PFA fixed and stained as in Figure 3B. Representative images are shown from 2 experiments (duplicate wells/condition).
ll by hookworm larvae as an immuno-evasion mechanism employed by the parasite to evade NET-mediated killing.

Bacteria, protozoa, and fungi have all been reported to have evolved strategies to overcome ensnarement by NETs via the secretion of nucleases that degrade the DNA backbone structure of these structures (de Buhr et al., 2014; Jhelum et al., 2018; Sumby et al., 2005; Zhang et al., 2017). Interestingly, DNase activity has also been described in the excretory and secretory products of various infectious stages of helminths, such as Trichinella spiralis and the hookworm Ancylostoma caninum (Liu et al., 2007; Yun et al., 2012). In the latter report, the function of the identified DNase activity was not investigated; however, the authors proposed that it may facilitate tissue penetration (Yun et al., 2012). 27 DNase II enzymes have been identified in Trichinella spiralis but were reported to miss the DHSKW motif, necessary for DNase II enzymatic activity. Our work identifies another function of parasite-secreted DNase, namely the degradation of NETs and evasion of NET-mediated larval killing. It is intriguing that a DNase II, rather than a DNase I, would be secreted by a pathogen. Indeed, the secretion of a DNase II by hookworm larvae was unexpected given that these enzymes are normally restricted to the cytoplasm of all other species investigated to date.

Several recent reports have described the ability of neutrophils contributing to the killing of various nematode larvae (Bonne-Annee et al., 2014; McCoy et al., 2017; Pionnier et al., 2016; Sutherland et al., 2014; Tamarozzi et al., 2016). Yet, whether NETs contribute to this killing has remained unclear with some reports describing an association between NETs and the presence of Strongyloides stercoralis larvae (Bonne-Annee et al., 2014) or microfilariae (Tamarozzi et al., 2016), while others report the presence of neutrophils but not NETs around Nb larvae (Sutherland et al., 2014) and Brugia malayi (McCoy et al., 2017). The finding that Nb secretes a DNase II that makes the detection of NETs following natural infection difficult may explain these inconsistencies. Alternatively, different nematode species may exhibit differential abilities to either induce or degrade NETs. In those studies where NETs were reported to surround nematode larvae (Bonne-Annee et al., 2014; McCoy et al., 2017; Pionnier et al., 2016; Sutherland et al., 2014; Tamarozzi et al., 2016), it was hypothesized that NETs would not impact on helminth viability directly but that they may instead act to ensnare motile larvae, and thus facilitate their killing by other immune cells (Bonne-Annee et al., 2014). Indeed, S. stercoralis larvae were reported to be efficiently trapped by NETs, and to be subsequently killed by monocytes (Bonne-Annee et al., 2014). Although other cells may well contribute to the killing of hookworm larvae in vitro, we provide evidence that NETs alone can directly kill hookworms in vitro.

To date, pathogen killing by NETs has been demonstrated for gram-negative and gram-positive bacteria, as well as fungi (Brinkmann et al., 2004; Yipp and Kubies, 2013). Two distinct mechanisms of NET-mediated toxicity have been described. In the first, NETs mediate direct toxicity to bacterial outer membranes via their DNA backbone (Brinkmann and Zychlinsky, 2012; Halverson et al., 2015; Marsman et al., 2016). In the second, killing of fungal pathogens, such as Candida and Aspergillus, has been shown to be mediated by anti-microbials that decorate the DNA backbone of NETs (Parker et al., 2012; Urban et al., 2009). Of note, it has been recently been demonstrated that the proteins found within the structure of NETs differ depending on the stimulus used to induce NETosis (Chapman et al., 2019; Lim et al., 2018; Petretto et al., 2019). It would therefore be interesting to elucidate the means by which NETs are toxic to nematode larvae and, if relevant, to identify the proteins that are associated with NETs released in response to these parasites.

How neutrophils are attracted to nematode larvae and what triggers NETosis remains unclear. It has previously been suggested that C5a might be important for recruitment (Giacomin et al., 2008), however, addition of serum was not found to be required in vitro to trigger neutrophil chemotaxis toward Nb L3. Rather, our data indicate the neutrophils can respond to larvae directly. However, it is not clear whether neutrophils recognized Nb larvae per se or whether they detect bacteria associated with the parasite. Indeed, for B. malayi, the symbiont bacterium Wolbachia has been shown to be required to trigger NETosis (Tamarozzi et al., 2016). For soil-transmitted helminths like hookworm, bacterial contamination of larvae is common, both in nature and in the laboratory, as L3s emerge from eggs passed out in the feces of infected animals. Given that NETosis in response to nematodes has only been described for soil-transmitted helminths or by filariae containing an endosymbiont bacteria, it is likely that bacterial association is important to this mechanism (Bonne-Annee et al., 2014; McCoy et al., 2017; Pionnier et al., 2016; Sutherland et al., 2014; Tamarozzi et al., 2016). In our own experiments, we utilized larvae that were first washed extensively with an antibiotic cocktail, however, residual contamination of bacteria and/or their products is likely making it impossible to distinguish between these possibilities.

Hookworm vaccines currently in development target the adult stage of the parasite, with the lead candidate, Aspartic protease-1 (APR-1), being involved in the ability of the parasite to feed on host blood. While it decreases adult worm survival and associated egg release, it fails to confer sterilizing immunity (Zhan et al., 2014). Here, we show that NETosis can affect both establishment and survival of Nb, despite the fact that the parasite evades the bulk of the immune-mediated attack. Furthermore, the human hookworm Na expresses a homolog of Nb-DNase II that confers the parasite with an evasion strategy. Thus, a useful vaccination approach could be to target the hookworm DNase, in combination with APR-1. This would allow the host to target both larval and adult stages of the parasite and could represent an efficient strategy to both alleviate the pathology induced by the parasite and to decrease the likelihood of establishment and subsequent transmission.

In summary, our findings unveil the ability of NETs to trap and kill infective hookworm larvae in the skin and identify hookworm production of a DNase II as an immune-evasion mechanism employed by these insidious parasites. These findings expand our understanding of mammalian-helminth interactions and open avenues for the development of a successful anti-helminth vaccine, as well as a potential intervention drug for NET-mediated diseases, such as sterile injury, gallstones, septic shock, and autoimmunity.
STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chom.2020.01.011.

ACKNOWLEDGMENTS

We thank the MICU animal facility at Monash AMREP and the Monash Micro Imaging platform for support with confocal imaging. We thank the Ecole Polytechnique Fédérale de Lausanne (EPFL) animal facility, and Jessica Sordet-Desimoz and EPFL Histology Core Facility, for providing invaluable help and useful suggestions for the experiments outlined. A special thanks to Jose Artacho from the Bio-Imaging and Optics Platform. We also thank Derre-Bobillot, A., Cortes-Perez, N.G., Yamamoto, Y., Kharrat, P., Couvé, E., Da Cunha, V., Decker, P., Boissier, M.C., Escartin, F., Cesselin, B., et al. (2013). An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to Candida albicans. J. Immunol. 190, 4136–4148.

AUTHOR CONTRIBUTIONS

T.B., T.-D.T., T.B., and S.S performed infection experiments. T.B. and M.K. prepared the ES of the parasite. T.B., N.L.H., V.P., and G.L.G. conceived of the idea and participated in the experiments. T.B., N.L.H., and V.P. analyzed the data. T.B. and G.L.G. wrote the manuscript. All authors discussed the results and commented on the paper.

DECLARATION OF INTERESTS

A.L. and P.G. are shareholders in Paragen Bio Pty Ltd, a biotechnology company focusing on the use of hookworm proteins to treat inflammation.

Received: October 29, 2019
Revised: December 15, 2019
Accepted: January 17, 2020
Published: February 12, 2020
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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Human/Mouse anti-Myeloperoxidase | R&D system | Cat#AF3667; RRID:AB_2250866 |
| Rabbit polyclonal to Histone H3 (citrulline R2 + R8 + R17) | Abcam | Cat#ab5103; RRID:AB_304752 |
| anti-Gr1, clone RB6-8C5 (depletion) | BioXcell | Cat#BE0075; RRID:AB_10312146 |
| Anti-Ly6G, clone 1A8 (depletion) | BioXcell | Cat#BE0075-1; RRID:AB_1107721 |
| InVivoMAb rat IgG2a isotype control, clone 2A3 (depletion) | InVivoPlus | Cat#BE0089; RRID:AB_1107789 |
| InVivoPlus rat IgG2b isotype control, clone LT-2 | BioXcell | Cat#BE0090; RRID:AB_1107780 |
| BV421 anti-Ly6G, clone AL-21 (in vivo staining) | BD | Cat#562727; RRID:AB_2737748 |
| AlexaFluor 647 anti-Gr1, clone RB6-8C5 (in vivo staining) | BD | Cat#108420; RRID:AB_493481 |
| AlexaFluor 647 anti-Ly6G, clone 1A8 (in vivo staining) | BD | Cat#127609; RRID:AB_1134162 |
| **Bacterial and Virus Strains** |        |            |
| E. coli BL21 (DE3) | Thermo-Fisher Scientific | Cat#C600003 |
| **Biological Samples** |        |            |
| Nippostrongylus excretory Secretory (NES) products | Cambersis et al., 2003 | N/A |
| Mouse polyclonal serum anti-NES | This paper | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| DNase I, bovine recombinant | Sigma-Aldrich (Roche) | Cat#04536282001 |
| Sytox green nucleic ac | Life technologies | Cat#57020 |
| Sytox blue nucleic acid | Life technologies | Cat#511348 |
| Phorbol 12-myristate 13-acetate | Sigma-Aldrich | Cat#79346-1MG |
| NE-inhibitor, sivelestat sodium hydrate | Sigma-Aldrich | Cat#57198-5MG |
| Carboxy fluorescein succinimidyl ester | Sigma-Aldrich | Cat#92846-5MG-F |
| Fluorobrite YO carboxylate microsphere | Polysciences, Inc | Cat#18720-10 |
| Fluorobrite | Thermo-fisher | Cat#A1896701 |
| CUBIC mount | Epp et al., 2015 | N/A |
| **Critical Commercial Assays** |        |            |
| MACS Neutrophil Isolation Kit, mouse | Miltenyi Biotec | Cat#130-097-658 |
| Lympholyte-Poly | Cedarlane | Cat#CL5070 |
| **Deposited Data** |        |            |
| Fiji Macro for measuring distance of cells to a an helminth parasite | This paper | 10.5281/zenodo.3596520 |
| Nb_DNase_II | This paper | GenBank: M938457 |
| **Experimental Models: Organisms/Strains** |        |            |
| Nippostrongylus brasiliensis | Monash University - original strain Laboratory of Lindsay Dent | N/A |
| Necator americanus | JICU | N/A |
| Mouse: C57BL/6 | Charles River | N/A |
| Mouse: C57BL/6 | Monash University | N/A |
| Mouse: C57BL/6 | EPFL | N/A |
| Mouse: PAD4-KO (C57BL/6 background) | The Francis Crick Institute | N/A |
| Candida albicans | SC5314 clinical isolate (The Francis Crick Institute) | N/A |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. Nicola Harris (nicola.harris@monash.edu).

Requests for the plasmid encoding the recombinant Nb-DNase II generated in this study should be made to Prof. Nicola Harris and Prof. Alex Loukas (alex.loukas@jcu.edu.au). Note that it will be made available on request but we may require a completed Materials Transfer Agreement if there is potential for commercial application.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
C57BL/6J (WT) mice were obtained from Charles River Laboratories. All mice were maintained under specific pathogen-free (SPF) conditions at École Polytechnique Fédérale de Lausanne (EPFL), Switzerland or by the Monash Intensive Care Unit Facility at Monash University, AMREP campus Melbourne, Australia or by the Biomedical Research Unit, Malaghan Institute of Medical Research, Wellington, New Zealand. PAD4-KO mice were maintained at the Francis Crick institute. All mice were age and sex-matched and used between 6-14 weeks of age. Littermates of the same sex were randomly assigned to experimental groups. Mice were maintained at 3-5 animal per cage and ad libidum access to water and food. All animal experiments were approved by the Service de la consommation et des affaires vétérinaires (1066 Epalinges, Canton of Vaud, Switzerland) with the authorization number VD3001, or by the AEC committee of the Alfred campus, Melbourne Australia with authorization number E/1846/2018/M or by the Victoria University of Wellington, Wellington, New Zealand.

Preparation and Isolation of *N. brasiliensis* Larvae
Nb was maintained by monthly passage in Lewis rats. Infective larvae (L3) were prepared from 2 week old rat fecal cultures as previously described (Camberis et al., 2003). Prior to infection, larvae were washed in a mix of (Penicillin/Streptomycin 1000 U/mL (GIBCO), Gentamicin 300 U/mL (Sigma) in PBS), for 30 min and then rinsed in sterile PBS. 250 L3 were delivered by id. injection in 10 μL of sterile PBS to the belly skin. Of note, because of the low volume for infection, the dose of infection is more variable what is classically expected for this parasite. For injection into the belly skin, mice were prepared one day earlier by removing a small area of hair from the injection site using tweezers.

Isolation of Neutrophils or Monocytes

**Mouse**
Neutrophils and monocytes were isolated from the mouse bone marrow of naive mice using negative selection MACS separation kits.
**Human**

Neutrophils were isolated from fresh blood of healthy donors (the sex is unknown) using Lympholyte-Poly, as recommended by the manufacturer. Cells were plated at 1 million per well in a 24 well plate and cultured 10% FBS, RPMI at 37°C, 5% CO2. Purity was assessed by cytopsin followed by a Diff-quick stain.

For experiments that took place in EPFL (Switzerland), the samples were provided via the blood center of the Transfusion Interregionale CRS (Epalinges, Switzerland) in accordance with the Cantonal Ethics committee of the Canton of Vaud (Vaud Switzerland). Written consent from the donors was obtained by the Lausanne blood transfusion center, the donors agreed that after absolute anonymity that their blood be used for medical research purposes. For experiments that took place in Monash University (Australia), blood was taking by an authorized person in the department from anonymous donors in agreement with the application CF07/0141-2007/0025, approved by Monash University (Medicine and Dentistry Human Ethics Sub-Committee). Written consent from the donors was obtained prior to drawing blood, and the donors agreed that after absolute anonymity that their blood be used for medical research purposes.

**METHOD DETAILS**

**In Vitro Co-culture of Neutrophils or Monocytes and Larvae**

Primary cells were cultured at 1 million per well in a 24 well plate in presence of 100 antibiotic-treated Nb or Necator L3 in complete RPMI. Cellular adherence to the larvae was assessed 24 h later using an inverted bright-field microscope. To measure viability, Sytox Green was added at 1:100 dilution 15 min prior to imaging on a fluorescent inverted microscope.

For chemotaxis assays, neutrophils were placed on the upper chamber formed by a 4 μm pore transwell (Ibidi), while larvae were placed in the lower chamber. The number of neutrophils that migrated from the upper to the lower chamber was assessed after 24 h using an inverted brightfield microscope.

**L3 NES Preparation**

For the generation of Nb excretory/secretory (NES) products, L3 were washed extensively in sterile PBS supplemented with penicillin and streptomycin, incubated for a further 1 h in RPMI (GIBCO) supplemented with penicillin and streptomycin then cultured at 37°C, 5% CO2 in RPMI containing antibiotics (penicillin, streptomycin, gentamicin and tetracycline; Sigma–Aldrich) and 1% glucose (Sigma–Aldrich). The supernatant was collected every 2 days for a period of 2 weeks and was subjected to sterile filtration then concentrated by centrifugation through a 10,000 MWCO cellulose membrane (Centriprep; Millipore). Contaminating LPS was removed using an EndoTrap Blue LPS-binding affinity column (Hyglos GmbH, Germany). The concentration of residual endotoxin was determined using the Limulus Assay (Lonza), and only those batches found to contain less than 1 U LPS per 1 μg protein were used for experiments.

**In Vivo Treatment**

Where indicated anti-Gr1 mAb (RB6-8C5, 0.250mg) or anti-Ly6G mAb (1A8, 0.5 mg) were administered intraperitoneally at day –1, 0 and 1 of infection. Cellular depletion efficiency was evaluated by flow cytometry in the blood and by imaging of the infected skin.

For imaging granulocyte recruitment, anti-Gr1 mAb (RB6-8C5, 10 μg), anti-Ly6G a mAb (1A8, 5 μg) or anti-LyGC mAb (AL-21, 5 μg) were injected intravenously 1 h before infection.

For experiments employing DNase I, mice were treated with 1000 U recombinant DNase I (Roche) intraperitoneally every 4 h for a total of 12 h starting at the time of infection.

For experiments using NES, mice were injected via the intradermal route with 10 μL of 100 μg LPS-depleted NES together with Nb L3.

**Dye Labeling of Live Parasites**

For external labeling of the larval sheath L3 were washed several times in PBS then incubated at room temperature for 8 min in 2.5 mM carboxyl fluorescein succinimidyl ester (CFSE, Sigma-Aldrich, Ex. 492 nm /Em. 517 nm), and washed in PBS three times prior to use for infection. For internal larval labeling, L3 were sterilized by washing in antibiotics and then fed with Fluorosphrite YO carboxylate microsphere (Polysciences, Inc, Ex. 529nm/ Em. 546 nm) beads for 4 h. Larvae were then washed in PBS containing 0.05% tween prior to use for infection. The efficiency of staining and the viability of larvae was verified by microscopy prior to infection.

**Ex Vivo Imaging of Infected Skin by Laser Scanning Confocal Microscopy**

Fluorescence imaging of neutrophils, monocytes and/or NETs was performed using imaging by immunofluorescence microscopy of skin flaps immediately following their removal from sacrificed animals. At the time of necropsy, a skin flap of the site of injection was removed and mounted in complete Fluorobrite on a slide with a 1 mm thick holding chamber. The subcutaneous tissue facing upward was covered with a coverslip and place to image upside down on a heating pad. Images were acquired on Zeiss LSM700 laser scanning confocal microscope mounted in an inverted microscope equipped with 20 x objective, 1.2 N.A. using regular photomultiplier tubes (PMTs), 1-a.u. pinhole. Each image was acquired using the indicated fluorescent channels and the same acquisition settings across different samples.
To control for differences in background fluorescence between experiments and antibody/dye batches, the contrast was adjusted to minimize autofluorescence and a minimum brightness threshold was set such that only positive staining could be visualized. The same contrast and threshold values were applied to all images taken across all treatment groups within a single experiment using Fiji (Schindelin et al., 2012).

For the quantitative analysis of myeloid cell recruitment around larvae, including a measure of their distance from the larvae, a custom-made macro was generated as described in Figure S2. Briefly, in Fiji, the ‘Region of Interest’ was defined as the outline of the worm. A distance map was defined and binarized signal for each fluorescent dye used to measure the percentage of area of stain depending of the distance to the larvae.

Extracellular DNA was labeled by iv. injection of 100 μL of Sytox Green (Ex. 504 nm/ Em. 523 nm) or Sytox blue (Ex. 444 nm/ Em. 480 nm) DNA dye (50 μM). Monocyte and neutrophil recruitment were visualized by iv. injection of AlexaFluor 647-anti-mouse Gr1 antibody (Clone RB6-8C5), AlexaFluor 647-anti-mouse Ly6G antibody (Clone 1A8) and BV421-anti-mouse Ly6C antibody (Clone AL-21). All antibodies and dyes were injected intravenously 1 h prior to infection. For imaging of myeloid cell recruitment following the topical application of larvae, L3 were applied to untreated skin at 40 min intervals for a total of 12 applications. Infected skin was removed and imaged 1 h following the last larval application, and washed extensively with water to remove any remaining extracellular DNA.

Whole Mount Imaging of Infected Skin by Confocal Microscopy

Whole mount imaging was performed at one cm² skin area using the indicated fluorescent channels and the same acquisition setting employed across different samples. Images were analyzed using Fiji (Schindelin et al., 2012). The same contrast and threshold values were applied to all images from all treatment groups within a given experiment. For quantification of the area of positive signal relative to the distance to the larvae, a custom-made macro was used as described in the previous section.

In Vitro NETosis Imaging and Analysis

Human neutrophils were isolated using a lympholyte Poly gradient separation (Cedarlane). 50,000 cells were co-cultured with 100 L3 antibiotic treated Nb or Necator larvae in complete RPMI containing 10% serum and 1% penicillin/streptomycin/tetracyclin in a 8 well μ-Slide (Ibidi®). Cells were incubated at 37°C, 5% CO₂ for 1 hr prior to imaging. Just before imaging 1 μL/well of sytox Blue or Green (5mM) was added to each well. Images were acquired on Zeiss LSM700 laser scanning confocal microscope equipped with 20 x objective, 1.2 N.A. using regular PMTs, 1-a.u. pinhole or Nikon A1r laser scanning confocal microscope mounted on an inverted microscope equipped with 20 x objective, 1.2 NA using regular PMTs, 1-a.u. pinhole. Each image was acquired using the indicated fluorescent channels and the same acquisition setting across different samples. Images were analyzed using Fiji (Schindelin et al., 2012). The same contrast and threshold values were applied to all images from all treatment groups within the experiment. For quantification of the area of positive signal relative to the distance to the larvae, a custom-made macro was used as described in the previous section.

NET Formation and Lifetime Assays

Neutrophils were isolated from human peripheral blood over Histopaque-/Percoll- gradient as described elsewhere (Aga et al., 2002). 2 x 10⁶ neutrophils per well were plated in an 8 well μ-Slide (Ibidi®) in HBSS +Ca/Mg +10mM HEPES +3% plasma. Cells were stimulated with an MOI of 0.1 of C. albicans (SC5314 clinical isolate) pre-formed hyphae in the presence of 0.1 μM SYTOX Green (Invitrogen, S7020) and in the absence or presence of 1, 10 or 100 μg/mL of NES. Timelapse images were obtained every 10 min.
for 14 h using a LEICA DMIRB microscope (20x objective) and analyzed using Fiji/ImageJ software. NET formation was counted dynamically over 14 h and NET lifetime was measured as the elapsed time until NET Sytox signal disappeared (events per 1500 cells per condition, 24 events per condition analyzed for NET lifetime).

To assess the potential of Nb-DNase II to degrade NETs, 28 000 neutrophils per well were plated in a 96 well plate in RPMI+10% serum in presence of 100 uM PMA and 100 ug of NES of DNase II for 3 h. Sytox Green was added at 1:1000 dilution prior to imaging by confocal imaging as described for the ex vivo skin samples.

NES Endonuclease Activity Assay

1 µg of pMAL-c2X plasmid (size: 6700bp, Addgene, (Walker et al., 2010)) was incubated in the absence or presence of 1, 10 or 100 µg/mL NES or 1 U/mL of DNase I (D5025, SIGMA) and in the absence or presence of 40 µg/mL of G-actin (A2522, SIGMA) in HBSS +Ca/Mg +10 mM HEPES. After 15 or 45 min of incubation at 37°C, the samples were deactivated at 75°C for 5 min and 300 ng of DNA were analyzed via agarose electrophoresis.

Monocyte Viability Assay

Human peripheral blood monocytes were isolated over a Histopaque 1119 gradient (Sigma-Aldrich) and the CD14-positive monocytes isolated using MACS CD14 micro beads (Miltenyi Biotec) according to manufacturer’s instructions. The CD14-positive monocytes were plated at 5 x 10⁴ per well on an 8 well µ-Plate (ibidi®) and neutrophils were plated at 2 x 10⁵ per well on a 24 well plate in HBSS +Ca/Mg +10 mM HEPES +10% FCS. Neutrophils were then incubated in the absence or presence of NES (30 µg/mL) and in the absence or presence or PMA (50 nM). After 5 h of incubation, the medium was transferred from the neutrophils onto the monocytes (after removal of the monocyte medium). Monocyte viability was quantitated 18 h later using 140-250 cells per condition with SYTOX green staining (0.1 µM). Values were normalized against the viability of monocytes that had not received a medium change.

Protein Selection, RNA Extraction and cDNA Synthesis

For the screening of proteins secreted by N. brasiliensis L3 larvae (NbL3) with DNase activity, a Pfam analysis was performed on the NbL3 excretory/secretory products described previously (Sotillo et al., 2014) using HMMERv3.2 (Finn et al., 2011) to identify proteins containing a DNase domain.

Total RNA from N. brasiliensis was extracted using TRI reagent (Sigma-Aldrich, USA) as per manufacturer’s instructions. Briefly, N. brasiliensis adult worms were homogenized in 500 µL of TRI reagent for 2 min on ice using a pellet pestle followed by addition of 500 µL of TRI reagent and incubated at RT for 5 min. Then, 0.2 mL of chloroform (Sigma-Aldrich, USA) was added, shaken vigorously for 15 s, incubated for 5 min at RT and pelleted at 12,000 g for 15 min at 4°C. The aqueous phase was transferred to a fresh clean tube and 0.5 mL of isopropyl alcohol (Sigma-Aldrich, USA) and 3 µL of Glycoblue (Thermo Fisher Scientific, USA) was added. The mixture was mixed gently by inverting the sample 5 times, incubated for 5 min at RT and centrifuged at 12,000 g for 10 min at 4°C. The RNA pellet was washed with 1 mL of 75% ethanol in RNase-free water (Sigma-Aldrich, USA) and the pellet was air-dried. The RNA pellet was finally resuspended in 12 µL of RNase-free water and kept at ~80°C until use.

First strand cDNA was synthesized using reverse transcriptase as follows. One (1) µl of oligo (dT) primers (500 µg/mL) (Life Technologies, USA), 1 µl NTP mix (10 mM) (Bioline, UK) and 11 µl of RNA were mixed and incubated at 65°C for 5 min in a 96-well thermal cycler (Applied Biosystems, USA) and on ice for 2 min. Then, 4 µl (5x) of first strand buffer (Invitrogen, USA), 1 µl 0.1 M dithiothreitol (Invitrogen, USA), 0.5 µl RNaseOUT (40 units/µl) (Invitrogen, USA), 0.25 µl (200 units) of SuperScript III reverse transcriptase (Invitrogen, USA) and 0.75 µl water was added. Finally, the solution was incubated for 1 h at 55°C, then 15 min at 70°C in a thermal cycler and frozen at ~20°C until use.

Gene Cloning

Cloning of N. brasiliensis cDNA Encoding for Nb-DNase II

The cDNA sequence encoding for the protein of interest (m.13872) was obtained from previous studies (Sotillo et al., 2014). The presence of a signal peptide was predicted using the online software SignalP v4.0 (http://www.cbs.dtu.dk/services/SignalP/) and sequence without the signal peptide (amino acid residues 22–378) was amplified by PCR using the presence of a signal peptide was predicted using the online software SignalP v4.0 (http://www.cbs.dtu.dk/services/SignalP/).

The PCR reaction was performed as follows: 1 µl (50 ng) adult worm cDNA, 3 µl (10 µM) each of forward primer and reverse primer, 10 µl MyTaq red reaction buffer (Bioline, UK), 32.5 µl water and 0.5 µl MyTaq DNA polymerase (Bioline, UK). PCR conditions were 35 cycles of denaturation at 95°C for 15 sec, annealing at 50°C for 15 sec, extension at 72°C for 45 sec, and final extension at 72°C for 7 minutes. PCR products were subsequently digested with Ndel and Xhol (Biolabs, USA) to clone in frame into pET-41a (Novagen, USA). Recombinant plasmid was transformed into E. coli BL21 (DE3), plated onto Luria Bertani agar plates supplemented with 50 µg/mL kanamycin (LBkan) and incubated overnight at 37°C. The recombinant clones were PCR amplified using T7 and reverse specific primers for confirmation of insertion of sequence.

Protein Expression

Expression of recombinant protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM and cultured in LB supplemented with kanamycin for 24 h. The recombinant protein was located in inclusion bodies and purified as follows. Triton X-100 was added to a final concentration of 3% after sonication, the mixture incubated for 1 h at 4°C with gentle shaking and then
pelleted at 20,000 g for 20 min at 4°C. The supernatant was removed, the pellet washed twice with 30 mL of lysis buffer (with centrifugation at 20,000 g for 20 min at 4°C after each wash) and the final pellet resuspended in 20 mL of solubilization buffer (50 mM sodium phosphate, 40 mM imidazole, 300 mM NaCl and 8 M urea). The resuspension was incubated at 4°C overnight with gentle shaking, centrifuged at 20,000 g for 20 min at 4°C and the supernatant decanted and stored at −80°C.

Recombinant proteins were purified by immunoaffinity chromatography (IMAC) using 1 mL His-Trap Ni²⁺ columns using an AKTA Prime UPC FPLC system (GE Healthcare, USA) and eluted with an increasing linear gradient of imidazole (100-500 mM). Fractions containing purified recombinant proteins were combined and buffer exchanged into 1x PBS containing 300 mM NaCl and 8 M urea using a 3 kDa MWCO Amicon Ultra-15 centrifugal unit. The identity of expressed proteins was confirmed by SDS-PAGE and western blot using anti-His monoclonal antibodies.

**Polyclonal Antibody Production**

Male BALB/c mice were purchased from the Animal Resource Center, Perth, Australia and maintained at the AITHM animal facilities on the James Cook University, Cairns campus. Mice were kept in cages under controlled temperature and light with free access to pelleted food and water. All experimental procedures performed on animals in this study were approved by the James Cook University (JCU) animal ethics committee (A2433). All experiments were performed in accordance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act.

Three male BALB/c mice (6 weeks old) were immunized intraperitoneally with 50 μg of recombinant protein emulsified with 50 μl Alum adjuvant (Thermo Fisher Scientific, USA) and boosted twice at two weekly intervals using same amount of protein/adjuvant. Blood was collected at necropsy by cardiac puncture, allowed to clot and then serum was removed by centrifugation at 10,000 g for 10 min and kept at −20°C until use.

**Homolog Identification and Phylogram Analysis**

BlastP was used to identify protein sequences presenting high similarity to Nb-DNase II. Top-scoring hits with alignments covering at least 95% of the Nb proteins were considered for further analysis. A multiple sequence alignment was carried out using the alignment program MUSCLE using default parameters (Edgar, 2004). PhyML, a phylogeny software (Guindon et al., 2010), was used for a maximum-likelihood (ML) phylogenetic analyses of aminoacid sequences using approximate likelihood ratio test [aLRT] and Shimodaira–Hasegawa [SH]-aLRT for a fast-approximate likelihood-based measure of branch support and Nearest-neighbor interchanges (NNI) for tree improvement. The tree was finally visualized with The Interactive Tree of Life (iTOL) online phylogeny tool (https://itol.embl.de/, Letunic and Bork, 2019). For identification of putative Nb-DNase II homolog in Na, all sequences annotated in the genome (Tang et al., 2014) with a DNase motif were aligned to Nb-DNase II with Clustal Omega. The Multiple sequence alignments were visualized using the JalView 2 Desktop application (http://bioinformatics.oxfordjournals.org/content/25/9/1189). Finally, sequence features of interest were manually highlighted in the alignments.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The choice of statistical tests was based on sample size and on Bartlett’s test when normal distributions of the errors were expected. Data from separate experiments were pooled when possible. Statistical parameters including the exact value of n, the definition of center, dispersion and precision measures (mean ± SEM) and statistical significance are reported in the Figures and Figure Legends.

Representation and data analysis were performed GraphPad Prism 8. Statistically significant values are indicated as follows: NS, p > 0.05; *, p < 0.05; **, p < 0.01; ***,p < 0.001. Spearman’s rank correlation statistical analysis was performed using Minitab 17 Statistical Software (2010). Of note, when multiple comparisons were performed in post hoc test, the comparisons reported on the figure are the only one that had been tested.

**DATA AND CODE AVAILABILITY**

The protein sequence of the DNase II from *Nippostrongylus brasiliensis* identified in this work is available in Data S1 and has been deposited in GenBank, accession code MN938457.

The macro designed to quantify the distance of neutrophils and monocytes to the larvae is detailed in concept in Figure S2 and has been available through Zenodo, https://doi.org/10.5281/zenodo.3596520.

All other original/source data for this paper are available from the corresponding author on request.