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The Role of Endocannabinoids in Host-Pathogen Interactions

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Chapter one, includes a reprint of material from “The Two Faces of Nematode Infection: Virulence and Immunomodulatory Molecules From Nematode Parasites of Mammals, Insects, and Plants” in Frontiers in Microbiology, 2020. Sarah Bobardt and Drs. Meera Nair and Adler Dillman wrote that article.

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Dedication

For Mom, Dad, Mikey, Evan, Dave, and Nikki, for your unending support and love.

Abstract
The role of endocannabinoids in regulating host-parasitic interactions, particularly in the lungs, has previously not been well-characterized. The murine-infecting model for human hookworm, *N. brasiliensis*, has a spike in endocannabinoid production during the infectious larval stage, indicating the potential role for these lipid-derived molecules in staging an infection. The rates of endocannabinoid production decreases when the nematode transitions to the mandatory lung-infectious stage. At the same time, endocannabinoids have been shown to have an ameliorating effect on the immune system in the gut of mammals. In this project, we use mice lacking the genes for the mammalian endocannabinoid receptors, Cnr1 (CB1KO) and Cnr2 (CB2KO), in order to isolate the role of endocannabinoid signaling in the lung immune response.

The first chapter focuses on a survey of the small excreted/ secreted molecules that are involved in **host and parasite** interactions. The second chapter focuses on the **immune response to the pathogenic nematode**, in which macrophages and eosinophils are co-cultured with live nematode larvae. We found that macrophages isolated from the *N. brasiliensis*-infected lungs of CB1KO mice, but not CB2KO mice, had increased Relmα expression, indicating an increase in macrophage alternative activation. Both lung macrophages and eosinophils from CB1KO mice exhibited excessive binding to *N. brasiliensis* larvae in co-culture. This indicates that endocannabinoid signaling is important for regulating the immune response to the nematode in the lung. This chapter informs the exploration of **host-nematode interactions *in vivo*,** in which CB1KO mice produced more eosinophils in response to a Nippo infection. The third chapter of this

project employs a bioinformatics approach to explore the **effect of the host immune system on the gene expression of the nematode.** The fourth chapter summarizes these findings.

This project implicates a role for the endocannabinoid system in regulating immune cell-helminth interactions in the lungs and is the premise for the exploration of endocannabinoids as a target to regulate the immune response to pulmonary helminth infection.

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Chapter One: Modeling Hookworm Infection in a Laboratory Setting

Epidemiology of Hookworm Infection

Approximately 500 million people worldwide are infected with hookworm, and while the parasite is not usually deadly, the effects of helminthiasis can still be severe [1]. Hookworm infection is often called a disease of poverty, because it is generally found in low- and middle-income countries, where it is a leading cause of anemia in pregnant women [2]. Anemia can have an impact on an individual's ability to work or attend school, further hindering people from obtaining upward social mobility. In a study with the model of hookworm, *N. brasiliensis*, infected mice showed impaired cognitive function [3]. Current anthelminthics kill adult nematodes in the gut, usually after they have had a chance to establish an infection [1]. This project seeks to better understand the host-pathogen interactions that occur during helminth infection, in order to identify novel targets for anthelminthics.

Recent research has identified an enzyme pathway for the digestion of heme in both human and murine parasites [4]. The genes APR and GST allow the nematode to breakdown and digest heme molecules. Na-APR-1 (the version of the enzyme produced by the human hookworm *N. americanus*) is currently being explored for efficacy as a vaccine against hookworm infection, highlighting the importance of identifying novel biologically relevant molecules [5]. We discuss other promising molecules in a later section of this introduction.

N. brasiliensis (*Nb*) is a useful model for hookworm infection because it produces a strong type 2 (Th2) immune response, similar to that of *A. ceylanicum* and *N. americanus*, which infect humans [6]. However the pulmonary immune response has not been well-characterized, in part because the nematode is only in the lung of the host for a short period of time [1]. However recent research on controlled infections have demonstrated an expansion of type-2 conventional dendritic cells in the lungs of humans infected with the helminth *S. mansoni* [7].

In addition, *Nb* is relatively easy to maintain in a laboratory setting in rats, which is its natural host [8]. The lifecycle of the nematode is short, with an infection resulting the characteristic lung eosinophilia within a matter of days. *Nb* can readily infect mice, allowing for further exploration of the interaction between host immune response and parasite through a variety of techniques, such as bronchoalveolar lavage (BAL) [9, 10]. *Nb* continues to be used as an important model of helminth infection, and the latest techniques include genetic manipulation through lentiviral transduction and sequencing of nucleic acids from secreted extracellular vesicles, greatly expanding the repertoire for research into this important nematode [11, 12].

Immune response to helminths

N. brasiliensis is a skin-penetrating nematode that briefly promotes neutrophilia in the blood and arrival of inflammatory monocytes within the first 24 hours post-infection [13]. Rodents and humans produce a type 2 immune response to helminth infections in the lungs and intestines, which is characterized by an increase in eosinophils and

alternatively activated macrophages [14]. Eosinophils produce IL-4, which further promotes a type 2 immune response. Alternatively activated macrophages secrete Relmα, which promotes wound repair over worm killing [15].

Nb infection in the gut results in tuft cell release of IL-25 promoting an increase in Th2 cytokines, which leads to an increase in mucins produced by goblet cells, resulting in nematode expulsion [16]. Rodents are generally immune to subsequent infections with *Nb* through lung-resident CD4+ T cells [17]. T_h2 cells promote the expansion of eosinophils and alternatively activated macrophages, which are able to rapidly neutralize secondary infections [14].

Role of secreted/ excreted molecules in host-pathogen interactions

Author's Note: This section was adapted from "The Two Faces of Nematode Infection: Virulence and Immunomodulatory Molecules From Nematode Parasites of Mammals, Insects and Plants" which was previously published in *Frontiers in Microbiology* [18].

While the mammalian immune system has adapted effective mechanisms for neutralizing, killing, and expelling parasites, helminths have also adapted novel strategies to increase damage to host tissue and evade attack. These immune-evasion molecules could be harnessed to capture the potential benefits of helminth infection, such as the restoration of gluten tolerance, without an actual infection [19]. At the same time, these molecules are important targets for the development of vaccines, such as the previously discussed blood-feeding enzyme APR and also anthelminthics, such as endocannabinoids. Below we discuss some of the ES molecules with the most promise for each of these aims.

Virulence Factors

Parasitic nematodes rely on host resources in order to establish a long-lasting niche and complete their lifecycle [20]. To this end, they can produce a wide variety of molecules to assist in their ability to attack, invade, and digest host tissue [21]. Their arsenal of host virulence molecules can be targeted to develop vaccines or design anthelminthics to reduce worm burden and mitigate host pathology in infected individuals. Significant research on anthelminthics and vaccines is performed in preclinical rodent models to find potential targets for vaccine development prior to the initiation of human research and clinical trials (Figure 1). Here, we highlight promising nematode-derived molecules for anthelminthic and vaccine targets. These include enzymes involved in host tissue invasion and parasite feeding, nematode-derived molecules necessary for parasite development, and immunogenic ES proteins as vaccine candidates.

Nematode-Derived Enzymes

Nematodes produce a variety of enzymes for host tissue digestion and feeding that provide useful targets for vaccine development or anthelminthics. For example, enolases are plasminogen-binding surface proteins that are involved in assisting parasites in invading host tissue by promoting the degradation of fibrin [22, 23]. Vaccination with a Trichinella spiralis enolase resulted in the production of specific antibodies in mice, however, this did not lead to a striking reduction in worm burden [24]. Additionally, parasite-derived enolases were found in the serum of *Brugia malayi*-infected individuals, indicating its potential as a diagnostic molecule [25]. For hookworms, enzymes involved

in processing blood hemoglobin have demonstrated promising vaccine potential [21, 26]. Specifically, the *N. americanus* hemoglobinase aspartic protease (Na-APR-1) and the heme detoxifying Na-Glutathione S-transferase (Na-GST-1), are currently in clinical trials for a hookworm vaccine, with early results showing the vaccine is safe and immunogenic [5, 27, 28]. Several phase 1 clinical trials have been completed with recombinant Na-APR-1 vaccine. Administration of the recombinant Na-GST-1 vaccine to hookworm-naïve individuals as well as those from hookworm endemic regions in Brazil was found to be safe and immunogenic. The success of these trials suggest that safe and effective vaccines could be developed by targeting enzymes integral to nematode feeding and survival. For example, in filarial nematodes, enzymes have provided promising targets for anthelminthic and vaccine development. These include GST, which protects the nematode parasite by neutralizing host cytotoxic products (e.g., reactive oxygen species) and mediating drug resistance, and UDP-glucuronosyltransferase (Bm-UGT), a detoxifying enzyme expressed in the *B. malayi* intestinal lumen that was essential for its survival [29, 30].

Nematode-Derived Molecules Involved in Growth and Metabolism

Lipid-derived molecules are involved in a variety of biological functions in parasitic nematodes including metabolism and development, making them of particular interest as potential novel anthelminthics that target parasite fitness and development [31]. Endocannabinoids are lipid-derived molecules important for metabolic homeostasis and immunity, among other functions, and are produced by both mammals and nematodes

[32]. The specific function of parasitic nematode-derived endocannabinoids in the host is unclear. However, functional studies for endocannabinoids have been possible in the free-living nematode *C. elegans*, where endocannabinoids, 2-arachidonoyl glycerol (2-AG) and anandamide (AEA) played a significant role in metabolism and aversion to pain [33, 34]. Understanding the interplay between host and parasitic nematode-derived endocannabinoids might reveal new immune and metabolic targets to reduce parasite fitness or improve the host response. The steroidal hormone dafachronic acid (DA) modulates nematode lipid metabolism and development, and the ligand-binding domain for the steroid hormone nuclear receptor for DA (DAF-12) is highly conserved among nematode species, such as *C. elegans*, *N. brasiliensis*, *Haemonchus contortus*, and *Strongyloides stercoralis*, therefore targeting these receptors may have therapeutic potential to impair parasite fitness [31, 35-38]. In *C. elegans*, the DAF-12 system acts to inhibit dauer formation, and in *H. contortus*, DA promoted transition from free-living to parasitism by modulating dauer-like signaling [31, 35, 37]. Recent investigation of the DAF-12 system in *S. stercoralis* hyperinfection supports the therapeutic potential of inducing this steroid hormone pathway. In a mouse model of *S. stercoralis* hyperinfection, which is an often fatal condition in immunocompromised individuals, DA treatment was protective and reduced *S. stercoralis* parasite burdens by suppressing the development of auto-infective L3a larvae [36]. A later chapter of this dissertation focuses on the role of endocannabinoids in the host immune response to nematode infection in the lung.

Immunogenic Nematode ES Proteins

Molecules integral to the parasite's growth and ability to colonize and feed on the host offer promising vaccine and anthelminthic targets. However other weapons of warfare employed by the worms, such as excreted molecules that are immunogenic and promote a protective anti-helminthic immune response can also be considered as vaccines or adjuvants. The family of venom allergen-like proteins (VAL) family is one such example. This family has been extensively studied especially given their high expression in many parasitic nematodes [39]. While these proteins are conserved among a wide variety of nematodes, their functions are diverse—including examples of both pro-inflammatory and immunosuppressive molecules. Here we explore some of the most pertinent examples from this family, focusing on the VAL proteins that have demonstrated immunogenic properties for vaccine potential. VAL proteins are homologs of vespid (wasp) venom proteins, the latter of which are locally toxic and can induce allergic and inflammatory responses in humans [40-42]. This makes nematode-derived homologs of these proteins of particular interest in understanding host-nematode interactions that lead to the excessive pathology for the host [39-42]. VAL proteins are conserved in several parasitic nematodes, including *Heligmosomoides polygyrus*, *B. malayi*, *Trichinella pseudospiralis*, and *Teladorsagia circumcincta* [39, 43-46]. Notably, VALs are highly expressed: a study of the secreted products from the gastrointestinal murine parasite *H. polygyrus* revealed that members of the VAL protein family were the most abundant product [47]. Due to their abundance and conserved structure, VALs have been considered as vaccine candidates. For instance, the *B. malayi* protein BmVAL-1 is highly

immunogenic, promoting antibody and T cell responses in humans, and conferring protection in vaccination models in mice and jirds [46, 48, 49]. To target larval stages, Bm-VAL-1 and *B. malayi* abundant larval transcript2 (Bm-ALT-2) were combined in a multivalent vaccine, which successfully increased antibody titers and provided enhanced worm killing in a challenge infection in mice [49, 50]. The importance of combining antigens as a vaccine strategy for a more effective immune response is increasingly being recognized. In *H. polygyrus* infection, immunization with a cocktail of three *H. polygyrus* VALs induced antibody production that protected mice from challenge *H. polygyrus* infection [51]. The biological function of VALs in infection is unclear, however, given that they are secreted sterol-binding proteins, they may bind immunomodulatory molecules such as prostaglandins and leukotrienes, which have both immune stimulatory and regulatory roles [52]. The immunogenic properties of VALs have made them potential targets for vaccine development. Na-ASP-2 (Ancylostoma-secreted protein), another member of the VAL family, initially showed promise as a hookworm vaccine [53, 54]. This allergen-like protein was associated with the production of IgE and IgG4 antibody responses that correlate with reduced risk of high *Necator americanus* burdens in endemically affected areas. Further, validation studies in dogs confirmed that Na-ASP-2 specific antibodies were protective in challenge infections [53]. However, early clinical trials resulted in generalized urticarial reactions in many individuals, associated with pre-existing Na-ASP-2-specific IgE [54]. This failed clinical trial is a cautionary tale for vaccine development against parasitic nematodes. First, the potential for non-protective allergic-immune responses in previously exposed individuals in endemic areas needs to

be considered. Additionally, anti-inflammatory nematode-derived molecules that are necessary to mitigate host tissue damage and inflammation may need to be carefully considered before being used as vaccine or therapeutic targets, since inhibiting these may be more pathogenic than beneficial for the host. Although the effectiveness of VALs as vaccine targets for helminths is challenged by these recent studies, their immunogenic potential may be harnessed for use as adjuvants against other infectious pathogens. For example, recombinant ASP-1 derived from the filarial nematode *Onchocerca volvulus*, Ov-ASP-1, has shown promise as an adjuvant for vaccines against viral infections, such as HIV, SARS-CoV, and influenza, augmenting viral antigen-specific antibody titers in immunization studies in mice [55].

Serine protease inhibitors (serpins) constitute another highly conserved family of nematode ES proteins, identified in many nematodes, including *B. malayi*, *Anisakis simplex*, and *H. contortus* [56-59]. In vitro studies of a serpin derived from *H. contortus* showed that it reduced blood coagulation [58]. The anti-coagulation function of serpins is likely an important feeding mechanism for blood-feeding nematodes. *B. malayi* microfilariae secrete serpins, perhaps to mitigate a coagulation response to excess circulating microfilariae in the bloodstream during chronic infection [56]. *B. malayi* serpins are also immunogenic and stimulate mouse and human B and T cell responses, however, this immune response is short-lived, suggesting that serpins alone are not effective vaccine candidates for long-term immunity [56]. It is interesting to note that despite many preclinical studies on nematode-secreted proteins, only nematode enzymes are currently the subject of ongoing vaccine clinical trials. This suggests that targeting

virulence factors, which are integral components of the worm's physiology may offer the most promising vaccination targets.

Immunomodulatory Molecules

Parasitic nematodes have evolved multiple mechanisms to evade the host immune system, allowing persistence in their host, in some cases for decades, without being killed [60]. Nematode-derived products are key to the immunomodulatory capabilities of the parasites, and investigating their mechanism of action may identify novel therapies for allergic and inflammatory diseases. Reviewing current research into which molecules show the most promise for the development of immunotherapies is an ongoing conversation that has already received a significant amount of attention [60-65]. Here we contribute to that conversation by contextualizing the most current advances in our understanding of immunomodulatory capabilities of nematodes and identifying the molecules that appear to show the most promise for further research. Identifying the specific molecules within parasitic nematode ES or extract that have the strongest immunomodulatory potential is a main focus in the field of "helminth therapeutics" [64]. Nematode-derived immunomodulatory molecules include mimics of host immune mediators as well as novel molecules that are unique to parasites themselves. Many of the products with extensive characterization are proteins. However non-proteins products, such as carbohydrates and small RNAs, are currently being studied for their potential role in immunomodulation [60, 66, 67]. Mechanisms of immunomodulation for the main nematode-derived molecules discussed here are summarized in Figure 1. Glycans The differential glycosylation of lipids and proteins during the lifecycle of parasitic

nematodes provides a unique opportunity for the development of vaccines and novel anthelminthics [66, 67]. Glycosylation patterns unique to the parasite are potential vaccine targets, because they are distinct from host glycosylation patterns and potentially more immunogenic, acting as pathogen-associated molecular patterns. On the other hand, glycosylation patterns that mimic the host can be explored for their immunomodulatory potential, providing novel immunotherapies. Gala1–3GalNAc-R (α-Gal), a parasite specific glycan epitope produced by the sheep pathogen *H. contortus* induced an IgG response in lambs and is implicated in protection against *H. contortus* challenge infections [68]. Similarly, glycosylation patterns are essential for the host to recognize the glycans on the surfaces of mucin-like proteins expressed by *T. canis*, and led to pro-inflammatory cytokine expression by human THP-1 macrophages [69]. In the anaphylactic reaction known as α-Gal Syndrome (AGS), humans produce IgE in response to α-Gal present in red meat. However, humans infected with *T. canis* had reduced IgE antibodies to α-Gal caps on N-glycans, indicating that the parasites may be able to downregulate the allergic response, even though this is not an epitope that the worms themselves make [70]. The ability of the parasite to suppress the immune response to oligosaccharides provides evidence for the "hygiene hypothesis" which argues that an increased sensitivity to a wide variety of allergens may result from a reduction in helminth infections in countries with stronger sanitation infrastructure [71]. In another example of immunomodulation, N-glycans produced by the canine heartworm *Dirofilaria immitis* allowed the worm to hide from the host immune system, by imitating host glycosylation patterns and also using unique glycosylation patterns that interfered with

host binding to other nematode-derived molecules, a technique known as glycol-gimmickry [72]. Further, changing the glycosylation patterns on proteins in *H. polygyrus* resulted in an increase in proinflammatory cytokines and a decrease in nematode-specific IgG1 in Balb/c mice [73]. Together these studies show the importance of glycosylation patterns in both immunogenicity and evasion of the host immune system by parasitic nematodes.

Proteins

ES proteins have systemic effects on the immune system, which could be harnessed as therapies for allergy and inflammatory diseases. For example, in vitro treatment of macrophages with *T. spiralis* ES generated a regulatory phenotype that prevented airway allergic inflammation in mice [74]. Similarly, in the dog hookworm *A. caninum*, the secreted anti-inflammatory protein-2 (AIP-2) suppressed airway inflammation in an asthma model in mice in a dendritic cell and Treg-dependent pathway [75]. The *H. polygyrus* ES proteins, *H. polygyrus* Alarmin Release Inhibitor (HpARI) and *H. polygyrus* Binds Alarmin Receptor and Inhibits (HpBARI), were identified due to their ability to downregulate the initiation of both type 2 allergic and parasitic responses through the IL-33-ST2 pathway. HpARI bound to the alarmin IL-33 in necrotic cells and prevented its release, while HpBARI binds IL-33 receptor ST2, preventing IL-33 engagement [76, 77].Intranasal administration of HpARI followed by infection with the skin-penetrating *Nippostrongylus brasiliensis* lead to greater intestinal worm burdens in comparison to untreated infected mice, indicating that a *H. polygryus*-specific product could impair immune responses to a different but related parasitic worm. HpBARI

administration suppressed Th2 inflammatory responses to the extract from the allergenic fungus Alternaria. While *H. polygyrus* is a nematode parasite of mice, and HpBARI targets murine ST2, a homolog of HpBARI (HpBARI_Hom2), was identified that could effectively suppress the human ST2, supporting the clinical relevance of these findings. A similar strategy to inhibit Th2 cytokine responses is employed by *T. muris* with p43, the most abundant protein in its excretome/secretome [78]. *T. muris* p43 contains structural domains homologous to thrombospondin and the IL-13 receptor, which allowed it to tether to matrix proteoglycans and bind and inhibit IL-13. Functionally, p43 inhibits its function in promoting worm expulsion. Another recently identified candidate for immune modulation is the enzyme *H. polygyrus*-derived Hpb glutamate dehydrogenase (GDH), which reduced allergic airway inflammation in mice by inducing a switch from pro-inflammatory to anti-inflammatory eicosanoids (e.g., prostaglandins). Hpb-GDH was effective at suppressing inflammatory pathways in both mouse and human macrophages and granulocytes by inhibiting the 5-lipooxygenase and instead promoting the cyclooxygenase pathway leading to the synthesis of prostanoids and the downregulation of 5-LOX metabolites [79]. Identifying nematode-derived enzymes that target host immune-metabolic pathways with the resulting effect of suppressing inflammatory responses is an exciting new research avenue that may offer novel immunotherapeutics of allergic diseases. Nematode-derived cysteine protease inhibitors (cystatins) also have demonstrated anti-inflammatory functions. Cystatin from the filarial nematode *Acanthocheilonema viteae* downregulated Th2 cytokine responses in an airway allergy model in Balb/c mice, including decreased IL-5 and IL-13 in the broncho-alveolar lavage

[80]. In in vitro microglial cultures from cells harvested from rat brains and stimulated with LPS, *A. viteae* cystatin downregulated nitric oxide and TNFα expression as well as mRNA expression of the pro-inflammatory cytokines iNOS and COX-2, providing promise for therapies for neurodegenerative diseases, such as Parkinson's disease [81].Cystatins from filarial nematode *B. malayi* were also immunosuppressive: treatment with recombinant Bm cystatin was able to reduce dextran sulfate sodium (DSS)- induced colitis in mice [82]. Specifically, Bm cystatin led to increased Tregs in the colon and alternative activation of peritoneal macrophages. Recently, cystatin from the ES products of the zoonotic nematode *T. spiralis*, rTsCstN, was discovered as structurally homologous to human cystatin [83]. Functionally, rTsCstN suppressed inflammatory cytokine production by LPS-treated mouse bone marrow derived macrophages. Cystatins are found in a wide variety of nematode species, including *O. volvulus*, *H. contortus*, and *B. malayi* [84-88] and their potential as immunomodulators is being explored. Cytokines are commonly mimicked by parasitic nematodes in their efforts to modulate the host immune system. One such ortholog is Macrophage Inhibitory Factor (MIF), which is produced by many nematodes including *B. malayi*, *Anisakis simplex*, *Wuchereria bancrofti*, and *H. contortus* [57, 89-91]. Murine MIF is important for alternative activation of macrophages, promotes the Th2 response during nematode infection, and is required for optimal worm clearance [92]. On the other hand, nematode MIF homologs appear to have an immunosuppressive effect [93]. MIF isolated from *A. simplex* increased Treg responses and reduced colitis severity. Here, mice treated with rAsMIF regained previously lost weight and had lower disease activity indices in DSS-induced colitis. Another modulator

of Tregs are TGF-β orthologs which are found in several parasitic nematodes, including *H. polygyrus*, *N. brasiliensis*, and *T. circumcincta* [94]. Hp-TGM was recently identified as a TGF-β mimic produced by *H. polygyrus* [95]. Interestingly, this mimic is structurally distinct from mammalian TGF-β, however, it is able to bind to mouse and human TGF-β receptors and induce Foxp3 expression in Treg cells. Furthermore, Hp-TGM was immunosuppressive in an allogenic skin graft model where it delayed graft rejection. Across the proteomes of parasitic nematodes, there is consistency in the presence of amino acid motifs recognizable by T-cell receptors, known as T-cell-exposed motifs or TCEMs [96]. Using bioinformatics to analyze the proteomes of a wide variety of nematodes, many proteins have been identified with extremely high indices of predicted immunosuppression, indicating that the protein is likely to promote Treg responses. For instance, the hookworm, *A. ceylanicum* alone had over 500 peptides with a highly suppressive index [96]. Given its rapidity and cost-effectiveness, the ability to screen in silico for immunotherapeutic nematode-derived proteins may constitute an important frontier for research in nematode immunomodulation. Some products have an effect on a wide range of immune cells. These include ES-62, a secreted protein from the nematode *A. viteae*, which interacts with B cells, dendritic cells, macrophages, and mast cells to downregulate inflammatory responses [97]. This protein's anti-inflammatory potential is reliant on post-translational modification including the attachment of phosphorylcholines. Current research on characterizing small molecule analogs for ES-62 is an example of the potential for nematode-derived products to be the impetus for the development of immunotherapies [98]. A recent study highlighted nematode-secreted DNases as a novel

mechanism for impairing neutrophil-mediated killing [99]. Within hours post-infection with rat hookworm *N. brasiliensis*, host neutrophils swarmed invading nematodes and released neutrophil extracellular traps (NETs) comprised of nucleic acids, histones, and granular proteins. This provides evidence that NETs, originally identified in bacterial killing, are also used against helminths. However, at the same time, hookworms have developed an excretory/secretory deoxyribonuclease protein, known as Nb-DNase II, that can degrade the NETs, in both in vitro and in vivo models. This new finding provides an exciting avenue of targeting parasitic nematode DNAses as vaccine or therapeutic targets to promote NET-mediated nematode killing.

Extracellular Vesicles

Extracellular vesicles (EVs) released by parasitic nematodes during infection may provide a powerful strategy for the parasitic nematode to generate widespread effects on host cells [100]. Of therapeutic promise, treatment with EVs from *T. spiralis* and *N. brasiliensis* suppressed colitis of mice and protection was associated with reduced proinflammatory cytokines and increased Th2 and Treg responses [101, 102]. In addition to containing lipids and proteins that may be immunomodulatory, EVs may also serve as cargos to deliver small RNAs to host cells, such as macrophages and intestinal cells, where they target and suppress host RNA. sRNAs have been identified in EVs generated by several parasitic nematodes, including *T. spiralis*, *N. brasiliensis*, *Trichuris muris*, and *H. polygyrus*, where they are predicted to target host immune gene networks [101-104]. For example, *H. polygyrus* EVs were able to suppress macrophage responses and IL-33 signaling, and contained miRNAs that specifically targeted host DUSP1 RNA, a

regulator of MAPK signaling [105, 106]. miRNA generation has been reported in several nematode species, including *Ascaris suum*, which infects pigs, where miRNA sequence analysis predicted that they targeted the host Th2 immune response (IL-13, IL-25, IL-33) [107]. Circulating filarial nematode-derived miRNAs were detected in the blood of *Litomosomoides sigmodontis*-infected mice, *O. volvulus*-infected humans, *Loa loa*-infected baboons and *Onchocerca ochengi*-infected cattle [105, 108, 109]), however, whether they were present in EVs, or targeted host gene expression, is unclear. The products included in EVs can differ by lifecycle stage and sex of the nematode [91, 110]. Examining the molecules found in EVs for all lifecycle stages will allow for the discovery of a wide variety of potential drug targets. Molecules found in all developmental stages associated with the host could be strong candidates for the development of vaccines, allowing the immune system to recognize parasites throughout an infection, such as Galectin2 [111]. On the other hand, molecules unique to adults may assist the worm in evading the host immune system in order to maintain a chronic infection, making them of particular interest for the development of anthelminthics and as immunotherapies. Interestingly, adult female *B. malayi* EVs had far more complex proteomes than males, with nearly four times as many proteins, including a MIF homolog, which may be involved in regulating the immune system [91]. EVs may offer containment and protection from degradation of a multitude of immunogenic nematode antigens that could allow for more effective host immune responses to helminths. For example, intact EVs, but not lysed EVs, from *T. muris* were able to reduce egg burden in a subsequent infection of this worm, making them potential vaccine candidates for

improved immunogenicity [106, 112]. EVs present a unique opportunity to study the parasitic lifecycle, allowing for a greater understanding of molecules that are required to initiate and maintain an infection.

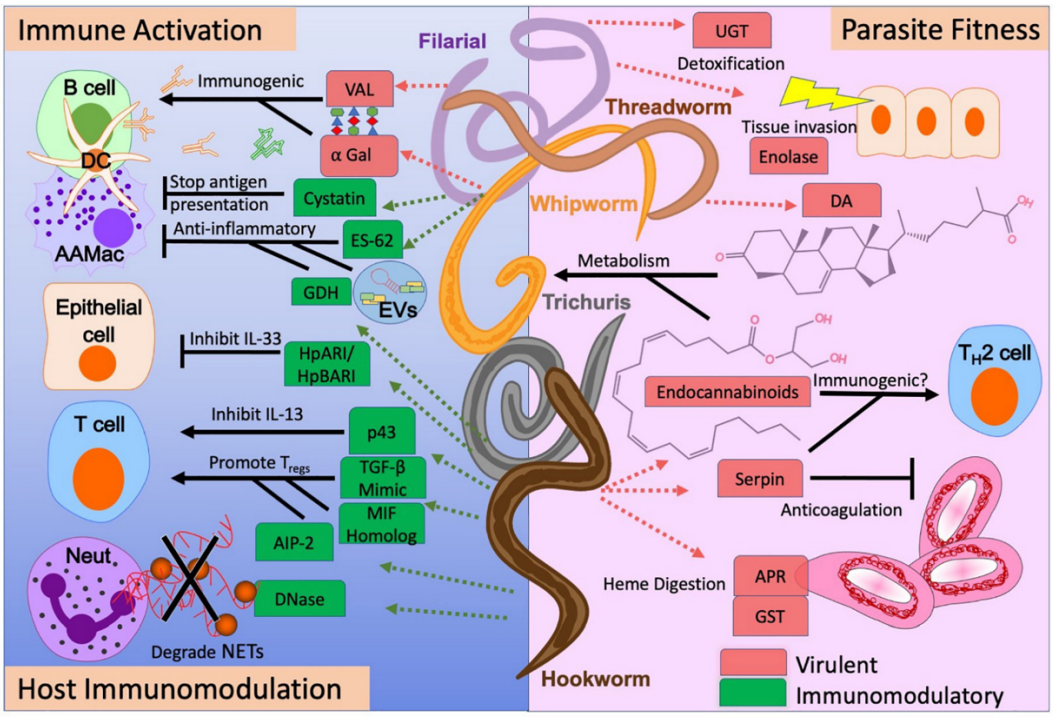

Figure 1: The pleiotropic functions of nematode-derived molecules. Functions range from promoting or inhibiting the host immune response (left), to providing essential physiologic functions for the nematode parasite (right). Understanding the virulence (red) and immunomodulatory (green) potential for specific nematode-derived molecules allows us to determine their utility as vaccines, anthelminthics, or new therapeutics for allergic or inflammatory diseases.

Non-mammalian model systems: entomopathogenic nematodes and plant parasitic nematodes

Model systems have proven to be valuable to the study of human disease [113]. For example, initial analysis of the Drosophila melanogaster genome identified that over 60% of human disease-associated genes had orthologs in the fly. Studies of fly immunity led to the discovery and description of the Toll pathway and subsequently Toll-like receptors

in mammals [114]. Similarly, determining the mechanistic function of conserved parasitic nematode effectors may benefit from the use of the entomopathogenic (EPN) insect model system, which is cheaper, faster, and allows for more individual hosts to be used per experiment than could possibly be done in a mammalian system. Effectors could be mechanistically described in the model system, providing an elevated starting point for experimentation in parasitic infections of mammals. EPNs form a mutualistic relationship with bacteria, carrying them inside their intestine when they infect their hosts, releasing the bacteria into the hemolymph of their insect host. The bacteria assist in killing the host, and, along with liquified host tissue, serve as a food source [115, 116]. Similar to skin-penetrating nematode parasites of mammals, the initial infection process is entirely dependent on the ability of the nematode to enter the host and suppress its immune system. EPNs suppress the host immune system early in infection, causing it to tolerate not only nematode parasites but their symbiotic bacteria, until the host succumbs to infection [117]. The specific molecules excreted/secreted from EPNs could be used for pest control in agricultural settings and also for immunoregulatory studies. Here we discuss the current research and known functions of EPN-derived virulence and immunomodulatory molecules, and how they relate to molecules employed by mammalian pathogenic nematodes. We also discuss main virulence factors that are present in plant parasitic nematodes, highlighting the striking conservation of these parasitic virulence mechanisms across the tree of life. The ES products from the EPN *Heterorhabditis bacteriophora* are lethal to their insect hosts at high concentrations [118]. Treatment of *Drosophila* with proteins extracted from the supernatant of activated

H. bacteriophora suppressed expression of antimicrobial protein diptericin, a product of the immune deficiency (Imd) pathway in insects [118]. This immunomodulatory mechanism is swift enough to allow for the infection of not just the nematode, but its mutualistic bacterial co-infector *Photorhabdus luminescens*, which would otherwise be killed by its insect host. In a similar manner, *S. carpocapsae* suppresses the immune response of its *Drosophila* host, allowing for the propagation of the endosymbiotic bacteria *Xenorhabdus nematophila* [119]. Shortly after infection, and before the bacteria is released from the gut of the infected nematode, there is a significant reduction in total insect hemocytes, suggesting that the nematode itself is capable of suppressing the host immune system, to the benefit of its endosymbiotic bacteria. The mechanism for immunomodulatory products from EPNs remains to be determined, however it appears to be time sensitive. After 3 hours of exposure to live *S. carpocapsae*, insect hemocytes had reduced phagocytic activity, which was not apparent after only 1 hour [120]. Identification of the specific EPN-derived molecules that target this innate immune Imd pathway in *Drosophila*, and whether they are conserved in mammalian parasitic nematodes could allow discovery of new anthelminthics and immunotherapies. There are striking differences between the EPN lifecycle and that of mammalian pathogenic nematodes, most importantly the fate of the host, which is swiftly killed by EPNs in contrast to mammalian parasitic nematodes which establish chronic infections [121]. In order to evade the host immune system, contribute to host killing, and then feed on the dead body, the parasite must be able to successfully suppress the host immune response, release toxins, and then digest host tissue, making EPNs a strong model for identifying

anti-inflammatory molecules as well as strong virulence factors. Recently, studies characterizing the specific proteins present in EPN ES revealed the remarkable resemblance to mammalian parasitic nematode-derived proteins with regards to structure and function. These include VAL proteins, enolases, serpins, and cystatins (Figure 2). For instance, *Steinernema glaseri* was shown to express enolases only at the activated infectious juveniles (IJs) stage, suggesting that the protein has a role in staging an infection, likely to digest the insect tissue [122]. In infected insects, this secreted enolase was present in the insect hemolymph, and alone was sufficient to allow for quicker propagation of the bacteria, *Xenorhabdus poinarii*. Many venom proteins, with similarity to mammalian parasitic nematode VAL proteins, were identified in the ES of activated *Steinernema carpocapsae* and *S. feltiae* infective juveniles (IJs), and may contribute to the high toxicity of these parasites to their insect hosts [123, 124]. *S. carpocapsae* also expressed the serpin-like inhibitor ScSRP-6. Sc-SRP-6 impaired clot formation in its insect host by preventing the incorporation of melanin, known as melanization, which is an important defense mechanism in insects [117]. Likewise, Sc-SRP-6 inhibited the hydrolysis of insect gut juices, a function that is thought to be conserved in *A. ceylanicum*. This serpin-like protein not only modulated the immune system, but inhibited digestion as the nematode passes through the gut of its host. Similar serpin-like genes were also found in mammalian-pathogenic nematodes, such as *B. malayi* [125]. ES products from EPNs therefore have similar functions to those from mammalian-pathogenic nematodes and may serve as powerful models for rapid discovery of useful

targets for anthelminthics, given the comparative affordability and shorter lifecycles of EPNs.

EPNs also produce cystatin, particularly when they detect insect hemolymph, as a location cue for their presence in the insect [126]. Further research comparing the similarities between cystatins from EPNs and mammalian pathogenic nematodes would be valuable in validating the connections between these models. Like nematode parasites of animals, plant-parasitic nematodes (PPNs) are masters of immune modulation, most of which is mediated by their secreted proteins and molecules. Because of their devastating effects in agriculture, PPNs are well-studied, and hundreds of secreted effectors have been identified, though, similar to other nematodes, few have been studied in mechanistic detail [127, 128]. A detailed discussion of PPN effectors is beyond the scope of this review, however several recent reviews focus on PPN virulence factors and host-pathogen interaction [127-129]. Here we discuss PPN virulence factors that are shared with EPN and mammalian parasitic nematodes, including VALs and cystatins. VAL genes have been identified in many PPNs, and their expression is associated with host invasion and migration through host tissues [39, 130]. PPN VALs have been shown to be important for modulating host immunity, especially in the early stages of infection [131, 132]. Several VAL family proteins have been characterized, and they appear to modulate similar processes in both plants and animals, suggesting that mechanistic studies in one model system will be valuable to our understanding of how these effectors work in general. Many cystatin genes have been predicted in plant-parasitic nematode genomes, but little is known about their role in parasitism. A recent description of a cystatin from

the pine wood nematodes (PWN) *Bursaphelenchus xylophilus*, found that Bxcpi-1 is involved in the development and pathogenic process of the nematode [133]. The shared ancestry and parasitic behavior of these nematodes could be an explanation for their similar strategies of immune modulation. The conservation of molecular mechanisms of parasitism could allow for the identification of more proteins as well as small molecules that could be used to optimize an immune response during nematode infections, balancing an inflammatory response with worm burden. Further research will allow for ES products to be harnessed for the modulation of the immune system in mammals beyond the context of nematode infections, with applications in allergy and inflammatory diseases, without the risks of infection with live worms. The early stages of EPN infections are of particular interest, as the parasite focuses on suppressing the immune system without killing its host, prior to the release of mutualistic bacteria. This highly immunosuppressive stage may have applications with the mammal-infecting parasites that persist in their hosts for months or even years, such as hookworms and filarial nematodes [20].

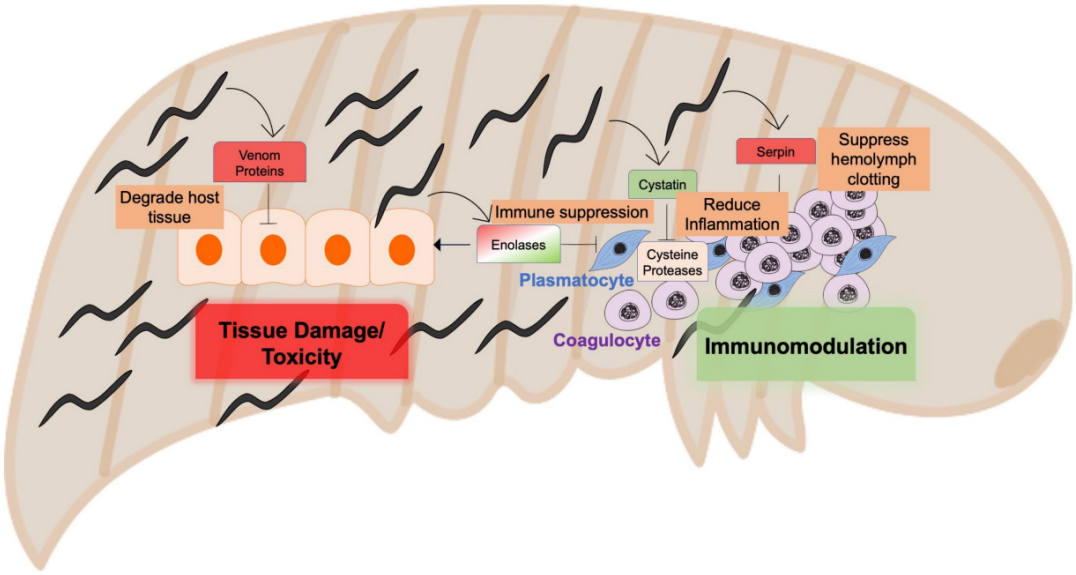

Figure 2: Entomopathogenic nematodes are a useful model for studying the role of excreted/ secreted proteins in host-pathogen interactions.

Conclusion

Significant research has been conducted into the role of excretory/ secretory proteins in the crosstalk between parasitic nematodes and their hosts. While many such molecules have promise as novel targets for anthelminthics, we found that endocannabinoids especially merit further exploration. Lipid-derived molecules are not well-characterized, yet they are highly conserved in both mammals and nematodes. In the next chapter of this thesis we explore the role of endocannabinoids in the host immune system's behavior. Then, in the third chapter, we rely on a bioinformatics approach to develop a preliminary understanding of the role of endocannabinoids in the parasitic nematode *N. brasiliensis.*

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Chapter Two— The Role of Endocannabinoids in Pulmonary Immune Response to Helminth Infection

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Chapter Two: The Role of Endocannabinoids in Pulmonary Immune Response to Helminth Infection

Abstract

Endocannabinoids are lipid-derived molecules that have a broad range of effects, including regulation of the immune system. While there has been significant research into the role of endocannabinoids in chronic pain and neurological disorders, there has not been strong conclusions about their role in the pulmonary immune response. We have utilized *N. brasiliensis*, a model of human hookworm infection, to produce a robust immune response in the lungs of wild-type (WT) mice and mice lacking the endocannabinoid receptor CB1 (CB1R-/-).

In this chapter, we explore the role of endocannabinoids in helminth infections in the lung, seeking to understand the biological process responsible for an increased inflammatory response in CB1R-/-. This includes characterization of the immune cells from the lungs of mice infected with *N. brasiliensis* via flow cytometry, nanoelements/ RNA-seq, and ELISA. We found that CB1R-/- M2 macrophages (IL-4 treated) had increased levels of Relmα, a marker of Type 2 (Th2) immune response, as well as CCL24, which is involved in the migration of eosinophils. This finding is suggestive of a mechanism behind the increased levels of eosinophils in the lungs of helminth-infected CB1R-/- mice. While we did not find a consistent difference in the parasite burden between WT and CB1R-/- mice, we did find that CB1R-/- macrophages and eosinophils from memory-infected mice showed increased binding to *N. brasiliensis in vitro*, indicating an overall more robust inflammatory response.

Introduction

Helminth infection induces a T helper type 2 (Th2) immune response, which is characterized by the alternative activation of macrophages and the promotion of eosinophils [1]. This study focused on both macrophages and eosinophils, which are crucial to trapping and killing parasitic nematodes in the lung and gut of the host [2]. Studies in humans have found that macrophages activated with immune serum had an increased expression of the chemokine CCL24, which is involved in the recruitment of eosinophils [3]. Our study found that IL-4 treated macrophages from CB1R-/- mice had increased levels of CCL24, both at the RNA and secreted protein level, potentially explaining the increase in eosinophils found in the lung after infection.

Expansion of eosinophils are a hallmark of helminth infection and an essential part of the immune response to these large parasites [4]. Functionally, they produce IL-4, a cytokine that promotes the alternative activation of macrophages and they can also act as antigen-presenting cells, signaling to Th2 cells and promoting a response to helminth infections [5]. **We propose that CB1R-/- mice have an increase in eosinophils due to a loss of endocannabinoid signaling through this receptor (Figure 1).**

In this study we employed cocultures of infectious juvenile *Nb* larvae (L3) with macrophages and eosinophils from a memory model of infection, in which mice were infected twice (18-30 days apart) in order amplify the immune response to the helminths [2, 6]. This had the effect of producing sufficient eosinophils in the lungs to set up cocultures with infectious larvae and replicated clinical hookworm re-infection, which is common in endemic areas [7].

Here we explore the role of endocannabinoids in the immune response to nematode infections. In order to do this, we employed mice lacking cannabinoid receptor 1 (CB1R), a Class A G-protein receptor (rhodopsin GPCR family). We employed the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) because they both have high binding affinity to the cannabinoid receptors (CB1 and CB2) [8]. Notably, AEA is only a partial agonist of CBRs and 2-AG is a full agonist of CBRs [9]. In addition, cannabinoid molecules can act on other receptors besides CB1R and CB2R, including the vanilloid receptors, such as TRPV1 [8].

The endocannabinoid system is involved in energy homeostasis and pain sensation, but also in regulating the immune system [8, 10]. The role of endocannabinoids in regulating the immune system in the lungs has not been clearly characterized.

Previous research in the Nair Lab has demonstrated that lungs and intestines in mice infected with *N. brasiliensis* had increased levels of endocannabinoids in comparison to naïve mice, indicating a potential role for these lipid-derived molecules in responding to helminth infection [11]. Furthermore, nematodes produce endocannabinoids, particularly during the infectious larvae stage of the lifecycle, indicating a potential role for endocannabinoids in regulating the murine immune response to helminth infections. This study was followed up by demonstrating that peripheral restriction of CB1R with the drug AM6545 resulted in increased damage to the alveolar space and eosinophilia in the airway, indicating that endocannabinoids have an ameliorating infect on the immune

response in the lung [12]. This chapter further characterizes the way in which signaling through CB1R, but not CB2R regulates the Th2 immune response in the lung (Figure 1).

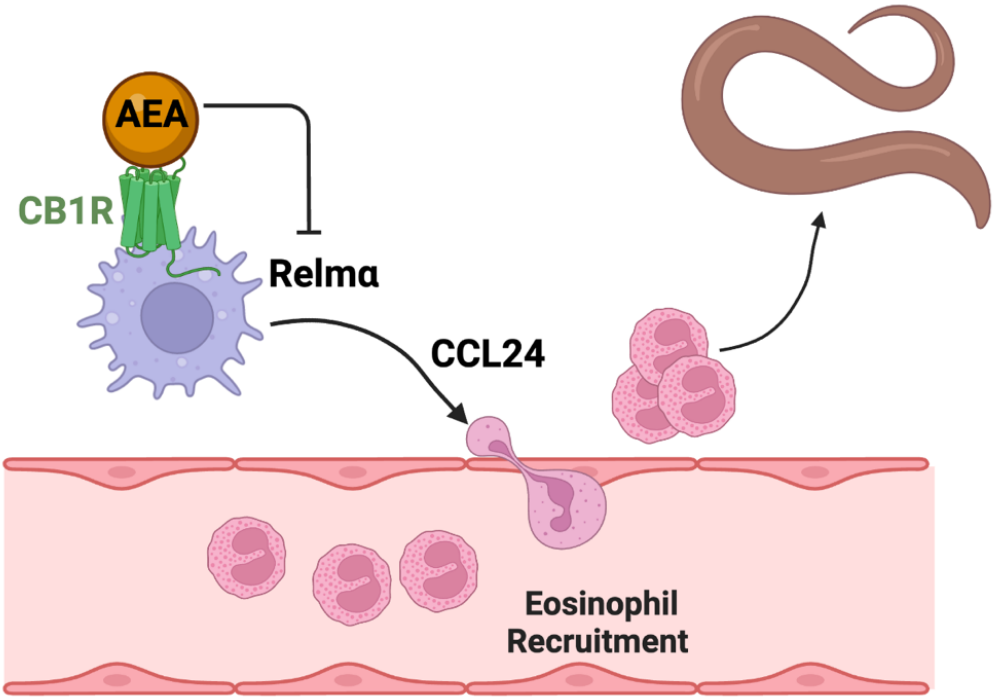


Figure 1: Graphical summary of the effects of the endocannabinoid AEA on the immune system. CB1R-/- macrophages had increased levels of Relmα and CCL24. In WT macrophages, AEA suppresses production of Relmα and CCL24 in a dose-dependent manner. This figure was created with Biorender.

Results

In line with Wiley et al., which peripherally restricted CB1R using the drug AM6545, the fully-body knockout of CB1R did not yield a statistically significant difference in weight loss, egg burden, or worm burden. This indicates that worm killing is not an inherent function of endocannabinoid signaling through CB1R (Figure 2).

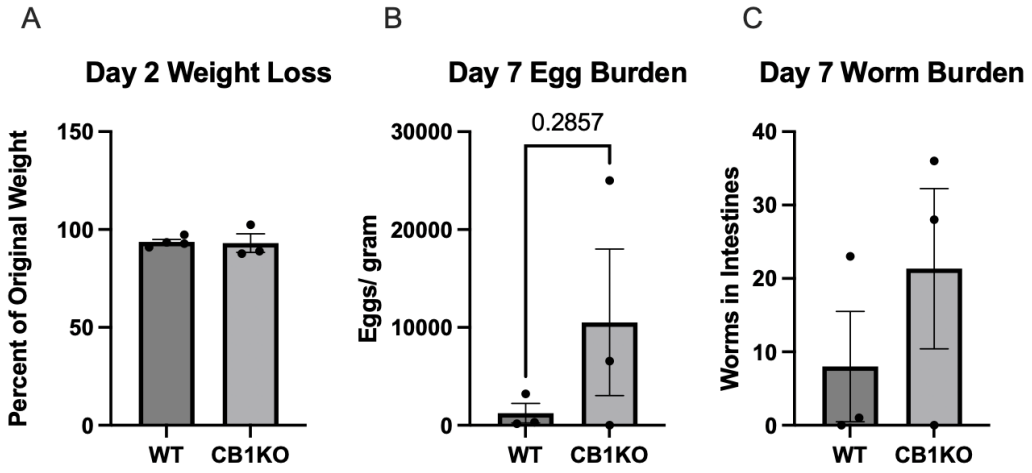


Figure 2: Lack of CB1R does not influence weight loss at D2 post-infection and egg/ worm burden in *N. brasiliensis* infected mice. WT and CB1R-/- (CB1KO) mice were infected with 600 *Nb* L3. (A) Mice were weighed at days 0 and 2 post-infection. (B) Nb egg burden at day 7 post-infection. (C) Intestinal parasite burden at day 7 post-infection. n=3 each for WT and CB1R-/- mice.

While worm killing is not affected, endocannabinoid signaling through CB1R has a significant impact on the immune cell profile. Similar to previous work in which CB1R was peripherally restricted through the drug AM6545, we found an increase in eosinophils in *Nb*-infected CB1R-/- mice.

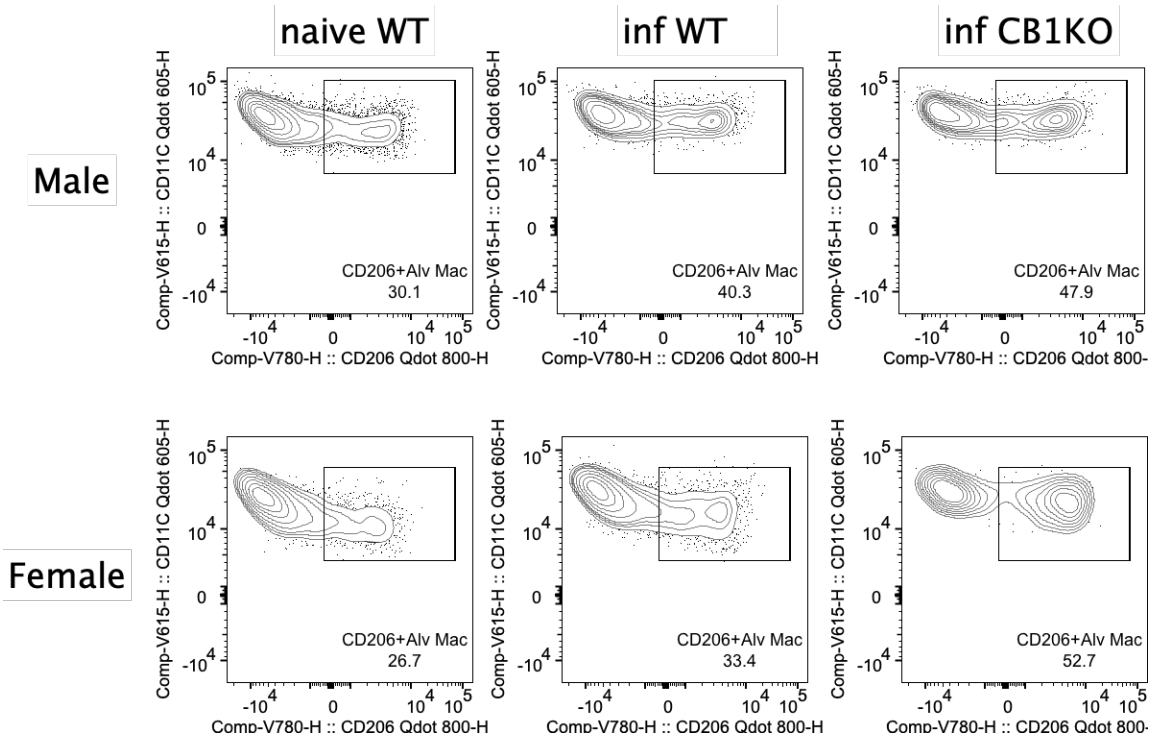


Figure 3: Flow cytometry of immune cells collected from the Bronchoalveolar Lavage (BAL) of memory-infected mice show an increase in CD206+ (M2) macrophages. BAL fluid was collected from WT and CB1R-/- mice were infected with 500 L3 *Nb* and characterized using flow cytometry. CB1R-/- mice showed an increase in CD206+ alveolar macrophages and eosinophils. Flow plots are representative of two experiments of males and females, where WT n=3 males and 3 females and CB1R-/- n= 3 males and 2 females.

To demonstrate the cell-intrinsic role of CB1R in immune cells, bone marrow was isolated from both WT and CB1R-/- mice and grown into macrophages using previously described methods [11]. These macrophages were then treated with either 1x PBS or 20ng/mL of IL-4, in order to induce alternative activation. IL-4 treatment resulted in an increase in the production of Relmα, as determined by ELISA of the supernatant from the treated wells, demonstrating a Th2 immune response had been initiated. Wells treated with IL-4 were also treated with vehicle and the endocannabinoid AEA and 2-AG in order to determine the effect of the endocannabinoid on gene expression of the treated

macrophages. RNA was isolated from the macrophages and analyzed using nanoelements (Figure 4). In CB1R-/- -derived macrophages treated with IL-4 and AEA had increased expression of both Relmα and CCL24. Previous research has demonstrated that Relmα is a secreted protein that is involved in dampening the Th2 cytokine response to helminth infection [13]. Thus it is possible that the increased inflammatory response in the CB1R-/- mice is met by increased dampening of the immune system. Additionally, the overexpression of CCL24 by these cells potentially explains the mechanism involved in the eosinophilia in *Nb*-infected mice.

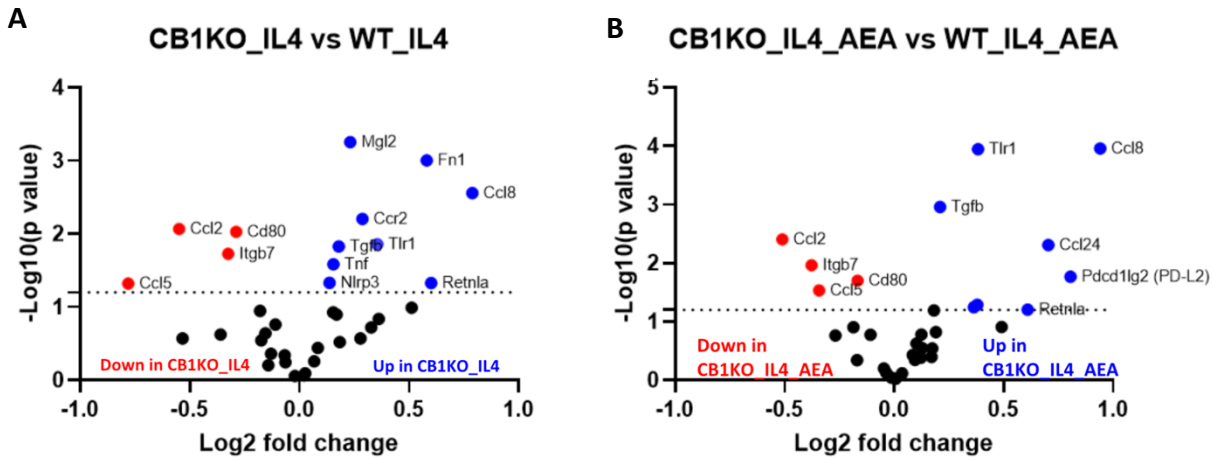
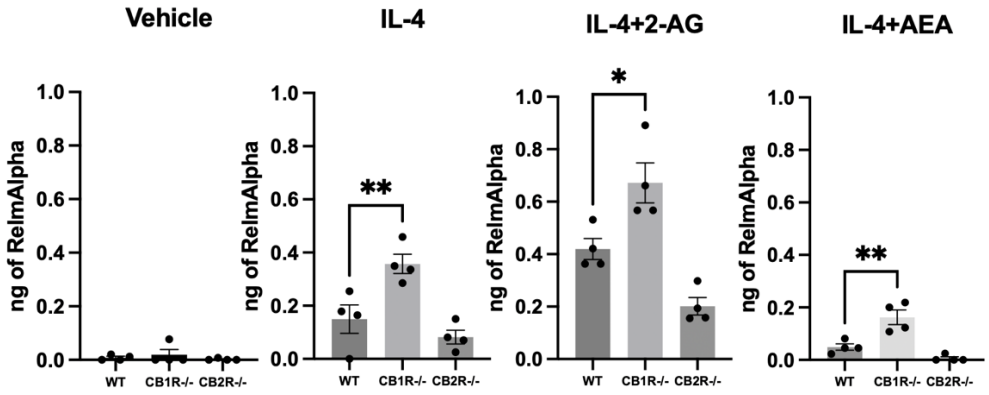


Figure 4: Nanoelements data of bone marrow-derived macrophages from WT and CB1R-/- mice demonstrate the role of endocannabinoid signaling in the Th2 immune response. Treatment of AEA resulted in an increase in the expression of Relmα, a marker of Th2 immune response, and CCL24, also known as Eotaxin2, which is involved in the attraction of eosinophils, a potential source for the eosinophilia observed in these mice when infected with *N. brasiliensis* (Coakley, 2020). n=4 wells of treated cells for each group.

A


Vehicle
IL-4
IL-4+2-AG
IL-4+AEA
ng of RelmAlpha
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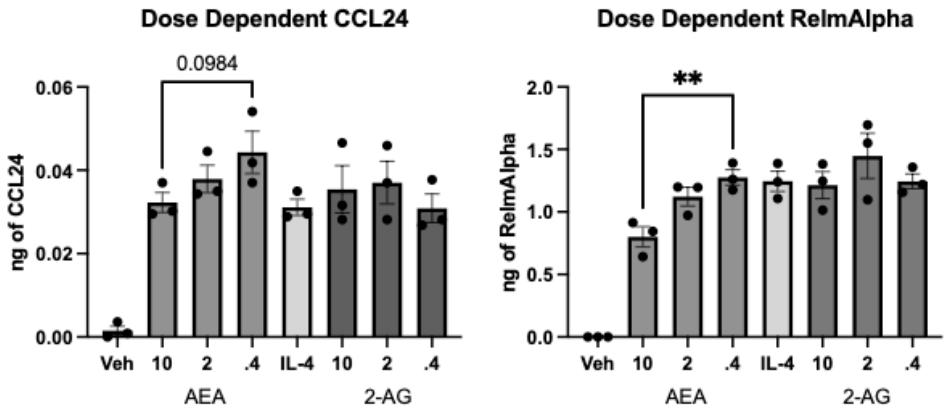
WT
CB1R-/-
CB2R-/-
*
**

B


Dose Dependent CCL24
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0.0984
ng of CCL24
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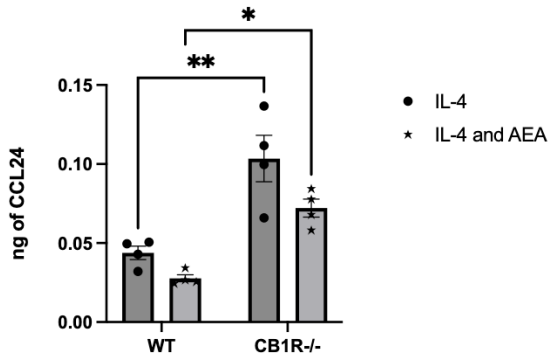
AEA Treatment
ng of CCL24
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WT
CB1R-/-
*
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IL-4
IL-4 and AEA

Figure 5: RELMα and CCL24 ELISA of supernatant from bone-marrow derived macrophages treated with IL-4 and AEA. (A) RELMα was increased in CB1R-/- bone-marrow derived macrophages, in all 3 groups, which were each treated with 20 ng/ mL of IL-4, and either 5μM 2-AG or 10 μM AEA. This demonstrates the robust production of the secreted protein in mice lacking the CB1 receptor. n=4 wells each for WT and CB1R-/-. (B) AEA but not 2-AG reduced the expression of RELMα in WT bone-marrow derived macrophages treated with IL-4 in a dose-dependent manner. n= 3 wells for each treatment group, including vehicle, 10, 2, and .4 μM of AEA/ 2-AG. All wells except the vehicle well were treated with 20 ng/ mL of IL-4. (C) CCL24 was upregulated in the supernatant of CB1R-/- macrophages, regardless of treatment group. n=4 wells each for WT and CB1R-/-. All wells were treated with 20 ng/ mL of IL-4 and 10 μM of AEA if indicated.

In order to further explore the effect of AEA on bone-marrow derived macrophages, RELMα and CCL24 ELISAs were performed on the supernatant from the wells of the treated cells. AEA but not 2-AG reduced RELMα expression in a dose-dependent manner. This is especially interesting given that AEA is only a partial agonist of the cannabinoid receptors but 2-AG is a complete agonist. This could be explained by a difference in required dosage and administration timing to see clearer immunomodulatory effects of 2-AG. This also suggests that a potential role of helminth-derived endocannabinoids could be immunomodulation of the host.

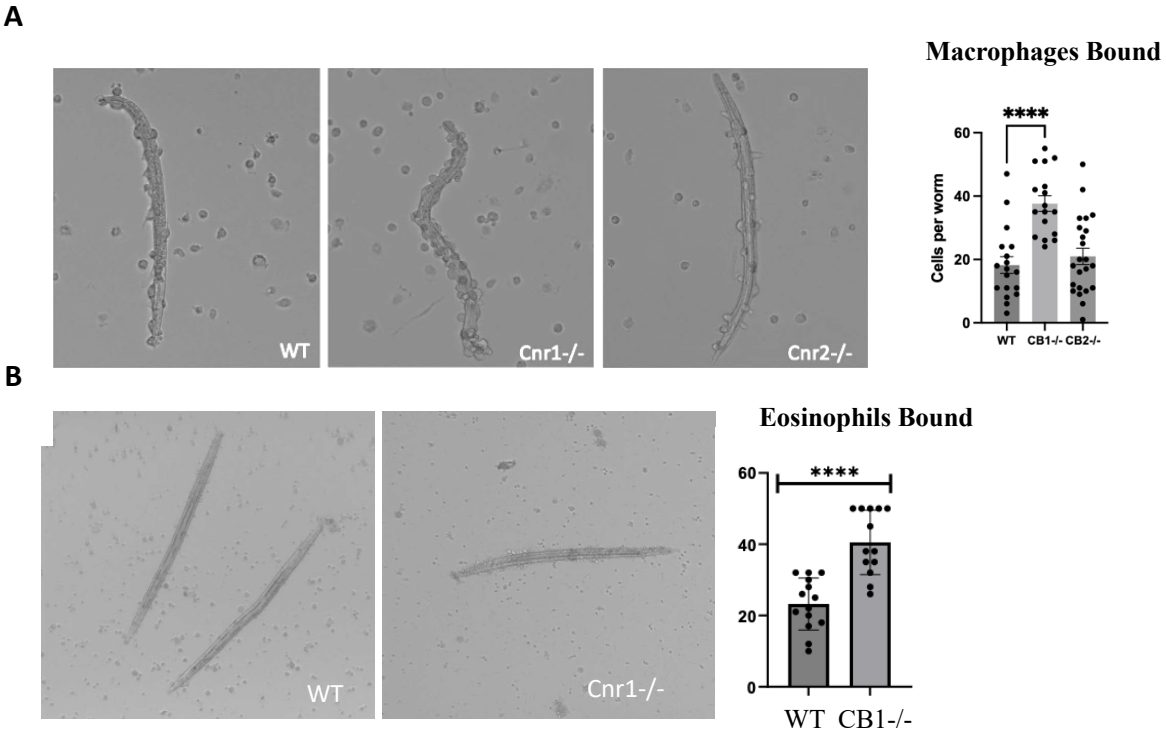

Figure 6: Coculture of macrophages and eosinophils isolated from memory-infected *N. brasiliensis* demonstrated increased binding of CB1R-/- mice to L3 larvae *in vitro.* (A) Macrophages and (B)eosinophils were isolated from the lungs of memory-infected mice and cocultured with infectious L3 larvae. Number of cells bound to each worm were counted, revealing an increased affinity for worms by these immune cells derived from CB1R-/- mice. n=10 or more worms per group.

In order to explore cell behavior, we set up cocultures of macrophages and eosinophils from memory-infected mice (Figure 6). While there was no clear difference in worm clearance *in vivo*, we saw that CB1R-/- mice had significantly more binding to worms *in vitro.* This supports the upregulation of CCL24 in the IL-4 treated macrophages of these mice, promoting the movement of eosinophils to the parasite.

Materials and Methods

Mice

C57BL/6J mice were purchased from the Jackson laboratory and bred in-house in pairs and trios. CB1R-/- were received from the DiPatrizio lab (originating from the Kunos lab) and bred in-house. Mice were age- and sex-matched and kept at ambient temperature with a 12-hour light/ dark cycle. All animal protocols were used by the Institutional Animal Care and Use Committee at the University of California, Riverside (protocol 20210017).

Infection

Nippostrongylus brasiliensis (*Nb*) nematodes received from the laboratory of Graham Le Gros (Malaghan Institute, New Zealand) were maintained in Sprague-Dawley rats purchased from Taconic. Mice were subcutaneously injected with 500-600 *Nb* L3 larvae and sacrificed at day 7 post-infection for primary infections and 4-5 days post-secondary infection. Secondary “memory” infections occurred 18-30 days after the primary infection. Egg and worm burden were quantified as previously described [11].

Bone Marrow Macrophages

Bone marrow was isolated from the tibia and femur of C57BL/6J (WT) and CB1R-/- mice and grown for six days in bone marrow macrophage media (30% L929 supernatant and 70% macrophage media—10% FBS, 1x Pen/Strep, 1 mM sodium pyruvate, and 25mM HEPES in DMEM) in petri dishes to promote the development of macrophages. Macrophages were then treated with PBS, 20ng/mL of IL-4, 2-AG and AEA.

Gene Expression analysis with NanoString

Cell lysates of treated bone marrow macrophages were prepared according to the manufacturer instructions and analyzed with a custom panel of 48 genes relevant to the Th2 immune response. Gene expression analysis was conducted using nSolver4.0, NanoString's gene analysis software.

CCL24 and RELMα Quantification

Sandwich Enzyme-linked immunosorbent assay (ELISA) was used to quantify CCL24 and RELMα from the supernatant of treated bone marrow macrophages. RELMα was quantified using the previously described protocol, with capture and biotintylated antibodies were obtained from Peprotech (Batugedara, 2018). CCL24 was quantified following the manufacturer's protocol for an ELISA kit obtained from R & D Systems, Inc.

Nippostrongylus brasiliensis- Immune CellCoculture

Macrophages and eosinophils from the lungs of primary and secondary-infected mice were isolated using CD11c and SiglecF microbeads (Miltenyi). L3 larvae were isolated from rat fecal plates, exsheathed with 0.25% NaOCl (Fisher Scientific), and treated with the antibiotics neomycin (400 ug/mL, Fisher Scientific), and penicillin/ streptomycin (400 U/mL, Fisher Scientific) for two hours. All cocultures included immune serum from *Nb* memory-infected RELMα-/- mice and maintained in a standard incubator at 37°C with 5% CO_2.

Statistical Analysis

All statistics, including student's t-test and one-way ANOVA were performed using GraphPad Prism. *= $p \leq .05$, **= $p \leq .01$, ***= $p \leq .0001$,

Discussion

It is well known that parasitic nematodes produce a variety of excreted/ secreted molecules in order to evade the host immune system and establish an infection [14, 15]. In fact, the immunomodulatory effects of helminths are so powerful, live helminth infections are currently researched as a source of novel molecules for fighting auto-immune disease [7, 16, 17]. Several studies have found benefits of these infections in metabolic diseases, such as Celiac's disease and diabetes [18-21]. By identifying the molecules produced by the nematodes that have these immunomodulatory effects, it

would be possible to develop therapeutics that can be administered in lieu of live helminths.

Endocannabinoids are one of the few molecules that are produced by both the host and the parasitic nematode during infection, making them highly valuable candidates for the identification of novel anthelminthics and immunomodulatory therapeutics [11]. These molecules are already being considered as novel therapeutics for a variety of diseases, in part because of their known effects on modulating inflammation and the nervous system [22-25]. While there has been significant research into the role of endocannabinoids in a variety of biological processes, including in the immune system, the role of these molecules in parasitic infections in the lungs has not been well-characterized [26-30]. The lungs are a key location for studying host-pathogen interactions, because of the vast and diverse immune landscape in this vital organ and because halting the pathogen in the lungs would prevent it from reproducing, which occurs in the gut [7, 31], 2023.

This study determined that endocannabinoid signaling through the CB1 receptor was not directly involved in the killing of nematodes, as CB1R-/- mice showed no statistically significant difference in weight loss, egg burden, or worm burden. However, these mice showed an increased immune response including an increase in eosinophils in the lungs [12]. Our project expanded on this previous research by considering the cell-intrinsic properties of alternatively activated macrophages (AAM), grown from the bone marrow of WT and CB1R-/- mice. We found that this macrophage population from CB1R-/- mice had increased levels of both RELMα and CCL24. The increased RELMα is an interesting result, because previous research demonstrates that RELMα is involved in modulating the

Th2 immune response [11]. However, CB1R-/- mice in fact have a more inflammatory response, with the increased airway eosinophilia [12]. This could be explained by a negative feedback loop, in which increased levels of inflammation promotes increased levels of RELMα. The increase in CCL24 levels by CB1R-/- alternatively activated macrophages suggests at the functional mechanism behind the eosinophilia identified in the lungs of *Nb*-infected mice. We also found that AEA reduced the expression of RELMα in a dose-dependent manner in wild type (WT) bone marrow derived macrophages. Further research is needed to explore the effect of AEA on macrophages through the CB1 receptor, such as its role in promoting phagocytosis [32]. While the CB1 receptor is not directly involved in regulating the severity of parasitic infection, the role of endocannabinoids in signaling through the CB1R to modulate the immune response in the lungs could have a role in other disease models, such as allergies. The effects of host endocannabinoids on the parasites themselves will be important, in order to determine if they would serve as effective anthelminthics [33, 34].

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Chapter Three – Introduction: A Bioinformatics Approach to Studying the Endocannabinoid Pathway in Nematodes

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Chapter Three: A Bioinformatics Approach to Studying the Endocannabinoid Pathway in Nematodes

Abstract

The endocannabinoid system is a well-conserved pathway found in both mammals and the parasitic nematodes that infect them. The previous chapter focused on the role of the endocannabinoid system in the mammalian host immune response, finding that loss of the endocannabinoid receptor CB1R results in an increase in the Th2 immune response. This chapter explores the role of the endocannabinoid system in the lifecycle of the parasite, in order to better understand how endocannabinoids can serve as targets for novel anthelminthics. Current research has demonstrated that *C. elegans* responds to the two major endocannabinoids, 2-AG and AEA, though there is not significant research in the role of endocannabinoids in parasitic nematodes. Through the use of bioinformatics tools including phylogeny and re-sequencing of the *N. brasiliensis* genome, we identify novel genes that are strong candidates for endocannabinoid pathway-related genes. In addition, we employed single-worm RNA-seq to identify potential targets for anthelminthics, such as the synthetic enzyme NAPE, which is upregulated in the infectious juvenile stage of the *Nb* lifecycle.

Introduction

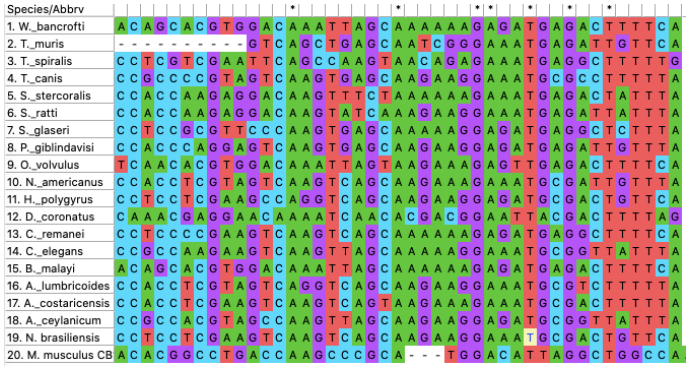
Hookworm infection is a neglected tropical disease that affects 500 million people and worldwide, causing over 3 million disability adjusted life years annually [1]. Infected individuals often suffer from iron deficiency anemia due to the ability of the parasite to feed on the host's blood [2]. Despite the severity of the effects of this disease, parasite resistance to current anthelminthics such as albendazole and mebendazole are on the rise, causing frequent rates of re-infection in endemic populations [3-6]. Identifying molecular targets for novel anthelminthics is essential to developing more effective mechanisms for targeting the parasite, particularly during the L3 larval stage, before the parasite has the opportunity to reproduce.

In *C. elegans*, the model organism nematode, the endocannabinoid system has recently received increased attention for its role in regulating physiological processes, such as nociception, metabolism, and even lifespan [7, 8]. This nematode has several genes that are orthologous to genes in the mammalian endocannabinoid pathway, including the synthetic enzymes NAPE-1/2, the degradative enzyme FAAH-1, and the endocannabinoid receptor NPR-19, which is a GPCR similar to CB1, and is involved in nociception [9, 10]. Nematodes also have endocannabinoid receptors besides those orthologous to CB1/2R, such as vanilloid subtype (TRPV) ion channels, which are involved in cholesterol uptake in response to the endocannabinoid 2-AG [11].

The endocannabinoid pathway is highly conserved among parasitic nematodes, indicating that it is a promising target for novel anthelminthics. For example, an ortholog to the gene *faah-1* was found in almost every parasitic nematode genome in a recent survey [12]. Preliminary analysis of the *Nippostrongylus brasiliensis* (*Nb*) genome found

that this nematode had genes orthologous to the *C. elegans nape-1* and *faah-1* and that these genes were upregulated during the infectious juvenile stage of infection [13]. However, a gene orthologous to *npr-19* was not identified [12]. In collaboration with the Dillman and Schwarz labs, the *Nb* genome was re-sequenced and annotated, allowing for greater clarity in identifying relevant genes. We explored the genes found in this newly annotated genome in order to identify novel endocannabinoid pathway genes, including a candidate ortholog for *npr-19*, which is highly similar in its amino acid sequence. We also used the software MEGA to align cDNA sequences and generate phylogenetic trees in order to explore how well the endocannabinoid system is conserved across parasitic nematode species, including those infecting humans. Altogether, this work indicates that the endocannabinoid system could be a potent target for arresting development in parasitic nematodes of a variety of hosts.

Results


Species/Abbrv
1. W._bancrofti
2. T._muris
3. T._spiralis
4. T._canis
5. S._stercoralis
6. S._ratti
7. S._glaseri
8. P._giblindavisi
9. O._volvulus
10. N._americanus
11. H._polygyrus
12. D._coronatus
13. C._remanei
14. C._elegans
15. B._malayi
16. A._lumbricoides
17. A._costaricensis
18. A._ceylanicum
19. N. brasiliensis
20. M. musculus CB

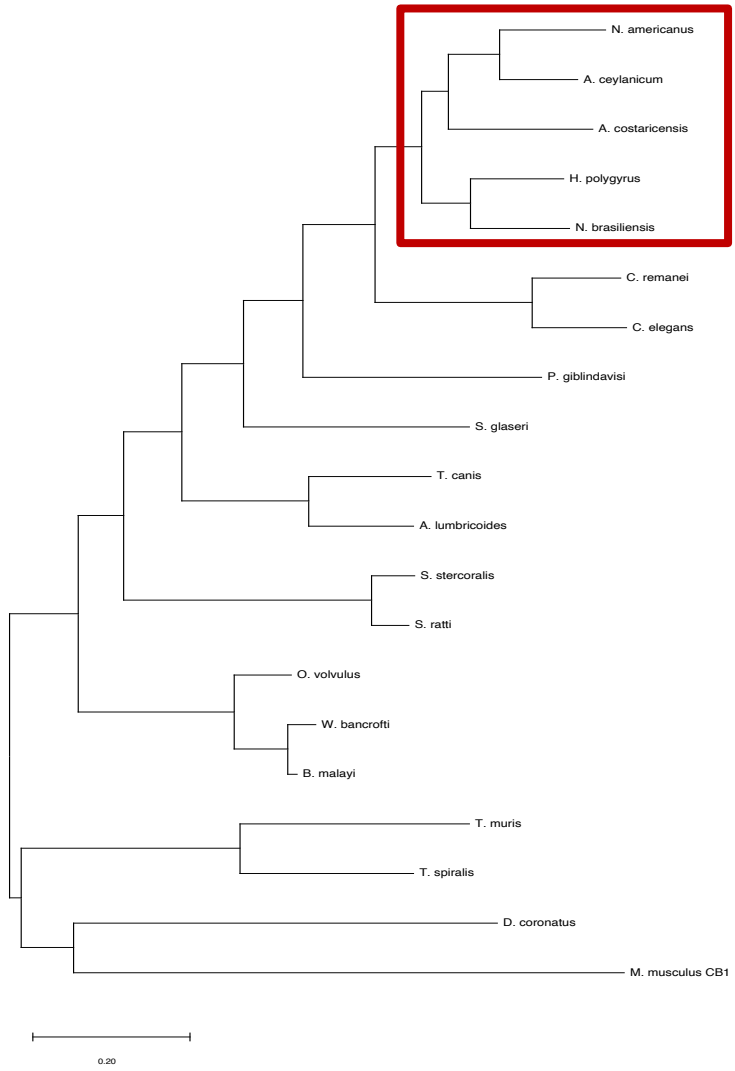

N. americanus
A. ceylanicum
A. costaricensis
H. polygyrus
N. brasiliensis
C. remanei
C. elegans
P. giblindavisi
S. glaseri
T. canis
A. lumbricoides
S. stercoralis
S. ratti
O. volvulus
W. bancrofti
B. malayi
T. muris
T. spiralis
D. coronatus
M. musculus CB1
0.20

Figure 1: Alignment of cDNA sequences of predicted orthologs of the *C. elegans* gene *npr-19*. The software MEGA was used to generate an alignment and the subsequent phylogenetic tree. The murine endocannabinoid receptor CB1R was used as an outgroup, suggesting that the receptor is not likely a direct ortholog of *NPR-19*. Indicated by the red box, the major species of hookworm and its models group together, indicating a potential shared origin of the receptor.

A phylogenetic tree of previously identified orthologs to NPR-19 shows that the receptor is highly conserved in parasitic nematodes. Previous analysis of the *N. brasiliensis* transcriptome did not result in the identification of an NPR-19 ortholog. In collaboration with the Dillman and Schwarz labs, we were able to re-sequence the *Nb* genome, generating more accurate gene predictions. As a result, we were able to identify an ortholog to NPR-19 with 87% positive matches (Figure 5).

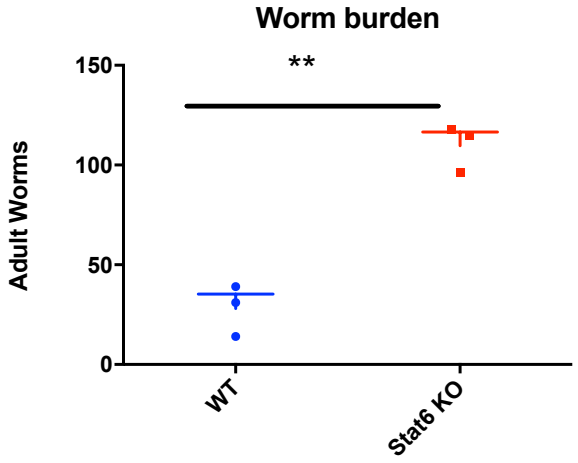


Figure 2: Mice lacking the transcription factor STAT6 show increased worm burden at D5 post-infection. Adult worms were counted in the intestines of wild type (WT) and STAT6-/- (Stat6 KO) mice in order to assess parasite burden prior to RNA isolation from the adult nematodes. n=3 for both groups.

The transcription factor STAT6 is involved in inducing the type 2 (Th2) immune response to parasitic nematodes [14]. Mice lacking STAT6 have a compromised immune response to *Nb* infection, giving the nematodes the opportunity to proliferate more readily (Figure 2). Genes that are upregulated in adult *Nb* isolated from these knockout mice are potentially involved in the nematode lifecycle, including staging an infection and reproducing in the gut of the host. These genes could be used to target reproduction,

thereby halting an infection in its early stages. We performed single worm RNA-seq and identified biologically relevant genes that are potential targets for novel anthelminthics [15, 16]. We used the quantification tool Kallisto in order to generate transcripts per million (TPM) due to its previously proven accuracy [17].

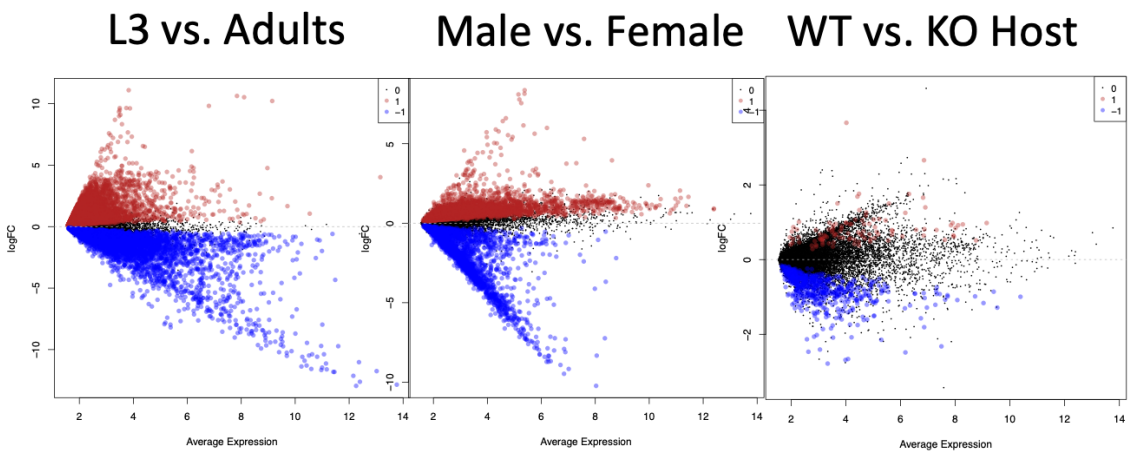


Figure 3: Volcano plots of differential gene expression. Comparisons were made between infectious larvae (L3) and adult *Nb*, Male and Female adult *Nb* isolated at D5 post-infection, and between adult *Nb* from wild type (WT) and STAT6-/- (KO) mice. n= 12 for the infectious juveniles and 6 for each group of adults.

We found that lifecycle stage had the biggest influence over differential gene expression in parasitic nematodes (Figure 3). Thousands of genes were differentially expressed between the larval stage and the adult (reproducing) stage. Next, the sex of the adult nematode also had a large influence on differential gene expression. Still, many genes were differentially regulated between adult nematodes that developed to maturity in either an immunocompetent, wildtype (WT) host and a STAT6-/- (KO) host. There was one gene, *nippo_chrIV.g8969,* from the newly sequenced *Nb* transcriptome that is significantly downregulated in nematodes from the KO host, with a p-value of 1.67 E-06

(Table 1). As of now, this gene has not been well-characterized, though a BLAST search on WormBase ParaSite shows the gene to have many orthologs in parasitic nematodes. We propose that this gene should be researched further in order to determine if it is a biologically relevant gene that can be targeted to reduce worm proliferation.

Gene ID	Annotation
nippo_chrIII.g6278	**G protein-coupled receptor, rhodopsin-like**
nippo_chrII.g4119	**GPCR, rhodopsin-like**
nippo_chrIV.g8969	**Transcription Factor-- Uncharacterized protein**
nippo_chrII.g3256	**G protein-coupled receptor, rhodopsin-like**
nippo_chrX.g15250	**G protein-coupled receptor**
nippo_chrIV.g9063	**G protein-coupled receptor**
nippo_chrIV.g10446	**Serpentine type 7TM GPCR chemoreceptor**
nippo_chrI.g2534	**GPCR, rhodopsin-like, 7TM [IPR017452]**
nippo_chrIV.prelim.g15210.t1	***C. elegans nape-1*** **ortholog**
nippo_chrX.prelim.g20996.t1	***C. elegans npr-19*** **ortholog**
nippo_chrII.prelim.g4061.t1	***H. contortus gst*** **ortholog**
nippo_chrX.prelim.g22278.t1/ nippo_chrII.g5262	***apr*** **identified by (Bouchery, 2018)**
nippo_chrIV.prelim.g12223.t1	***C. elegans faah-1*** **ortholog**
nippo_chrX.prelim.g20996.t1	***C. elegans npr-19 ortholog***

Table 1: Table showing endocannabinoid relevant genes identified in new genome based on preliminary annotations. The *N. brasiliensis* genome contains a significant number of GPCR's in the Rhodospin family, which could be potential endocannabinoid receptors. One uncharacterized transcription factor is downregulated in adult *Nb* from STAT6-/- mice, a potential new gene of interest.

Nippo_chrX.prelim.g20996.t1

Sequence ID: Query_388418 **Length:** 369 **Number of Matches:** 1

Range 1: 7 to 272 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
428 bits(1100)	2e-149	Compositional matrix adjust.	207/266(78%)	233/266(87%)	0/266(0%)

```
Query  10   NVSFYTLQALFTSANRRDDFIAVSIWTIMLLYALISNMLILAGIARSSTMRSATSYWFII  69
            N S+YTL AL ++  RRDD I V +W +ML YALISN+LIL GIARS+TMRSATSYWFII
Sbjct  7    NGSYYTLHALLSTRERRDDLIVVCVWLVMLSYALISNVLILVGIARSATMRSATSYWFII  66

Query  70   SIAICDILMTFISLGHLVPATAFHEEYVQFKSIRNIVMIFFYDLFWYTGVVQLGLMAGNR  129
            S+A+CDI+MT ISL HLVPATAFH+ YV+F S RNI+MIF YDLFWYTGVVQLGLMAGNR
Sbjct  67   SLAVCDIVMTSISLIHLVPATAFHDAYVEFYSHRNILMIFLYDLFWYTGVVQLGLMAGNR  126

Query  130  FVSIVYPMEYKHIFSRTRSLYLILFGYFLGFLVSLPTLFDCCHTLWDSNYYITVYEKPDT  189
            FVSIVYPMEYK++FSR RS+YLI+FGY LGFLVSLPTLF CCHTLW+S+YYITVY   DT
Sbjct  127  FVSIVYPMEYKNMFSRKRSVYLIVFGYVLGFLVSLPTLFPCCHTLWNSDYYITVYLPMDT  186

Query  190  LYKYVDMAVNSISLCMMIISYAVIILKVRASGRAMAKYQLTIRTRQQNALVNGVSLSCQM  249
             YKYVDM VNS SL MMI+SYAVII KVR SGRAMAKYQLTIRTRQQ+AL++G+SLS Q+
Sbjct  187  WYKYVDMGVNSASLFMMIMSYAVIIYKVRESGRAMAKYQLTIRTRQQHALMSGISLSSQL  246

Query  250  SECGRTSSVRPPRSQVSKKEMRLFIQ  275
            + C R SSVRPPRSQVSKKEMRLFIQ
Sbjct  247  NGCTRVSSVRPPRSQVSKKEMRLFIQ  272
```

unnamed protein product [Nippostrongylus brasiliensis]

Sequence ID: VDL75595.1 **Length:** 290 **Number of Matches:** 1

Range 1: 72 to 277 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
54.3 bits(129)	2e-08	Compositional matrix adjust.	57/246(23%)	101/246(41%)	48/246(19%)

```
Query  115  WYTGVVQLGLMAGNRFVSIVYPMEYKHIFSRTRSLYLILFGYF-LGFLVSLPTLFD-CCH  172
            WY G +   L A NRFV I +P   +  F+R R + +I+        +V+   L   CC
Sbjct  72   WYLGSLVQILFATNRFVVIFFPN--RIFFTRQRLIGMIIVCCLGAAAMVTYSQLLSPCCR  129

Query  173  TLWDSNYYITVY-----EKPDTLYKYVDMAVNSISLCMMIISYAVIILKVRASGRAMAKY  227
               D  Y+   Y     +  +    Y+D+ ++SI+    + SY             +A +
Sbjct  130  ITPDYRYFGYSYLIFPNQTSNPSMNYIDVPLDSITSAYCLGSY-------------VALF  176

Query  228  QLTIRTRQQNALVNGVSLSCQMSECGRTSSVRPPRSQVSKKEMRLFIQFFVVSLVFLLTW  287
               IR    N                          ++  K+E+R  +QF ++   + +TW
Sbjct  177  AYIIRMGTLN-------------------------NRAGKREVRCCVQFLLMFCTYTVTW  211

Query  288  TTWQWLPYMSESKWAYFVMTS-LFFINNSVNPTVYIIFNTQLRRELHYLICRHHVITTAQ  346
             T+   P +  ++   FV+T+ +F +N  +N T+Y+  N ++R   + LI R+
Sbjct  212  VTFFVYPAIGITQPEAFVVTTVMFMLNCGINSTIYLAMNREIRSAANKLIGRNLFGGQQS  271

Query  347  NKRKQT  352
            N  K T
Sbjct  272  NAEKST  277
```

Figure 4: New *Nb* transcriptome reveals a stronger candidate ortholog for NPR-19. (A) Alignment between *C. elegans* NPR-19 amino acid sequence (downloaded from WormBase ParaSite) and proposed NPR-19 sequence from the newly predicted transcriptome. The two amino acid sequences have high similarity, with an E-value of 2E-149, demonstrating the high likelihood that this is a true ortholog to *npr-19*. (B) Alignment of *C. elegans* NPR-19 amino acid sequence to the best match from the currently available *Nb* transcriptome.

Discussion

C. elegans is a potent model for studying the endocannabinoid system, due to significant similarities in synthetic and degradative enzymes and receptors to mammals [18, 19]. Several studies have demonstrated functional similarities in the response to endocannabinoids in both this model nematode and humans [9]. Feeding behavior for example, is modulated in both nematodes and mammals through the endocannabinoid pathway [20-22]. In this chapter we employ a bioinformatics approach to compare the endocannabinoid system in *C. elegans* to parasitic nematodes, many of which are often difficult or impossible to maintain in a lab, due to their need for a host [23]. For the most part, we find a high level of similarity between *C. elegans* and its parasitic cousins, through use of the genomics database WormBase ParaSite to compare relevant endocannabinoid pathway genes [24, 25]. The currently available *Nb* genome on WormBase has a short N50, due to the large and highly repetitive nature of its sequence [26]. In order to better analyze the endocannabinoid pathway in this model parasite, we collaborated with the Dillman and Schwarz labs to re-sequence the *Nb* genome and generate new transcriptome predictions. The role of endocannabinoids in host-pathogen interactions has shown a potential protective role for the molecules [27]. We use mice lacking the type 2 immune response in order to explore the role of endocannabinoids in responding to nematodes, however we did not find many genes in *Nb* that were relevant to the endocannabinoid system and differentially expressed in adult worms extracted from these immunocompromised mice [28].

The new transcriptome contains many new genes of interest that should be explored for their role in the endocannabinoid system and as targets for novel anthelminthics. This new transcriptome still had the previously identified endocannabinoid and blood-digestion genes (Table 1), but also had an ortholog to the well-characterized *C. elegans npr-19* gene, which was not found in the previous transcriptome, as well as other GPCR's in the Rhodospin family [29]. This preliminary review of the transcriptome indicates significant promise for future analysis, which could yield many biologically relevant genes not previously characterized.

Materials and Methods

Mice

C57BL/6J and STAT6-/- mice were purchased from the Jackson laboratory and bred in-house in pairs and trios. Mice were age- and sex-matched and kept at ambient temperature with a 12-hour light/ dark cycle. All animal protocols were used by the Institutional Animal Care and Use Committee at the University of California, Riverside (protocol 20210017).

Nb infection and worm burden

Nippostrongylus brasiliensis (*Nb*) nematodes received from the laboratory of Graham Le Gros (Malaghan Institute, New Zealand) were maintained in Sprague-Dawley rats purchased from Taconic. Mice were subcutaneously injected with 500-600 *Nb* L3 larvae

and sacrificed at day 5 post-infection in order to quantify adult *Nb* and collect specimens for single worm RNA-seq.

Sequence alignment and phylogenetic tree

cDNA sequences of orthologs to *C. elegans npr-19* were downloaded from WormBase ParaSite to compare to a predicted cDNA sequence from the genome of *N. brasiliensis.* The software MEGA was used to generate an alignment of the cDNA sequences and a phylogenetic tree.

Single Worm RNA-seq and Differential Gene Expression Analysis

RNA from individual worms was isolated as previously described [15, 16] Transcript per million (TPM) was quantified using Kallisto and differential gene expression analysis was performed using limma trend tools, as previously described.

Gene Annotation

Gene predictions were made by using the BLAST (Basic Local Alignment Search Tool) to align *C. elegans* and *H. contortus* amino acid sequences downloaded from WormBase ParaSite to the amino acid sequences of the predicted transcriptome from the newly re-sequenced *Nb* genome (courtesy of Eric Schwarz and collaborators).

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Chapter Four: Conclusion and Next Steps

The role of endocannabinoids in the immune system is a story that is still only partially told, though this dissertation seeks to shed some light on the effects of endocannabinoid signaling through the CB1 receptor in the context of helminth infection. Previous research in the Nair, DiPatrizio, and Dillman labs found that endocannabinoids are upregulated in the tissue of both the host and the parasitic nematode during infection. This was followed up by the discovery that peripheral restriction of the CB1 receptor in wild type (WT) resulted in airway eosinophilia during *Nb* infection. We found that this distinct eosinophilia is similarly found in whole body CB1 knockout mice (CB1-/-). This project seeks to link the reception of endocannabinoids through the CB1 receptor and a greater inflammatory response in the lungs of infected mice. Through the treatment of bone-marrow derived macrophages with endocannabinoids, we discovered the upregulation of the chemokine CCL24, also known as Eotaxin 2, which is involved in promoting the recruitment of eosinophils. We propose that this gene is downregulated in response to endocannabinoid signaling through the CB1 receptor. One major challenge of identifying the distinct role for a single endocannabinoid receptor is that endocannabinoids can signal through multiple receptors, not only CB1 and CB2, but also the TRP-V channels. This means that blocking a single receptor does not completely block endocannabinoid signaling. Blocking multiple endocannabinoid receptors at once is possible at the cellular level but presents a challenge at the organismal level because it presents a significant health burden on the individual, making clear experimentation difficult.

Transitory restriction of multiple endocannabinoid receptors through the use of chemical interventions could be an important future step in isolating the role of endocannabinoid signaling in the immune response to parasitic nematodes. We did not find that the CB2 receptor had an important role in worm killing or any other aspect of the host immune response to parasitic nematodes. This is somewhat surprising given that the CB2 receptor is more commonly associated with the peripheral nervous system and the immune system. Reception of endocannabinoids through CB2 has a role in the immune response to other pathogens and is worth further consideration. In terms of cell behavior, endocannabinoids may not be directly involved in promoting the killing of parasites by host immune cells but could reduce an inflammatory response and influence the nematode's health directly.

We found that the endocannabinoid receptor previously unknown in the model of hookworm *N. brasiliensis* is indeed present in the transcriptome, as a result of the newly sequenced and annotated genome produced in collaboration with the Schwarz and Dillman labs. This new resource for the immunology and nematology communities opens more lines of questioning than it closes.

We are now aware of several newly discovered genes that share similarities to NPR-19 in *C. elegans* and CB1R in mice that have not been functionally characterized.

One such gene, *nippo_chrIV.g8969* is differentially expressed in adult *Nb* dependent on their host's ability to initiate a Th2 immune response. This gene could potentially serve as a novel anthelminthic drug target, blocking the ability of the nematode to respond to host immune cells.

We hope this research is a starting point for further exploring how endocannabinoids can be used to modulate the interaction between the mammalian immune system and parasitic nematodes.