The analysis and characterization of the excretory/secretory products of parasitic helminths as immunomodulators of inflammatory bowel disease

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Abstract

Parasitic helminths infect more than 1.5 billion people worldwide. While helminths are well studied from a public health standpoint, these parasites are thought to be an important part in shaping the host microbiome and are organisms that have co-evolved with their hosts. The hygiene hypothesis postulates that the loss of colonization by helminths in westernized societies, because of increased sanitation, may contribute to diseases of immune dysregulation including autoimmune and autoinflammatory diseases. While the increase in hygiene has resulted in an immeasurable reduction in morbidity and mortality, the loss of our co-evolutionary partners (helminths) may contribute, in part, along with genetic and environmental factors, to the increased prevalence in diseases such as asthma, inflammatory bowel diseases, rheumatoid arthritis, and others. This thesis focuses on the specific effects of helminth excreted and secreted products (ESP) on inflammation *in vitro* and *in vivo*, and how these factors modulate the immune system. We hypothesize that immune mechanisms causing inflammation can be selectively interrupted by ESP. To study this, we produced ESP from different worm parasites and examined the activity in cell culture and *in vivo* mouse experiments to validate the immunomodulatory potential of these parasite products. Specifically, ESP from *Trichuris suis, Ascaris suum, Heligmosomoides polygyrus bakeri, Hymenolepis diminuta* and *Dirofilaria immitis* all produced ESP that reduced LPS-dependent TNFα secretion by bone marrow-derived macrophages (BMDM), and independently induce IL-10. Moreover, ESP metabolites from *A. suum* were found to improve dextran sodium sulfate colitis in C57BL6 mice. Ion exchange of bioactive proteins and liquid chromatography-tandem mass spectrometry analyses revealed candidate proteins that exhibited immunomodulatory activity *in vitro*. These anti-inflammatory profiles were recapitulated in BMDM using single or multiple recombinant proteins which included *T. suis*; nucleoside diphosphate kinase, triose phosphate isomerase, and glucose-6-phosphate isomerase. Fractionation of the small molecular weight metabolites present in the ESP of *T. suis*, *A. suum*, *Heligmosomoides polygyrus bakeri* and *Dirofilaria immitis* revealed a bioactive compound a mass of 686.215 amu that downregulated a pro-inflammatory response in BMDM and BMDC. This bioactive fraction was analyzed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). Future studies will investigate the precise structure of this metabolite and its target in macrophage with the goal to determining the mechanism in which modulate the inflammatory signalling**.** This research presents the isolation of molecule found in helminth-conditioned media that suppresses inflammatory cytokines and induces anti-

inflammatory cytokines *in vitro* and *in vivo*, which holds therapeutic potential in autoinflammatory diseases.

Abrégé

Les helminthes parasitiques infectent plus de 1,5 milliard de personnes dans le monde. Bien que les helminthes soient bien étudiés du point de vue de la santé publique, il a été démontré que ces parasites jouent un rôle important sur la composition du microbiome de l'hôte et que ces organismes ont coévolué avec leurs hôtes. L'hypothèse hygiéniste postule que la perte de la colonisation par les helminthes dans la société occidentale, en raison de l'amélioration de l'hygiène, contribue à l'augmentation de l'incidence de maladies de dérèglement immunitaire, y compris les maladies auto-immunes et auto-inflammatoires. Alors que l'amélioration de l'hygiène a entrainé une réduction significative de la morbidité et de la mortalité liées aux infections, la perte de nos partenaires coévolutifs (helminthes) peut contribuer en partie avec des facteurs génétiques et environnementaux à la prévalence accrue de maladies telles que l'asthme, les maladies inflammatoires de l'intestin, l'arthrite rhumatoïde et autres. Ce mémoire focalise sur les effets des produits excrétés et sécrétés (PES; *excreted-secreted products*, ESP) des helminthes sur l'inflammation *in vitro* et *in vivo*, et sur la façon dont ces facteurs modulent le système immunitaire. Nous émettons l'hypothèse que les mécanismes immunitaires causant l'inflammation peuvent être abrogés de manière sélective par les PES. Pour étudier cette hypothèse, nous avons récolter les ESP de différents vers parasitaires et examiné leur activité dans des cultures cellulaires et des expériences *in vivo* dans des souris afin de valider le potentiel immunomodulateur de ces produits parasitaires. Plus précisément, les PES de *Trichuris suis*, *Ascaris suum*, *Heligmosomoides polygyrus bakeri*, *Hymenolepis diminuta* et *Dirofilaria immitis* ont tous produit des PES qui réduisent la sécrétion de TNFα dépendante du LPS par les macrophages dérivés de la moelle osseuse (*bone marrow-derived macrophages*, BMDM), et induisent indépendamment l'IL-10. En outre, nous avons constaté que les métabolites dans les PES d'*A. suum* amélioraient la colite expérimentale induite par le sulfate de dextran sodique chez les souris C57BL6. Des analyses par chromatographie à échange d'ions et par chromatographie en phase liquide avec spectrométrie de masse en tandem (LC-MS/MS) ont révélé des protéines candidates qui ont présenté une activité immunomodulatrice *in vitro*. Ces profils anti-inflammatoires ont été récapitulés dans les BMDM à l'aide de protéines recombinantes, dont le nucléoside diphosphate kinase, la triose phosphate

isomérase et la glucose-6-phosphate isomérase de *T. suis*. Le fractionnement des métabolites de petit poids moléculaire présents dans les PES de *T. suis*, *A. suum*, *Heligmosomoides polygyrus bakeri* et *Dirofilaria immitis* ont révélé un composé bioactif d'une masse de 686.215 amu qui a réduit la réponse pro-inflammatoire dans les BMDM et les BMDC (*bone marrow derived dendritic cells)*. Cette fraction bioactive a été analysée par résonance magnétique nucléaire (RMN) ^{1}H et ^{13}C. De futures études porteront sur la structure de ce métabolite et sa cible dans le macrophage afin de déterminer le mécanisme de modulation de la signalisation inflammatoire. Cette recherche présente l'isolement d'une molécule présente dans les milieux de cultures conditionnés par les helminthes qui supprime les cytokines inflammatoires et induit des cytokines anti-inflammatoires in vitro et in vivo, ce qui présente un potentiel thérapeutique dans les maladies auto-inflammatoires.

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Contribution to original knowledge

Inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis are known to cause debilitating gastrointestinal (GI) symptoms in affected patients. However, the etiology of IBD remain largely elusive. While there is evidence that disease can be attributed to genetic predisposition, environmental factors, and possibly the microbiome, a single causative agent or pathophysiological mechanism has yet to be identified. However, there is an inverse correlation between the prevalence of IBD and the incidence of helminth infection. For example, in countries that have a high helminth burden there is a low incidence of IBD. This observation has led to the development of the "hygiene hypothesis", which states that the lack of co-evolutionary partners, i.e., intestinal parasitic helminths, leads to the dysregulation of the gut immune system. This dysregulation leads to the inappropriate activation of inflammatory responses against the commensal bacteria of the gut. It is conjectured that parasitic helminths, being well known for their ability to modulate the immune system, are part of the balance that prevents dysregulation of the gut immune system. This hypothesis postulates that parasites communicate with host immune cells and gut environment and suppresses unwanted inflammatory activity.

Here we studied excretory/secretory products (ESP) of intestinal parasites and the modulation of immune cells by ESP *in vitro* and *in vivo*. ESP from parasitic helminths are presumed to be key regulators of the parasite's ability to colonize the gut through crosstalk with host cells and possibly the gut microbiome. Previous work including gel permeation chromatography (GPC) and mass spectrometry identified major immunomodulatory regulators in the ESP of *Trichuris suis.* Select *T. suis* proteins identified in ESP were expressed in *E. coli* and tested *in vitro* for immunomodulatory activity. We further investigated recombinant ESP proteins that specifically suppressed pro-inflammatory activity and induce anti-inflammatory, specifically, a *T. suis* nucleoside diphosphate kinase (NDK), triose phosphate isomerase (TPI), and a glucose-6-phosphate isomerase (G6PI). We have examined both proteins and small molecular weight metabolites that exhibit immunomodulatory bioactivity. This work establishes that metabolite(s) present in the helminth-derived ESP modulate and potentiate immune cells to an anti-inflammatory phenotype.

Specifically, an amphipathic compound with a MW of 686.215 amu (NP686, NP refers to nonpolar) that was characterized by LC-MS/MS and purified using preparative high-performance liquid chromatography (HPLC). Treatment of bone marrow-derived macrophages and dendritic cells (DCs) with crude deproteinated ESP fractions or purified NP686 suppressed secretion of TNFα, an inflammatory cytokine, in cells stimulated with LPS. NP686 was detected in the ESP of *Trichuris suis, D. immitis, H. polygyrus bakeri*, and to a lesser extent in *A. suum* conditioned medium.

This work describes a novel molecule (NP686) found in the ESP of parasitic helminths, which is immune regulatory in nature that we believe plays a key role in the host-parasite interaction via modulation of the immune system. The discovery of this molecule can be pivotal in understanding the host-parasite interaction. NP686 may also have a therapeutic application, as it has anti-inflammatory properties that can be of potential use in treating IBD. This research demonstrates the role of anti-inflammatory ESP in a mouse model of colitis, showing that the disease was ameliorated with the administration of preparation of purified NP686 to colitic mice by intraperitoneal injections. We postulate that the molecule may have an anti-inflammatory role in other autoinflammatory diseases as well, such as rheumatoid arthritis, allergies, and psoriasis. Therefore, this work generates the potential for more research in those areas.

Contribution of authors

All parts of this work were written by Elizabeth Alexandra Siciliani with correction and feedback from Professor Armando Jardim.

All experiments in the presented work were conducted by Elizabeth and UPLC-MS/MS was performed by Prof. Armando Jardim at the University of Victoria and sent to The Institute of Parasitology for bioassays. NMR experiments were performed by Normand Cyr at the Département de biochimie et médecine moléculaire of the Université de Montréal. *Ascaris suum*-conditioned media was produced at the University of Iowa and kindly provided by Professor Richard Martin, from the UDSA. *Dirofilaria immitis*-conditioned medium was provided by Professor Thavy Long and *Heligmosomoides polygyrus bakeri*-conditioned medium was provided by Professor Fernando Lopes, both from the Institute of Parasitology of McGill University. *H. diminuta*-conditioned medium was provided by Professor Derek McKay. *Trichuris suis*-conditioned medium was provided by Professor Joe Urban, from the USDA.

Abbreviations

1D, 2D	1 dimension, 2 dimension
AMA	Antimycin A
AsESP	*Ascaris suum* excretory/secretory products
BMDC	Bone marrow derived dendritic cells
BMDM	Bone marrow derived macrophages
CD	Crohn's disease
CD	Cluster of differentiation
CDAI	Crohn's disease activity index
CEBPβ	CCAAT enhancer binding protein-beta
CM	Carboxymethyl
COSY	Correlated spectroscopy
CRP	C-reactive protein
DC	Dendritic cells
ddPCR	Digital droplet PCR
DMEM	Dulbecco's Modified Eagle Medium
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ESP	Excretory/secretory products
ETC	Electron transport chain
EV	Extracellular vesicles
G6PI	Glocose-6-phosphate isomerase
GMCSF	Granulocyte-macrophage colony-stimulating factor
GPC	Gel permeation chromatography
H&E	Hematoxylin and eosin
H&E	Hematoxylin and eosin
H2DCFDA	2',7'-dichlorodihydroflurescein diacetate
H2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
HBSS	Hank's balanced salt solution

HCM	Helminth-conditioned media
Hpb	*Heligmosomoides polygyrus bakeri*
HpbESP	*Heligmosomoides polygyrus bakeri* excretory secretory products
HPLC/UPLC	High/ultra performance liquid chromatography
HSQC	Heteronuclear Single Quantum Coherence
IBD	Inflammatory bowel disease
IEX	Ion-exchange chromatography
IL-10, 12	Interleukin-10, 12
ILC	Innate lymphoid cells
IP	Intraperitoneal
IPTG	Isopropyl β-d-1-thiogalactopyranoside
L5	Adult *T. suis* parasites
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
LCCM	L929 fibroblast-conditioned culture medium
LPS	Lipopolysaccharide
MPO	Myeloperoxidase
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut off
NDK	Nucleoside diphosphate kinase
NDP	Nucleoside 5'-diphosphates
NFκB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
NOD2	Nucleotide binding oligomerization domain 2
NP	Nonpolar
NTP	Nucleoside triphosphates
ODS	Octadecyl-silica
P	Polar
p.i.	Post-infection
PBMC	Peripheral blood mononuclear cells

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS-T	PBS-Tween
ROS	Reactive oxygen species
RP	Reverse phase
RPMI	Rosewell Park Memorial Institute (medium)
RT-PCR	Reverse transcriptase-polymerase chain reaction
rTsSp	Recombinant *T. suis* serine protease
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SNRP	Small nuclear ribonuclear protein
STAT	Signal transducer and activator of transcription
TBS-T	Tris-buffered saline
TC	Tissue culture
TGF-β	Transforming growth factor beta
Th1, Th2	T-lymphocyte helper 1, 2
Th1, Th2, Th17	T-lymphocyte helper Type 1, 2, 17
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNFα	Tumour necrosis factor alpha
TPI	Triose phosphate isomerase
Treg	Regulatory T-lymphocyte
TsESP	*Trichuris suis* excretory/secretory products
TSO	*Trichuris suis* ova
TSO	*T suis* ova
UC	Ulcerative colitis
UV	Ultraviolet

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Chapter 1: General introduction and Literature Review

PROJECT OVERVIEW

Rationale and Hypothesis

Multiple *in vitro* and *in vivo* studies have shown promise that helminths and helminth-derived products modulate the immune system via a Type 2 response. This has stimulated interest in the field of autoinflammatory diseases as potential therapeutic strategy, as autoinflammatory diseases are characterized by a dysregulation of the Type 1 and Type 2 arms of the immune system that lead to an excessive Type 1 response. Helminth-dependent polarization of a Type 2 response allows for a balance of the immune system to be restored. This is supported by the hygiene hypothesis, which states that increased hygiene, results in a higher incidence of immune dysregulation diseases such as asthma, hay fever, IBDs, rheumatoid arthritis and more. Here we study the potential of parasite derived molecules as immunomodulators of colitis. While other studies have shown the ability of live parasites, whole worm extract, soluble protein, and recombinant proteins to modulate inflammatory diseases, this work studies immunomodulatory proteins and metabolites in helminth derived ESP. The small molecules of helminth-derived ESP have, to our knowledge, not yet been studied for their activity in autoinflammatory disease. We are also interested in the small molecules as we hypothesize that they might comprise key modulators of the host-parasite interaction, inducers of the Type 2-polarized response seen in helminth infection and might hold therapeutic potential for autoinflammatory diseases. Small molecules are of particular interest as they are more desirable for therapeutic applications. We hypothesize that an immunomodulatory molecule(s) can be isolated from the ES of parasitic helminths using our biochemical methods.

Specific aims

1. Optimize the isolation of helminth-derived ESP into its molecular components.

2. Identify and characterize key immunomodulatory proteins in helminth-derived ESP and study their immune activity.

3. Identify and characterize a key metabolite(s) or small molecule(s) involved in the induction of a type-2-like immune response to helminths and their therapeutic potential in autoinflammatory diseases.

LITERATURE REVIEW

Inflammatory Bowel Disease

Canada currently has the highest incidence and prevalence of inflammatory bowel disease (IBD) worldwide with an alarming incidence of 7/1000 individuals (0.7%) and the incidence is increasing steadily (1). In 2018, there were an estimated 270,000 Canadians living with IBD in a population of ~37 million. IBD is an umbrella term for autoinflammatory and autoimmune conditions of the gut. Notably, IBD is sometimes mistakenly classified as autoimmune diseases because they behave similarly phenotypically. However, the mechanisms of autoimmune disorders are different from autoinflammatory diseases. Autoinflammatory diseases are caused by antigen-independent dysregulated immune responses leading to inflammation, while autoimmune diseases by definition are antigen-dependent (2), as portrayed in Figure 1. The term autoimmune implies a mistake in self-tolerance, whereas there is a lack of evidence of autoantibodies or T-lymphocytes in IBD. Some common inflammatory diseases of the bowel are Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are also reported to be increasing in prevalence proportionally.

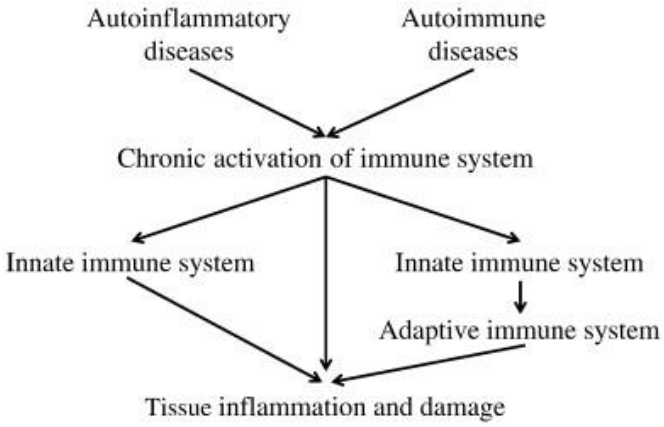


Figure 1. Mechanisms and differences between autoimmune and autoinflammatory diseases. (2)

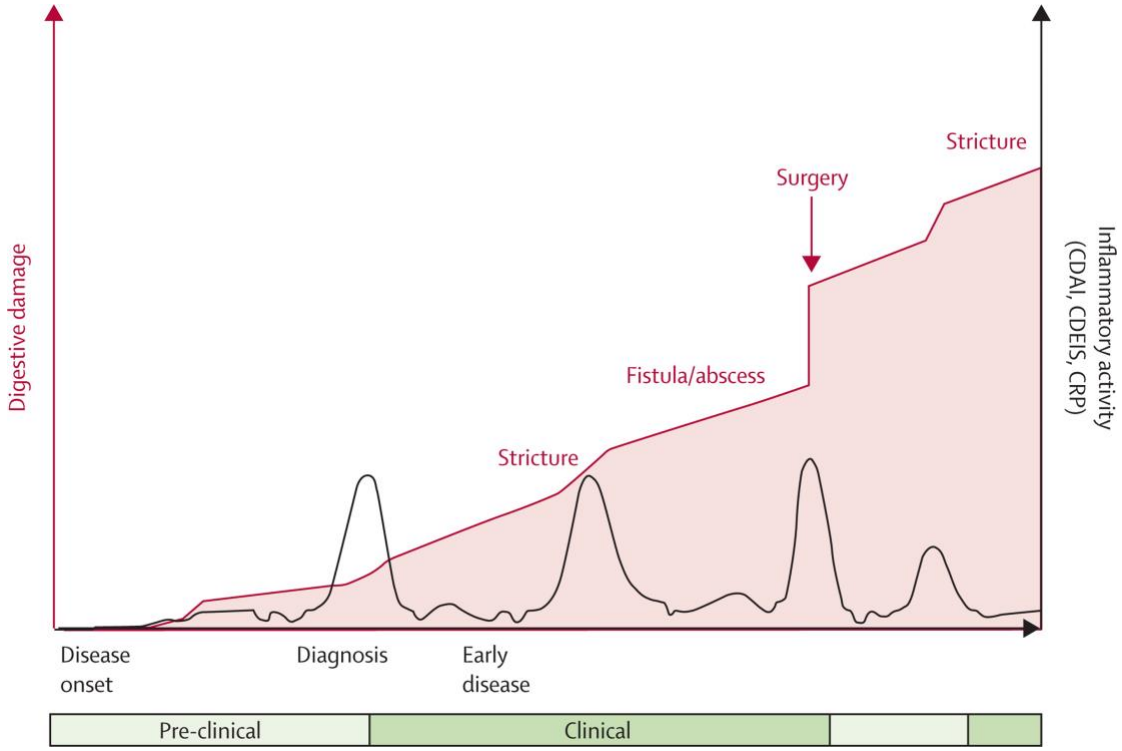


Figure 2. Natural history for a patient with inflammatory bowel disease over a lifetime. (3)

Pathophysiology of IBD

Inflammatory bowel disease is a lifelong struggle with episodes with symptoms, of abdominal pain, fever, bowel obstruction or diarrhea, dysentery, mucous secretion, and feelings of malaise (3). Diarrhea can lead to malabsorption, and subsequently malnutrition if persistent. It is known that the main driver of symptoms is dysregulated inflammatory activity. In Figure 2, the natural history of inflammatory bowel disease is exemplified to demonstrate how digestive damage scores are calculated. In general, digestive damage will increase over time and is, in part, irreversible. As seen above, the driver of this damage is the recurrent "flares" of inflammation which vary in length, severity, and treatability. These flares are unpredictable and are a significant cause of physical and mental stress in patients (4) and reduce quality of life

Inflammatory Bowel Disease

Inflammatory activity in the gut, which arises spontaneously or due to a particular trigger, results in an increase in digestive damage over time (3). IBD is of epidemiological importance as the occurrence is increasing worldwide (5), particularly in developed countries (6). IBD was originally postulated to affect mainly Caucasian and Ashkenazic Jews, until it was recently

observed that geographical status rather than race, attributed etiology more strongly to environmental factors (6).

UC and CD are disorders characterized by chronic, non-resolving inflammation that may result in lesions. According to Lennard-Jones (7), chronic inflammation can only be diagnosed once infection, ischemia, physical damage, or specific immunologic sensitivity can be excluded, because the cause of inflammation may be due to external causes. Once recognized causes of inflammation are excluded, other inclusive criteria are necessary for confirming a diagnosis. Criteria for the diagnosis of UC include chronic inflammation without the presence of granulomata, and inflammation should be localized to the rectum and some or the entire colon. Criteria for the diagnosis of CD are more complex, but in general include chronic inflammation along the entire digestive tract from the mouth to the anus, either continuous or separated by normal mucosal surfaces. CD can also include the presence of granulomata.

UC and CD are distinguished by the location of lesions in the GI tract with UC being characterized by lesions localized to the rectum and colon, and CD having lesions over the entire GI tract. UC consists of continuous lesions along the affected area, whereas CD can be patchy and discontinuous (7). The pathology in UC will be at the mucosal level, while CD can be transmural affecting the deeper layers of the GI tract. UC causes muscular thickening in the GI tract, whereas CD causes fibrosis. Finally, UC causes mucin depletion and glandular damage, while CD causes lymphoid ulcers and aggregates and granulomas.

Diagnosis and treatment of UC and CD

Diagnosis: CD and UC

Diagnosis of IBD is achieved both clinically and through laboratory testing, imaging, and gastroscopy/colonoscopy. There are overlapping findings between UC and CD, although differences exist to distinguish these disorders and treatment. Laboratory tests for CD diagnosis include: a complete blood count, a comprehensive metabolic panel, creatinine, liver function enzymes, and serum glucose. Importantly, c-reactive protein (CRP) is critical in differentiating between IBD and inflammatory bowel syndromes (IBS). The level of CRP can be used to estimate the severity of a flare up as they correlate with CD activity (8–10). CRP is the most sensitive marker for CD, although not specific, and less so for UC as it is not always elevated (10). Stool

inflammatory markers are not used routinely for diagnosis of UC or CD as they are elevated in both diseases, but these are useful in distinguishing from functional disorders such as IBS and remains useful to clinicians (11,12). Ileocolonoscopy is utilized in CD to look for aphthous ulcerations, cobblestone appearance, or discontinuous ulcerations (in between which have normal mucosa) with possible rectal sparing. Transmural inflammation found during interventions is highly suggestive of CD, while UC is limited to mucosal inflammation. Additionally, chronic UC will lead to thickening of the muscularis mucosa, which will result in chronic contracture of these muscle layers, shortening and narrowing the colon (13). This parameter is the basis of assessing colon length in experimental murine colitis experiments, as it is pathognomonic for colitis and muscularis mucosal inflammation or prevention and reversal, if treatment is successful.

Treatment: CD and UC

Since UC is localized to the colon and rectum, surgery is a possibility to remove the affected sections of the GI tract (7). Neither UC nor CD have a medicinal cure, largely because the etiology is unknown. For UC, a common treatment is an ileal pouch, which is necessary upon the removal of a section of the colon. Although this may provide the patient with relief from the pain associated with inflammation, their quality of life is drastically reduced. A comprehensive review by Panaccione *et al.* (14) demonstrated that a preferred method for a "step-up" treatment of IBD. This review describes the conventional, pyramidal approach to treatment, whereby treatments at the bottom are attempted first, and if remission is not achieved, the treatments further up the pyramid will be pursued. However, according to a systematic review (15), even the more common medications do not significantly induce remission of either UC or CD, while they may help prevent relapse. Overall, it is evident that treatments for inflammatory bowel disease are uncertain, may be debilitating, require a respectable amount of trial and error, and may greatly reduce the quality of life. The lack of a successful and safe treatment for such a prevalent and increasingly common disease calls for research to improve treatment.

Digestive damage can lead to a fistula, abscess, or scar tissue formation and causing a stricture. Worsening pathologies leads to necessary surgical removal of parts of the small intestine or colon, but this does not protect against recurrence in other parts of the GI tract. This can result in a vicious cycle that can propagate until there is not enough functional mucosa whereby malnutrition can occur, or not enough length and a colostomy bag might need to be placed.

Immune polarization: helminth-induced type II immunity

Parasites, and more specifically helminths, induce changes in immunomodulation during infection that are complicated and not well understood. However, a commonality across parasitic worms is the induction of a Type 2 immune response, whereby cells of the immune system are polarized and conditioned towards a damped inflammatory activity. This response is in opposition to the responses observed in patients suffering from IBD, whereby a Type 1 response is stimulated (16).

In addition, long-term infection with *H. polygyrus bakeri* in BALB/c mice induces T regulatory responses, *in vitro* and *in vivo*. Specifically, a subset of DCs known as tolerogenic DCs or $CD11c^{lo}$ cells are elevated following an *Hpb* infection lasting longer than 7 days (17). This subset of DCs are poor inducers of T-helper cells, but potent inducers of Foxp3 expression in spleen and mesenteric lymph node lymphocytes that favour a regulatory phenotype (T-regulatory cells) via cross-talk with $DC11^{lo}$, or tolerogenic DCs, and reduce the propensity of Type 1 inflammatory responses (17). These studies also showed that transforming growth factor (TGF-β) was required for induction of Foxp3+ cells in *Hpb* infected mice (17). Together, their data showed that induction of T-regs via tolerogenic DCs from chronic helminth infection led to a regulatory environment which could be responsible for the anti-inflammatory effects of helminths.

Helminth-mediated cellular immune regulation

Parasitic helminths modulate cellular immunity through different mechanisms and pathways. One group found that *T. suis* ESP obtained from the larval stages modulated several BMDC cytokines *in vitro* and suppressed airway hypersensitivity in an allergic airway inflammation/asthma mouse model (18). Other groups found immunomodulatory ESP in different parasites are required for the host-parasite interaction and successful establishment of an infection. For example, *Trichinella spiralis*, an intracellular myocytic parasite, is thought to utilize the proteins in ESP as a molecular mechanism to immunomodulate the host (19). For parasites causing disease, these proteins are an interesting diagnostic tool or a biomarker of infection. Other groups have found specific proteins that modulate chemically induced colitis through inhibition of neutrophil migration (20).

Evidence for helminth-derived ESP as immunomodulators of experimental disease

Numerous experimental colitis studies have demonstrated that IBD can be clinically improved with parasitic infections (16,21,22). These studies postulated that improvement of colitis was mediated by bioactive molecules in the parasite or secreted ESP responsible for immunomodulation or wound healing. We have previously shown that HPLC-fractionation of *T. suis* ESP protein identified specific bioactive proteins (23). Interestingly recombinant proteins expressed in *E. coli* recapitulated the immune modulation activity *in vitro*. These bioactive proteins included *T. suis*; nucleoside diphosphate kinase (NDK) and a triosephosphate isomerase (TPI) (23). Another group found that serine proteases (recombinant TsSp, rTsSp) constitutively expressed by *Trichinella spiralis* may be of therapeutic interest as these proteins ameliorate colitis in a murine model (24). These proteases are expressed in all life stages and likely have a crucial role in host-parasite interactions. Administration of rTsSp prior to inducing colitis diminished infiltration of colonic macrophages, reduced colonic TNF-α expression, and increased colonic IL-10 expression which correlates with decreased disease pathology. Another study showed that ESP from *T. spiralis* larvae reduced levels inflammatory cytokines secreted by DCs *in vitro*, and adoptive transfer of these ESP-treated DCs ameliorated TNBS-induced colitis in mice (25). Similarly, intraperitoneal injection of adult *T. spiralis* ESP shifted the immune response toward a T_{reg}-mediated phenotype and a reduction in inflammatory mediators. Histologically, ESP-treated mice had normal colon length and reduced intestinal disease pathology in mice (26).

Hygiene Hypothesis

In 1989, David Strachan published the initial proposal of the Hygiene Hypothesis suggesting that the correlation between increased autoimmune and autoinflammatory diseases in westernized countries could be attributed to, in part, increased hygiene (27). The rationale for this hypothesis was that human "co-evolutionary partners" have co-evolved for hundreds of thousands of years (28), are parasites and microbes present in the human gut, skin, and mouth microbiome are now lacking due to increased hygiene and changing environmental microbiota in industrialized countries. The diseases primarily on the uprise during the work by Strachan and colleagues were eczema and hay fever. This hypothesis does not recommend *reducing* hygiene, as increased hygiene has drastically reduced mortality globally by preventing pathogenic microbes (29). On the contrary, the recommendation from these authors is that the Hygiene Hypothesis should be

interpreted such that hygiene is necessary to prevent disease, while there should still be efforts made to preserve natural and essential microbes, as to not disrupt the process of immune tolerance by changing these natural balances. However, how this should be done is still not understood, and being that genetics also plays a large role, hygiene is not the only reason for the rise in dysregulated inflammation. Interestingly, a case-control study found that introduction of intestinal helminths in childhood primed immune cells in a Th2-dependent manner to protect the host from overactive Type 1 activation which would lead to inflammatory diseases later in life (30). Type 2 responses are not only responsible for parasite expulsion but help temper Type 1 responses; however, westernized lifestyles do not permit education of the immune system and possibly results in increased immune dysregulation. Consistent with this hypothesis Type 1 and Type 2 responses are thought to balance each other, and a lack of Type 2 responses may result in dysregulation seen in IBD and other autoinflammatory diseases. T-regulatory responses function in concert with Type 2 responses in dampening inflammation. Taken together, this information supports the hygiene hypothesis as an explanation for IBDs increasing at alarming rates, mainly in westernized countries which have low incidence of parasitic infections.

The hygiene hypothesis has extended further in recent decades to postulate that bacterial species have a beneficial effect in establishing a Type 1/Type 2 balance (31). Bacterial products are necessary to modulate crosstalk between the immune system and dysregulated inflammation in the body, and this can be affected by the environmental or genetic background of the host (31). This host environment includes the presence of parasites as part of the gut biome, which are absent for individuals in developed countries, dysregulating bacterial populations of the microbiome and immune tolerance.

Genetic predisposition to IBD

Epidemiological studies have shown genetics to be a risk factor in predisposition to IBD (32). However, genetics alone are not the sole predictive criteria, since both genetic and environmental factors play a role in IBD (32). Some genetic markers, nonetheless, have been shown to be associated with IBD. For example, the nucleotide binding oligomerization domain2 (*NOD2*) gene, which is involved in the innate immune system, has been significantly associated with IBD occurrence (33). NOD2 is involved in activating the nuclear factor kappa B (NFκB) which plays a role in the pathogenesis of CD, as it is upregulated in monocytic cell lineages. NFκB

initiates downstream inflammatory gene expression. Cuthbert *et al.* (34) demonstrated how individuals homozygous for genetic variants of *NOD2* (P268S, R702W, G908R, and 3020insC) have >20-fold increased risk of developing CD.

Aside from the genetic and environmental risk factors for IBD, tobacco smoking has been found to be a risk factor for UC and CD (6), while it reduces the symptoms of UC in case-control studies, with effects being dose-dependent (6,7). Conversely, development of disease favours smokers rather than non-smokers. Additionally, poor diet and white-collar occupation are risk factors for IBD (6).

Clinical trials

Clinical trials have investigated the safety and efficacy of non-human parasitic helminths as a therapeutic for IBD (35–39) or other immune disorders such as allergies, asthma, and multiple sclerosis. These studies investigated *Trichuris suis* ova (TSO) as a treatment, where parasitic ova or eggs of this parasite administered to patients that hatch to larvae and adult worms transiently colonize the gut were found to improve the US symptoms (40). Studies by Sandborn and colleagues showed that TSO treatments were safe (37).

Chapter 2: A standardized methodology to purify helminth-derived ESP was optimized to study and characterize helminth products

ABSTRACT

Parasite excreted and secreted products (ESP) have attracted increasing interest for studying different facets in parasite biology that include host-parasite interactions and understanding host immune mechanisms. However, the composition and preparation methods of ESP vary among research groups and may account for differences in experimental outcomes. To mitigate these issues in our studies, we aimed to standardize and optimize a protocol that we and collaborating labs use to assure reliability and experimental reproducibility. Healthy parasites were cultured using this standardized protocol in medium, referred to as helminth-conditioned medium (HCM). HCM was processed systematically, by sterilizing media by filtration and concentration of proteins with MW > 3,000 Da. Crude proteins were separated using gel-permeation chromatography and fractions tested using BMDM or BMDC culture prior to LC-MS/MS analysis to identify bioactive proteins. The small molecule metabolites in ESP (<3,000 Da) were collected in the flow through from the protein concentration and the subsequent purification is described in this chapter. This step yields the initial division between proteins and small molecules, which are both then purified independently as described in this chapter. The bioactivity of the small molecules or metabolites is described later, in chapter 4. Proteomic analysis allowed the characterization of candidate proteins to be studied for their immunogenicity. These proteins were produced as recombinants and tested on BMDM.

INTRODUCTION

Parasites have a remarkable immunomodulatory potential that is involved preventing worm expulsion from the host (41). To facilitate this biological activity, parasites secrete a myriad of molecules and components that are involved in for example, host cells interactions, degradation of host proteins, host nuclear targeting, and exosome communication. The complexity associated with parasite immune regulation is a strong contributor to the challenges faced in vaccine development against multicellular parasites. Conversely, helminth ESP opens doors to study the host-parasite interaction and host immune regulation. More importantly, ESP can reveal opportunities for exploitation of parasite-derived immune-stimulatory molecules as tools to better

understand immune dysregulation and expedite development of therapeutics for treating disorders such as autoimmune and autoinflammatory disease therapy. Studies on helminth-derived ESP have characterized the components of ESP, both proteins (25,42) or metabolites (43), the use of ESP in animal models (22,44,45), for example, for the treatment of experimental colitis.

Helminth-derived secreted and secreted products have been a point of interest for many years, for different purposes. Other studies have characterized the components of ESP, both proteins (25,42) or metabolites (43), as well as their use in animal models (22,44,45), for example, for the treatment of experimental colitis. Recently, studies have begun to emerge on the characterization of the metabolome of helminths. One group looked at the potential pharmacological targets of metabolomic players from *Nippostrongylus braziliensus* and *Trichuris muris* (43). Moreover, the study of these ESP benefits the understanding of the host-parasite interaction, which not only sheds light on the immune responses studied but also the potential drug targets for anti-parasitic medications. For example, one group studied ESP from *N. americanus* and revealed a response pathway which elucidated a mechanism for resistance to reinfection, which is rarely seen among helminths (46).

Other areas of interest in the realm of helminth-derived products include extracellular vesicles (EV). One group studied the EVs from *Fasciola hepatica* and *Echinostoma caproni* (47). They demonstrate both the existence and secretion of EVs from these parasites, as well as the EV uptake by host cells. This demonstrates a clear line of communication between the parasite and the host, which opens up new possibilities about the immunomodulatory ability of parasites.

METHODOLOGY

Isolation of helminths

T. suis larvae and adult worms were harvested from infected pigs (*Sus scrofa*), as previously described (23). Briefly, mixed-sex healthy pigs (crossbred: Landrace × Yorkshire × Poland China) were inoculated with a single dose of infective *T. suis* eggs (2×10^4 eggs/pig) (48) and following euthanasia, *T. suis* larval stages and adult worms were collected at various time points (10-54 days post-inoculation (p.i.)) from the cecum and proximal colon. Larval stages were isolated after pig tissues were washed free of adherent fecal material with warm tap water and then incubated for 3 h in PBS. Adult worms were manually removed from the tissue using forceps.

Larva (28-day) were purified by floatation on a 40% Percoll gradient and adults (L5) were picked individually free of debris. Worms were washed five times (10 min each), by sedimentation in wash buffer (HBSS pH 7.2, 500 U/ml penicillin G, 500 µg/ml streptomycin, 1.25 µg/ml amphotericin B, and 350 µg/ml chloramphenicol) at a ratio of 10 parts wash buffer to 1-part worms.

Adult *Heligmosomoides polygrus bakei* (*Hpb*) worms were isolated from infected BALB/C mice gastrointestinal tract at ~18-21 days p.i.(49). Briefly, the small intestine was placed in a minimal amount of sterile PBS with antibiotics (50 µg/ml gentamycin, 100 U/ml penicillin G and 100 µg/ml streptomycin) and opened longitudinally. The mucus layer was gently scraped free, and worms were removed from the intestinal tissue using forceps and placed 20–25 ml of PBS with antibiotics and incubated at 37 ^{0}C to dissociate host tissue. Tightly coiled viable adult worms were used to produce excretory/secretory products. Worms were extensively washed with multiple changes of PBS containing antibiotics to remove host tissue debris and fecal matter and cultured as previously described (49).

Dirofilaria immitis larva were isolated as previously described (50). Briefly, microfilaria (Missouri strain) were obtained from infected dog blood provided by the FR3 Molecular Resources and larva were isolated as previously described (50). Blood samples were diluted sodium carbonate and filtered through a 3.0 mm polycarbonate membrane filter and after several washes in PBS and 500 microfilariae were incubated in DMEM media containing 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 10 µg/ml gentamicin in a 24-well plate and the conditioned media was collected after 24 and 48 h.

H. diminuta conditioned media was provided by our collaborator Dr. Derek McKay at the University of Calgary. Briefly, adult *H. diminuta* worms were isolated form the small intestine from infected rats and then was several times with PBS to remove intestinal contents. Worms (1 worm/5 ml) were incubated at 37°C in RPMI 1640 containing penicillin and streptomycin (Sigma) for 18 h, with media replaced every 5 h. The conditioned media was filtered with a 0.2 mm filter and proteins were concentrated a 3K MWCO Amicon® Ultra Centrifugal Filter unit (Millipore).

Isolation of helminth derived excretory-secretory protein and metabolites

i. Isolation of protein and exosomes

To separate protein in solution from exosomes, ESP concentrated in a 3K MWCO Amicon Ultra Centrifugal Filter unit were centrifuged at 49, 000 rpm for 1 hour at 4°C on BeckmanCoulter tabletop ultracentrifuge using a TLA100.3 rotor. The protein concentration in the supernatant and the exosome pellet were quantified using a Bradford Assay (Cat#. 23236.) and proteins and exosomes were stored at -80°C.

ii. Isolation of nonpolar metabolites

Nonpolar metabolites in the 3 kDa MWCO filtrate were acidified (pH 4) by adding formic acid to a final concentration of 0.1% and passed through Strata C18-E reversed phase solid phase extraction column (Phenomenex (Cat#. 8B-S001-HCH-T)) activated with 10 ml of 100% methanol and then washed with 10 ml dH_2O. The C18-E column flow through was collected for future isolation of polar metabolites and the C18-E. washed with 5 column volumes of 0.1% formic acid and the bound metabolites were eluted using a step gradient of 10 ml of 25%, 50% or 100% acetonitrile (ACN) supplemented with 0.1% formic acid. The eluates were collected, and the organic solvent removed using a SpeedVac concentrator

iii. Isolation of polar metabolites

The flow through from the C18-E column was then passed through an Oasis HLB LP Extraction Cartridge column, for extraction of polar compounds. In the same manner, the column was eluted with 25%, 50% and 100% ACN each with 0.1% formic acid (10 ml each). The eluates were collected, and the organic solvent removed using a SpeedVac concentrator.

Prior to bioactivity analysis of nonpolar and polar metabolites dried fractions were sterilized by treating samples with 50 µl of with 70% ethanol and the ethanol was allowed to evaporate in a biosafety cabinet. Tissue culture grade water was used to re-suspend compounds to the desired concentration. Samples were stored at -20°C. Metabolite recoveries are listed in Table 2.

Identification of ESP protein and recombinant protein expression and purification.

From the *T. suis* excretome/secretome, ion exchange chromatography fractions were obtained, eluates were pooled, and these eluates were tested on BMDM for production of TNF-α and IL-10 as described here and in our previous work (23). Fractions with immunomodulatory activity were sent for LC-MS/MS analysis at the Proteomics platform of the CHU de Quebec Research Center. From these sequences, a shortlist was created of potentially bioactive proteins such that they can be expressed as recombinants for analysis. Proteins of interest include nucleoside diphosphate kinase (NDK, D918-00383), triose phosphate isomerase (TPI, D918-00560), and glucose-6-phosphate isomerase (G6PI), small nuclear ribonuclear protein (SNRP, D918-00505) and Niemann-Pick type C2 protein (NPC2). Parasite cDNA was amplified using specific primers and cloned into pET15b bacterial plasmids (Millipore). Recombinant proteins were produced using an *Escherichia coli* ER2566 chemically competent cells transformed with pET15b containing an open reading frame for the protein [28]. 15 mM of benzyl alcohol was added to mid-log phase bacterial cultures and protein expression was induced using 0.1-0.2 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) overnight at 16°C. Bacterial cells were pelleted and resuspended in lysis buffer (40 mM Tris -HCl pH 7.5 and 140 mM NaCl). The protein was purified by affinity chromatography, using nickel-NTA beads. Supernatants were concentrated on the beads, then the flow-through was passed through the column another 2 times for a total of 3 passes to allow for maximum binding. A stepwise elution gradient was used by sequentially washing with 40 mM, 80 mM, 160 mM, and 240 mM of Imidazole substituted with 40 mM Tris-HCl and 140 mM NaCl. Each fraction was collected separately, concentrated and the buffer was exchanged into 40 mM Tris-HCl pH 7.5, 140 mM NaCl 10 mM dithiothreitol (DTT), and 10% glycerol using a 3 kDa MWCO Amicon Stirred Cell filtration unit. The protein was then aliquoted and snap frozen with liquid nitrogen to be stored at -80°C.

Metabolite purification using high performance liquid chromatography (HPLC) or Ultra performance liquid chromatography (UPLC)

Parasite nonpolar metabolites isolated from conditioned culture media using solid phase extraction were further resolved using reversed HPLC chromatography or UPLC chromatography. Components in the *T. suis* and *Ascaris* crude metabolite (either 25% and 50% ACN from C18-E) were fractionated on a Beckman System Gold binary pump system equipped with a Beckman

reversed phase ODS column (5 µm, 4.6 ID x 15 cm), a photodiode array UV/Vis detector, and a 1.0 ml injection loop. For each experiment ~ 250 mg was injected and the column equilibrated with 0.1% formic acid and the column was developed with a 0-80 0.1% formic acid in acetonitrile of 60 minutes at a flowrate of 1.0 ml/min. The effluent was monitored at 220 and 280 nm and 1.0 ml fractions were collected, and the solvent was removed on a SpeedVac and the fractions were reconstituted in sterile PBS for screening in bioassays using BMDM.

Alternatively, crude metabolites from *Dirofilaria immitis* and *Heligmosomoides polygyrus bakeri* were fractionated on a ThermoFisher Scientific Ultimate 3000 RS UPLC system equipped Agilent Eclipse Plus C18 column (1.8 µm, 2.1 150 mm), a UV/Vis detector, and a 100 µl injection loop. The column was equilibrated with 0.05% formic acid and 200 µg of crude samples was injected and the column was developed with a 0-60% 0.05% formic acid in acetonitrile over 40 minutes at a flowrate of 0.3 ml/min and the effluent was monitored at 234 nm. Eighty 150 µl fractions were collected and the solvent was removed on a SpeedVac. Dried residue was then visualized using a 360 nm UV light to detect possible fluorescence emitting metabolites. The dried residue was sterilized with a mist of 70% ethanol and the ethanol was allowed to evaporate in a biosafety cabinet and the residue was dissolved in PBS and tested using a BMDM bioassay.

NMR

Analysis of the purified NP686 bioactive metabolite isolated from *D. immitis* conditioned culture media was analyzed on at the Université de Montréal at the Structural Biology Platform by Dr. Normand Cyr. The purified NP686 (~100 µg) was dissolved in 500 µl of 50% deuterated D_2O, deuterated methanol and transferred to a 5 mm NMR tube and the sample was analyzed 25°C on a Bruker Avance Neo 600 MHz with a TCI 5 mm cryo-probe. This instrument was used to perform a 1D, 2D double quantum filtered COSY, and ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) experiments. 1D ^{13}C NMR experiments were performed on a Bruker AV700 NMR spectrometer at the Université de Montreal, Department of Chemistry.

Bone marrow derived macrophage and dendritic cell differentiation and culture.

BMDM and BMDC were generated from 6–8-week-old C57BL/6 mice. BMDC were obtained by differentiating precursor cells, as previously described (23). In brief, bone marrow cells were differentiated into BMDC for 7 days in complete BMDC medium (RPMI supplemented with 10%

FBS, penicillin, 100 µg/mL streptomycin, 50 µg/mL) enriched with granulocyte-macrophage colony-stimulating factor (GMCSF), (Cat#. 78017.1). BMDM were obtained by differentiating precursor bone marrow cells resuspended in BMDM culture medium (DMEM, 10% FBS, penicillin, 100 µg/mL streptomycin, 50 µg/mL) supplemented with 15% L929 fibroblast-conditioned culture medium (LCCM) on day 0 in tissue culture (TC)-treated plates. Next day, non-adherent cells were collected to include only cells that are undifferentiated, cells were pelleted and resuspended in non-TC-treated plates in complete medium supplemented with 30% LCCM for 7 days, exchanging the medium on day 3 to remove any non-adherent precursors. On day 7, medium was removed from adherent cells and the cells were collected by adding ice-cold HBSS and scraping gently with a cell scraper. BMDC and BMDM culture purity was assessed by flow cytometry, monitoring CD11c, and CD11b and F4/80 co-expression, respectively.

PBMC monocyte isolation and cell culture.

PBMCs were obtained from fresh human blood using Ficoll-Paque density gradient media (Cat#. 17144002). The PBMC layer was obtained and stained with Wright's stain to assess monocyte percentage. Cells were plated to obtain a monocyte concentration of 1×10^6 cells/mL in culture medium (RPMI 1640, 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin), and incubated overnight at 37°C, 5% CO_2. The next day, medium was removed, and fresh medium was added, leaving only adherent monocytes with which assays were carried out.

BMDM culture treatment and stimulation.

Innate immune cell cultures were treated for 4 h with crude or purified ESP or recombinant proteins or left untreated, as indicated. Fresh medium was added with or without stimulation with LPS from *Salmonella enterica* (Sigma-Aldrich) to BMDM (10 ng/mL) and cultures were incubated for 18-20 h with the stimulating factor. Supernatants were collected after 20-24 and were assayed for secreted cytokines using enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay.

Secreted cytokines in the culture supernatants from treated BMDC, BMDM, or PBMC monocyte cultures were measured by sandwich ELISA. For analysis of cytokine secretion in murine experiments, plates were coated with the following capture antibodies: anti- IL-10 (clone JES5-

16E3), anti-IL-12p35 (clone C18.2), anti-TNFα (clone 1F3F3D4) (eBioscience), anti-IFNγ (clone R4-6A2), and anti-IL-4 (clone 11B11) (BioLegend). For analysis of cytokine secretion in human PBMC monocytes, plates were coated with anti-IL-10 (clone JES3-9D7) or anti-TNFα (clone Mab1) eBioscience. Wells were blocked with PBS 0.05% Tween 20 (PBS-T) with 1% BSA (Sigma-Aldrich) for 1 h at room temperature (RT), then culture supernatants were added to wells and incubated O/N at 4°C. Next day, the following biotinylated antibodies were used for murine experiments: anti-IL-10 (clone JES5-2A5), anti-IL-12/IL-23p40 (clone C17.8), anti-TNFα (clone XT3/XT22) (eBioscience), anti-IFNγ (clone XMG1.2), and anti-IL-4 (clone BVD6-24G2) (BioLegend). Similarly, the following biotinylated antibodies were used for experiments using PBMCs: anti-IL-10 (clone JES3-12G8) and anti-TNFα (clone MAb11) (eBioscience). Concentrations were calculated from standard curves generated using linear regression analysis of data obtained from serial dilutions using recombinant murine or human IL-10, IL-12p40, IL-12p70, and TNFα (eBioscience) standards.

RESULTS

Protocol for isolation and identification of ESP components was optimized

The workflow for the purification of ESP is described in detail in the methods. Briefly, crude culture medium was obtained from a 24 h culture of healthy parasites. The same methodology for culture and purification of ESP was applied for all helminths studied: *T. suis, A. suum, H. diminuta, H. polygyrus bakeri, and D. immitis.* Helminth-conditioned media was centrifuged or filtered with a large pore-filtration paper to remove insoluble debris, obtaining the soluble excreted and secreted products (ESP). Once the ESP was obtained, a series of steps were performed to identify and analyse the components of the ESP. Firstly, the ESP were filter sterilized and then separated into large and small molecule components using a 3 kDa molecular weight cut off (MWCO) centrifugal filter and/or Amicon stirred cell nitrogen concentrator, to concentrate the protein fraction (the retentate of the filtration device) and to obtain the flow-through containing the small molecules. Analysis of the large molecule fraction was done by various methods to characterize the proteins present (see next chapter).

The soluble and the insoluble ESP display different banding patterns on sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Figure 4). Well #1 contains

crude ESP, prior to removing insoluble debris or exosomes by centrifugation. The crude ESP was centrifuged at 49k x g yielding a soluble supernatant and a pellet containing insoluble materials as well as pelleted exosomes. Well #2 represents this supernatant while well #3 represents the exosome fraction. It can be observed that while there are bands which are present in both fractions, there are protein bands that appear in #2 that do not appear in #3 and vice versa. The following chapter refers to the varying immunomodulatory activity found in each of these fractions (Figure 9).

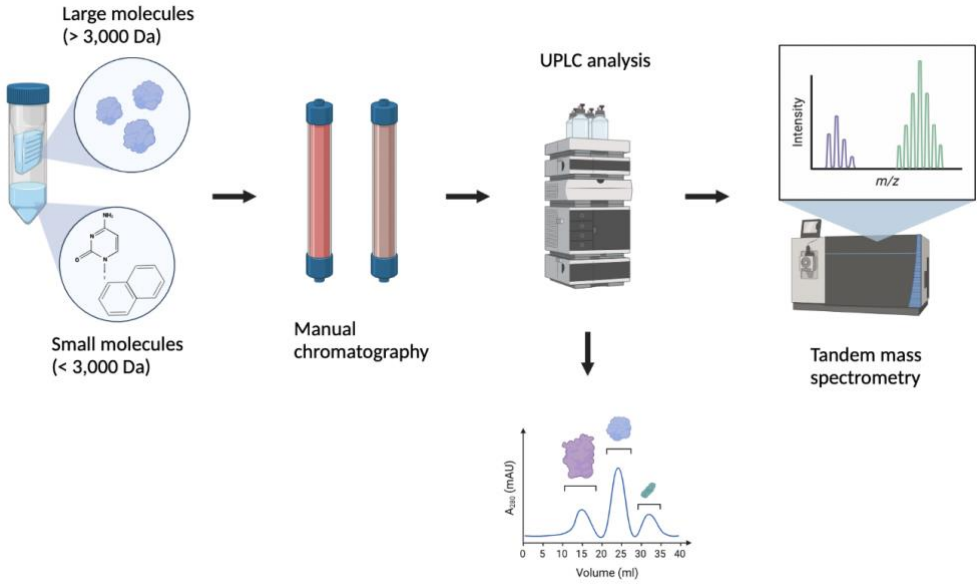


Figure 3. Workflow of separation of helminth-derived ESP using biochemical methods.
From left to right: Amicon separation of large from small molecules using a 3 kDa molecular weight cut off (MWCO) device. Manual chromatography using reversed phase followed by polar chromatography columns. UPLC-MS/MS separation of crude fractions that were obtained by manual chromatography, and collection of fractions. Tandem mass spectrometry analysis of crude or fractions.

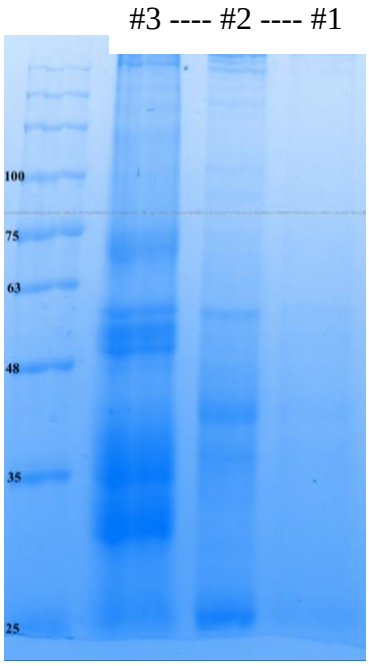


Figure 4. SDS-PAGE analysis of H. diminuta ESP.
Lane #1: Crude ESP. Lane #2: 49,000 rpm supernatant. Lane #3. Exosome fraction. SDS-PAGE analysis of H. diminuta shows differential protein composition in fractions from different stages of purification

Separation and purification of small molecule fraction allows further investigation of components

We have studied *T. suis* ESP protein previously (23), but we are also interested in the small molecular weight modulators of the host-parasite interaction, inducers of the Type 2-polarized response found in the ESP. We hypothesize that they are a key component of the response seen in helminth infection and might hold therapeutic potential for autoinflammatory diseases. We also hypothesize that bioactive proteins and metabolites secreted in the ESP synergize to modulate the host immune responses or alter the status of the host gut microbiome. The immunomodulatory potential of small molecules is discussed in subsequent chapters, however, the protocol used for purification of these molecules is presented here.

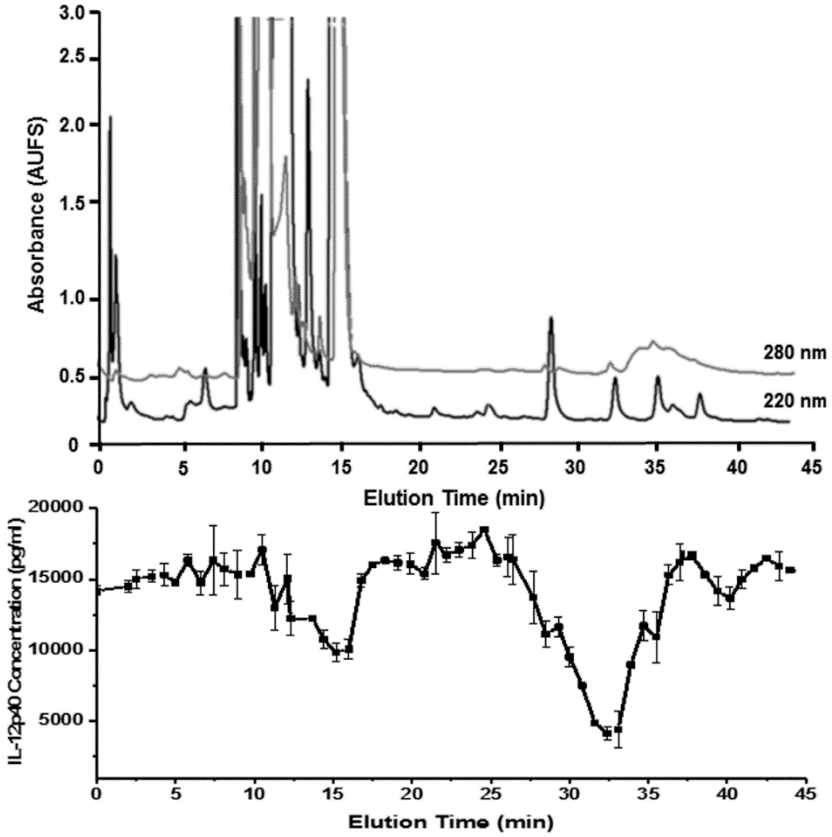


Figure 5. HPLC separation of T. suis 50% ACN fraction.
Top. Spectra for 220 and 280 nm of TsNP50. Bottom. Bioactivity screening for IL-12 secretion inhibiting fractions using the BMDM treatment assays described in the methods.

As described in the methods, the methodology focuses on separated polar and nonpolar molecules using manual chromatography and subsequently using preparatory HPLC to increase the specific activity of immunomodulatory molecules. First, nonpolar molecules or molecules with nonpolar moieties were bound to a reversed-phase, nonpolar column and eluted in a stepwise fraction to produce biochemically different fractions and further purify the compounds based on similar properties. Following this, the flow through from the reversed-phase purification was bound to a polar column to extract polar molecules or molecules with polar moieties which did not come out in the first extraction. This column was eluted in the same stepwise fashion as the reversed-phase, and fractions were collected.

Based on immunomodulatory activity as tested in biological assays described in subsequent chapters, candidate fractions were then advanced to further purifications. Namely, preparatory HPLC was utilized to obtained increasingly pure fractions, and these fractions were again tested for bioactivity, depicted in chapter 4. Fractions with bioactivity then were analysed for their

molecular components and their structures. These fractions are depicted by their 234 nm HPLC tracing (Figure 6, left panel) and the variable autofluorescence between fractions is represented (Figure 6, right panel) and suggests chemically different molecules among the fractions.

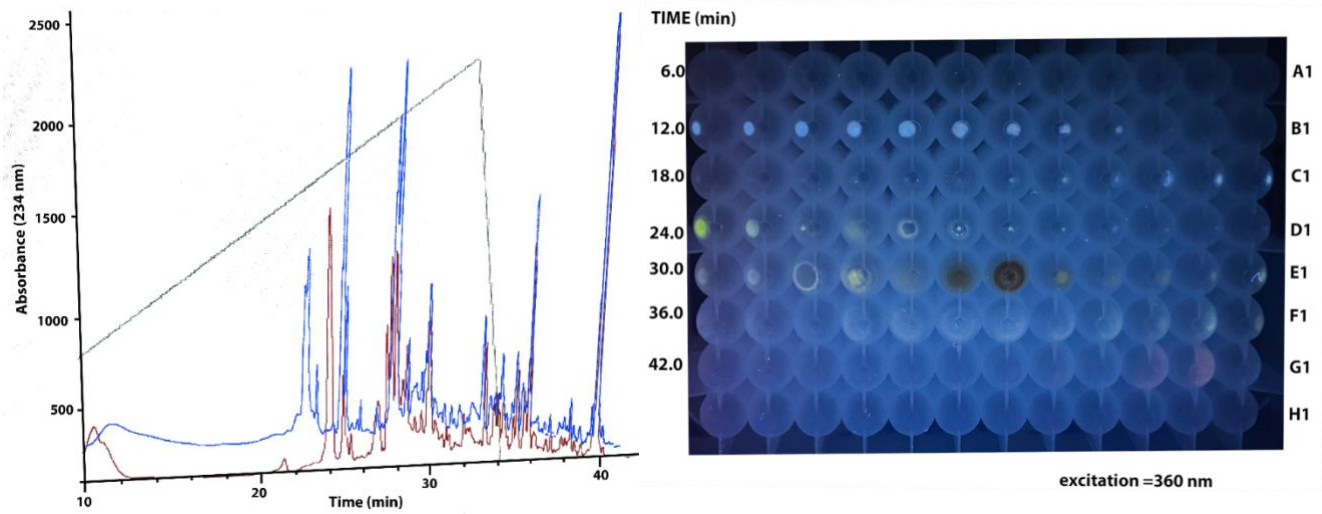


Figure 6. UPLC fractionation of the D. immitis 50% ACN fraction.
Left: UPLC tracing of two D. immitis nonpolar crude fraction batches. Right: 360 nm excitation of dehydrated, fractionated samples.

DISCUSSION

Standardized methods of parasite culture and purification of ESP are required to reliably and reproducibly draw conclusions about their immunomodulatory potential or other biologic processes. This work describes a protocol designed to isolate and extract the bioactive components of helminth-derived excreted and secreted products to study their immunomodulatory potential specifically for inflammatory bowel disease. However, studying the impact and mechanisms by which ESP modulate inflammation can be translated to other autoinflammatory or autoimmune diseases as well, such as rheumatoid arthritis. These methods describe a streamlined approach for the extraction of both ESP protein and small molecules as well as how to screen for immunostimulatory helminth-derived molecules *in vitro* and *in vivo* in IBD and inflammatory disease models.

In summary, parasites are cultured according to the methods described. The supernatant of the culture is isolated from the parasites and is referred to as helminth-condition medium (HCM). The barrier to studying the host-parasite interaction is that to study the parasite and its excreted

products, one has to remove them from the host environment. Therefore, HCM is analogous/mimics to the products secreted into the environment of the intestine during a helminth infection (excreted/secreted products; ESP); and the secretome of the parasite in this medium represents the products that are secreted in a parasitic infection, allowing us to study the players which on the immune system in a natural infection.

The medium is first separated from the crude into soluble and insoluble components. Following this, it is separated into large and small molecules, also referred to as metabolites, and then purified further based on their biochemical properties. The large molecules can be studied for the presence of proteins and exosomes. As shown in Figure 4, the stepwise fractionation of the protein impacts the makeup of the fractions, and consistency in the protocol is paramount to maintaining reliable results. In this separation, the fractions were molecularly different, and if these steps were not performed or were performed differently, the outcome of the analysis would be different. In the next chapter, the immunomodulatory potential of the fractions is tested.

The small molecules include metabolites of less than 3 kDa, which include metabolic breakdown products, signalling molecules, amino acids, and possibly anything produced by the organism under study. In this protocol, we describe the extraction of different components of the ESP of parasitic helminths, particularly, *Ascaris suum*. Large molecules are simply concentrated and tested in the bioactivity assays for immunomodulatory properties. The small molecules are separated based on polarity using reverse phase and normal phase chromatography, including manual column chromatography as well as high-performance liquid chromatography (HPLC). Reverse phase columns are used to produce a gradient from the least nonpolar to the most nonpolar compounds, and normal phase columns are used similarly to produce a gradient from the least polar to the most polar. These semi-crude fractions were investigated in the bioactivity assays described below to determine which fractions contain bioactivity, which were then advanced to a higher resolution of separation by RP-HPLC. These methods allow the investigation of small molecules based on their chemical properties, and since we were interested in investigating nonpolar small molecules these methods allow to increase the specific activity of those. Because of the multifaceted host-parasite interaction, it is important to increase specific activity because there may exist molecules with opposing effects in a crude sample of HCM. Therefore, if we do

not study the ESP in a systematic fashion such as the one described here, it is possible to mask the biological effects of potentially immunostimulatory molecules.

Different parasites studied in this work seem to have different patterns of production of metabolic by-products as well as excretion and secretion patterns. While we hypothesized that larger parasites would secrete proportionally more ESP protein and metabolites than the smaller parasites, this was not found to be the case. *T. suis*, *H. polygyrus bakeri* and *D. immitis* appeared to produce relatively more metabolites upon their purification than did *A. suum.* It is possible that the worm count plays a role in the amount of ESP produced, as we culture *A. suum* at a lower concentration than the smaller worms. However, of interest for our studies remains not the amount of ESP but the components. We are interested in studying the immunomodulatory capability of the parasites and therefor the crucial factor for our studies is the amount of immunomodulatory compound is secreted. Here, we describe the identification of a molecule of a molecular weight of 686.215 amu. This molecule is found in greatest quantities in ESP from *T. suis, H. polygyrus bakeri* as well as *D. immitis.* Future aims of this would be to attempt to detect the molecule in the blood of a *D. immitis* infected host and to further study the host target of the molecule.

In conclusion, we describe a methodology for the isolation of soluble extract, here we use the methods for the study of the host-parasite interaction for interest in immunomodulatory activity, particularly in IBD. These methods can be extrapolated to the isolation and purification of any organic isolate, such as plant matter, solubilized tissues, blood, etc. The same methodology can be applied to increase the specific activity of a particular or unknown compound in a crude extract in order to study the compound from its natural source, and subsequently it can be studied further as is described in the subsequent chapters of this work.

Chapter 3: Immunomodulatory proteins found in helminth-conditioned medium

ABSTRACT

Parasites have a complex and intimate interaction with their host. The complexity stems from the long history of co-evolution between every living mammal and their respective parasites. The selection pressure of the host immune system on parasite evolution has resulted in a modulation of the host immune system by the parasite with specific mechanisms to evade and modulate the immune attack on them. Here we study the protein component of helminth ESP for its immunomodulatory activity in BMDM, BMDC, and PBMC monocytes. We study the crude ESP of *T. suis, A. suum*, and *H. diminuta*, and, additionally, study the individual proteins of the *T. suis* ESP to isolate and characterize specific key modulators. Our methodology included obtaining the ESP using the protocol described in the first chapter. *T. suis* ESP were fractionated based on size with GPC. Fractions were collected from these purifications and assessed for bioactivity in BMDM. The modulation of secretion of TNFα in the supernatants by BMDM is measured using ELISA, and candidate fractions were analysed by LC-MS/MS and sequences that were present were analysed. The fractions contained both known and unknown sequences, and candidate proteins were produced in an *E. coli* expression system and purified for use in bioassays to test their individual immunomodulatory potential. Recombinant proteins found to have immunomodulatory activity in these bioassays include a nucleoside diphosphate kinase (NDK), triose phosphate isomerase (TPI), and glucose-6-phosphate isomerase (G6PI). Specifically, these three recombinant *T. suis* proteins suppressed LPS-dependent TNF-α secretion in myeloid-derived cells.

Moreover, this work studies the immunomodulatory activity of *A. suum* ESP. We found that *A. suum* had comparable bioactivity to *T. suis.* Specifically, AsESP suppressed LPS-dependent TNFα secretion and independently induced IL-10 secretion. Similarly, *H. diminuta* ESP were also tested for TNF-α modulation. Specifically, soluble HdESP were separated into a supernatant and pellet from a high-speed spin yielding a soluble protein fraction and exosome fraction. Notably, the only fraction that gave suppression was the supernatant of the high-speed spin, suggesting that subtraction of exosomes revealed the immunomodulatory potential of key players in the ESP, which were otherwise masked by the activity of components in the exosome fraction.

The work in this chapter work highlights the parasites which we found to have ESP that are immunomodulatory *in vitro*, as well as the specific protein components of ESP which have been specifically evidenced to modulate cytokine secretion in LPS-stimulated BMDM, BMDC, and PBMC monocytes.

INTRODUCTION

Parasites, along with the other organism's native to the human microbiome, have long evolved alongside their host, eliciting a co-evolutionary pressure in both directions. Throughout this evolution, helminths have developed the ability to secrete immune-regulatory factors that prevent expulsion of these large multicellular organisms. Thus, our immune system's ability to expel these organisms is quite limited (51). However, parasitism from helminths rarely results in death of the host even though it often does result in significant morbidity including malnutrition.

Clinical trials have shown the potential for parasites in their ability to modulate diseases of immune dysregulation. Weinstock and colleagues have shown the safety and efficacy of *T. suis* ova (TSO) in the remission of disease in Crohn's disease (35,39,52,53). Moreover, O'Neil and colleagues demonstrated similar effects of the hematophagous hookworm *Necator americanus* (54). Specifically, they found a decreased Crohn's disease activity index (CDAI) in patients receiving *N. americanus* as therapy via percutaneous administration.

It is postulated that the benefits seen in clinical trials can be explained by the fact that excreted and secreted products of the parasite have specific host targets which modulate immune dysregulation. It has been described that the most likely manner in which helminths communicate with the host on a molecular level is through the release of soluble mediators, and these can be enzymatic in that they ligate or degrade their target, interact with host receptors, target gene expression directly or indirectly, etc (55). One way this immune modulation is possible is through mimicry (56), such as in the case of a *Hpb* TGF-β mimic that drives T-reg production and other cytokine mimicry suppressing inflammatory mediators. There are several ways that secreted products can influence their targets, and each secreted product might exhibit a different mode of action and different target to orchestrate a balanced response and successful host-parasite interaction.

As a whole, it is well known and described that helminths induce a Type 2 response and exhibit their effects in this manner (57). For example, ESP from *Hpb* used to prime DCs induced differentiation of CD4+ T-regs such that they produce IL-10 (58). Additionally, the ESP reduces LPS-and other TLR-agonist-dependent IL-12p70 secretion (58). Another group studied *Hpb*-infected mice, and found that they had macrophages with reduced nitrous oxide production, as well as reduced cell proliferation in mesenteric lymph nodes (59). These findings suggest that the excretome produced by the parasites contains compounds which interact with the macrophages such that nitrous oxide pathways are affected. *Schistosoma mansoni* and *Ancylostoma caninum* ESP have the ability to improve colitis in TNBS treated mice (60). Namely, infected, colitic mice had a lower clinical score, improved macro and microscopic inflammation score, decreased MPO activity than non-infected colitic mice (60). *Toxocara canis* has also been shown to induce a Type 2 type response secondary to its ESP (61).

A large part of helminth's molecular communication with the host is accomplished through ESP, as these products clearly have specific targets which modulate biological pathways in the host. The purpose of this chapter is to exemplify the bioactivity of the ESP proteins of *T. suis*, *A. suum*, and *H. diminuta.* Their ESP were all produced in the same manner, and the proteins were the molecules of interest for the purpose of these data. Specifically, in this chapter, we aimed to show that the protein excreted from these parasites induce a Type 2 response *in vitro*, and individual proteins can be systematically teased out from the crude and their bioactivity can be revealed.

Many *Trichuris suis* proteins, including those discussed below, have homology with human proteins, which makes them of interest for studying their mechanism of action during *T. suis* infection. Nucleoside diphosphate kinase (NDK) catalyzes the phosphorylation of nucleoside 5'-diphosphates (NDP) to nucleoside triphosphates (NTP) (62). This enzyme is demonstrated to function in multiple pathways and has been implicated in tumor metastasis (63,64). Triosephosphate isomerase (TIM) catalyzes the interconversion dihydroxyacetone phosphate and glyceraldehyde-3-phosphate in glycolysis and gluconeogenesis (65). NDK and TIM have 75% and 83% sequence identity with their human homologues, respectively. *T. suis* small nuclear ribonucleoprotein (SNRP) has 61% sequence identity with the human small nuclear ribonucleoprotein homologue. Glucose-6-phosphate isomerase (G6PI) is a known glycolytic

enzyme in humans and other mammals, which plays the role of interconverting glucose-6-phosphate and fructose-6-phosphate. G6PI from *T. suis* has 70% sequence identity with the human G6PI. Interestingly, anti-G6PI antibodies are present in humans with inflammatory arthritis, such as rheumatoid arthritis, which is also an autoimmune disease (66). This suggests that some of these proteins may be immunostimulatory and play a role in immune dysregulation when they are missing or mutated. It is unclear what the role of parasite proteins may be in ameliorating disease, but studies to determine the underlying mechanism of action of the exogenous proteins may uncover such information.

METHODS

See the following methodology from Chapter 2:

- Isolation of helminth derived excretory-secretory protein and metabolites
- Identification of ESP protein and recombinant protein expression and purification.
- Metabolite purification using high performance liquid chromatography (HPLC) or Ultra performance liquid chromatography (UPLC)
- Bone marrow derived macrophage and dendritic cell differentiation and culture.
- PBMC monocyte isolation and cell culture.
- BMDM culture treatment and stimulation.
- Enzyme-linked immunosorbent assay.

RESULTS

T. suis ESP were purified and immunomodulatory proteins were identified

Excreted/secreted protein found in the large molecule fraction, or retentate of the 3 kDa MWCO filtration of the crude helminth conditioned media, as described in the methods (Figure 3). PBMC monocytes are treated with ESP protein as described in the methods, for 4 h and then stimulated with LPS or not overnight. Next day, cell culture supernatants are collected post-stimulation and are assayed for cytokine modulation via ELISA. Cells treated with ESP from *T. suis, A. suum,* or *H. diminuta* secreted significantly less TNF-α after being stimulated with immunostimulatory LPS than those which were stimulated with LPS alone.

Excreted and secreted proteins were studied in detail using *T. suis* ESP recombinant proteins. Specifically, TsESP were separated by GPC and ion exchange and analysed by LC-MS/MS. Ion exchange fractions of TsESP was previously tested in BMDM. From these treatment assays [data not shown], bioactive fractions were nominated, namely, 1, 5, 8, 10 and 14 and selected for LC-MS/MS to determine specific bioactive proteins. Fraction 5 was the only inflammatory fraction and was analysed alongside the others as a negative control and to compare protein sequences (Figure 7). A short list of sequences found in the anti-inflammatory fractions was created, and recombinant proteins were produced as described in the methods and tested in the PBMC monocyte treatment assay. The aim was to determine which of the proteins are anti-inflammatory, therefore cells were stimulated with LPS, an inflammatory molecule, and incubated overnight as described. Culture supernatants were subsequently analysed by ELISA for secreted TNFα and IL-10. Certain recombinant proteins were found to be immunomodulatory, specifically, downregulate TNF-α and upregulate IL-10. The immunomodulatory recombinant proteins were NDK, TPI and G6P (8).

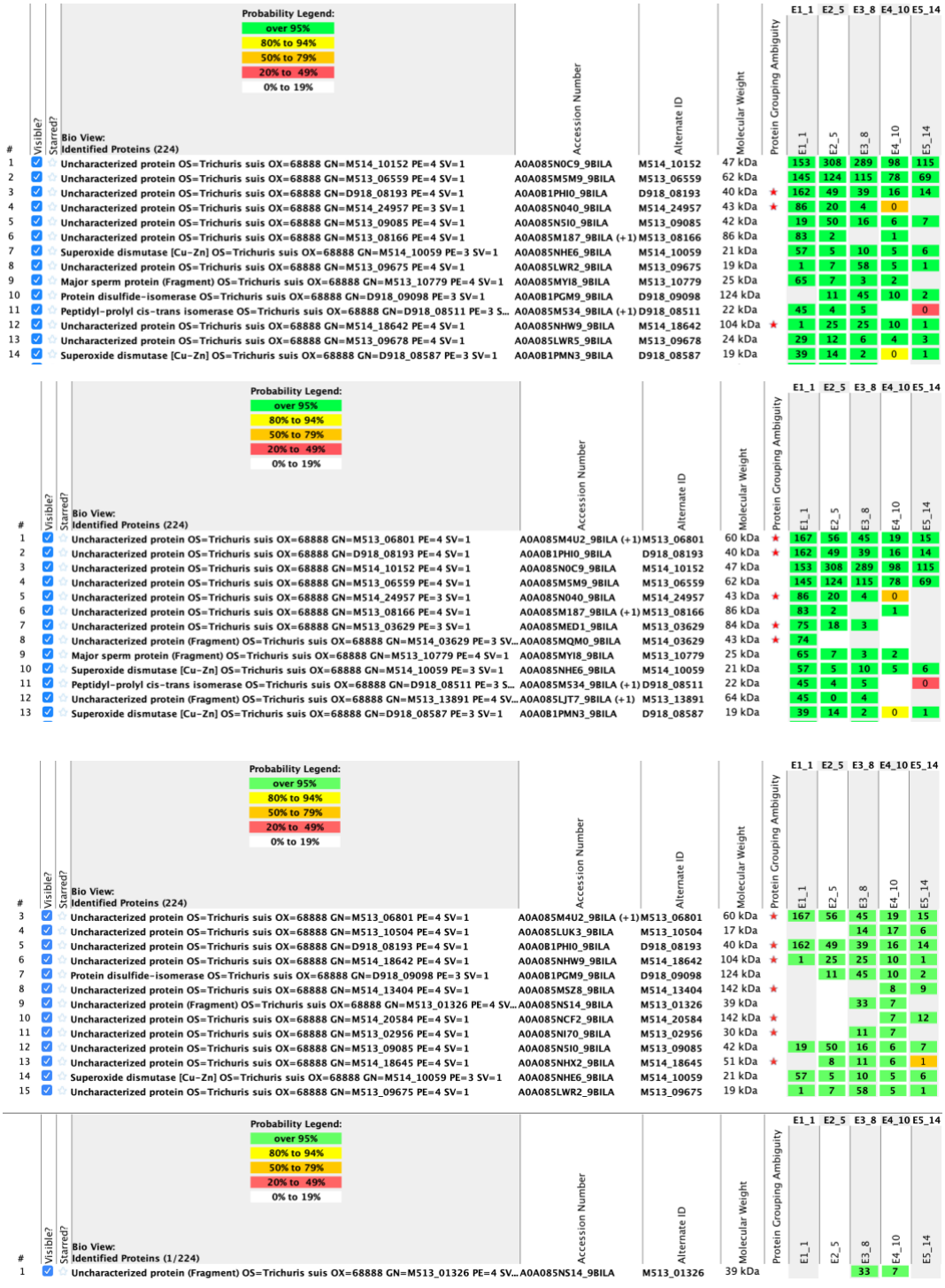


Figure 7. LC-MS/MS analyses of GPC fractions with immunomodulatory activity from TsESP. Fractions 1, 8, 10 and 14 were found to have anti-inflammatory activity in BMDM and fraction 5 was found to have inflammatory activity. The top counts of the proteins in each fraction are depicted in the 4 panels. Results were imported into Scaffold (Proteome Software, Inc., Montreal, QC, Canada; version 5.1.2.)

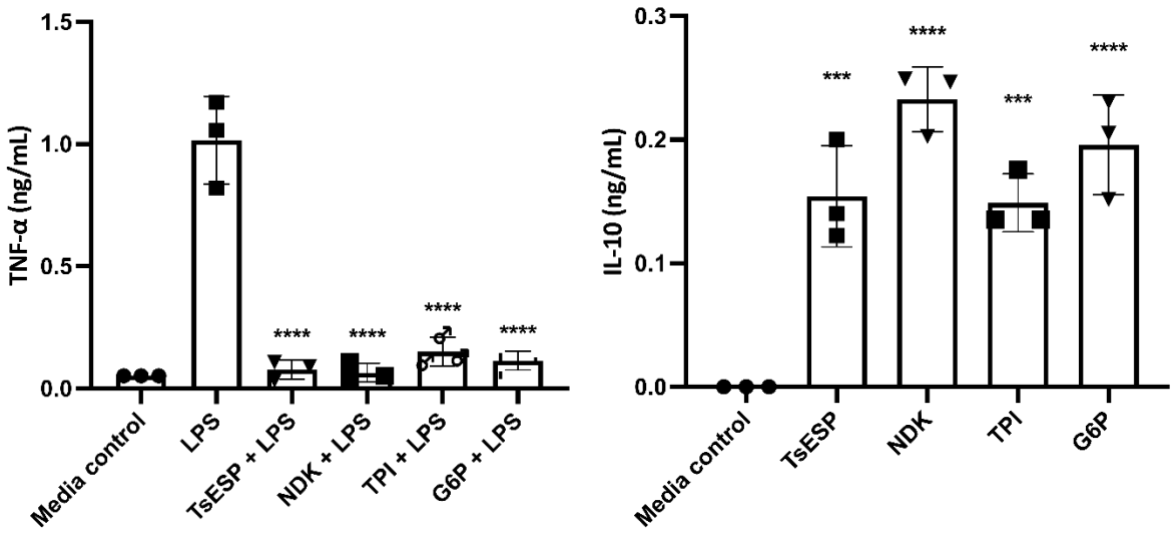


Figure 8. TNF-α and IL-10 secretion in PBMC monocytes treated with recombinant adult T. suis ESP protein.
Left: TNF-α secretion. Right: IL-10 secretion. Statistics were calculated using one-way ANOVA, where groups are compared to the LPS positive control. *** p < 0.001, **** p < 0.0001.

Other groups have studied ESP of various parasites and found that they contain immunostimulatory molecules that act to modulate the immune system of their host. After studying the immunomodulation by *T. suis* ESP, we decided to study the ESP of other parasitic helminths including *Ascaris suum*, *Dirofilaria immitis*, and *Hymenolepis diminuta*. Similarly, PBMC monocytes were isolated using Ficoll. Pure cultures were treated with or without ESP from *T. suis*, *A. suum* and *H. diminuta*, and were subsequently stimulated with LPS overnight as described in the methods. Cell supernatants were collected to analyse cytokine secretion using ELISA. *A. suum* and *H. diminuta*-treated PBMC downregulated TNF-α secretion to comparable levels as *T. suis*-treated PBMC (Figure 9).

Soluble, crude ESP proteins from *H. diminuta* demonstrate the ability to suppress LPS-induced TNF-α secretion on PBMC monocytes

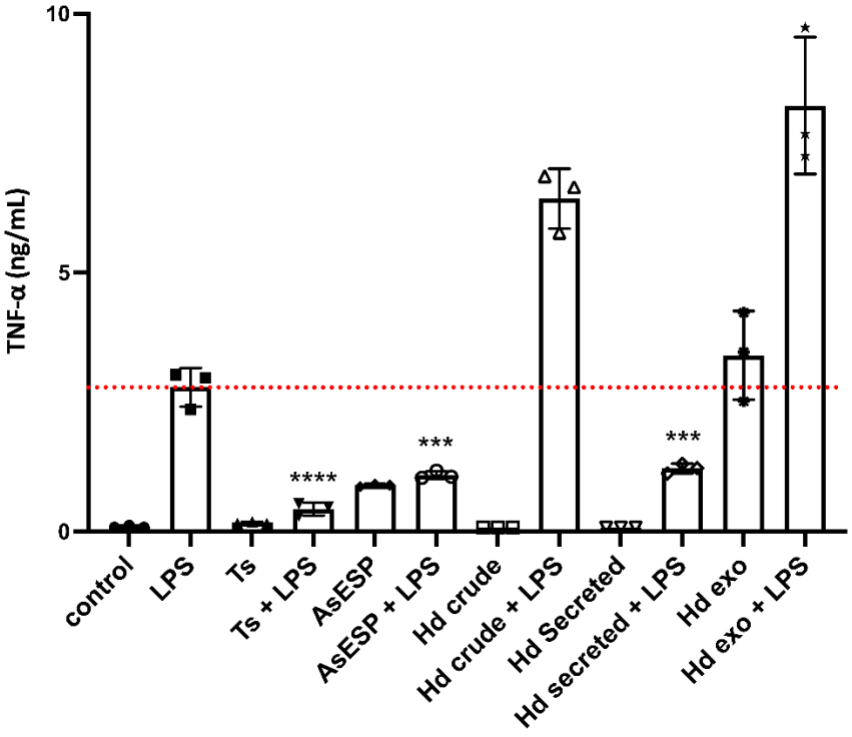


Figure 9. TNF-α secretion in PBMC monocyte treated with A. suum and H. diminuta protein fractions. Statistics were calculated using one-way ANOVA, where groups are compared to the LPS positive control. *** p < 0.001, **** p < 0.0001.

As demonstrated in Figure 4, crude *H. diminuta* ESP was separated into soluble and insoluble/exosome fractions by centrifugation. Different banding patterns appeared for these fractions, suggesting they have different proteins. This hypothesis was strengthened by testing their immunomodulatory activity; different levels of activity were found for the three different fractions (Figure 9). These data show that there is/are specific molecules or compounds involved in anti-inflammatory activity, and in this case the compound(s) of interest are found most abundantly in the soluble ESP.

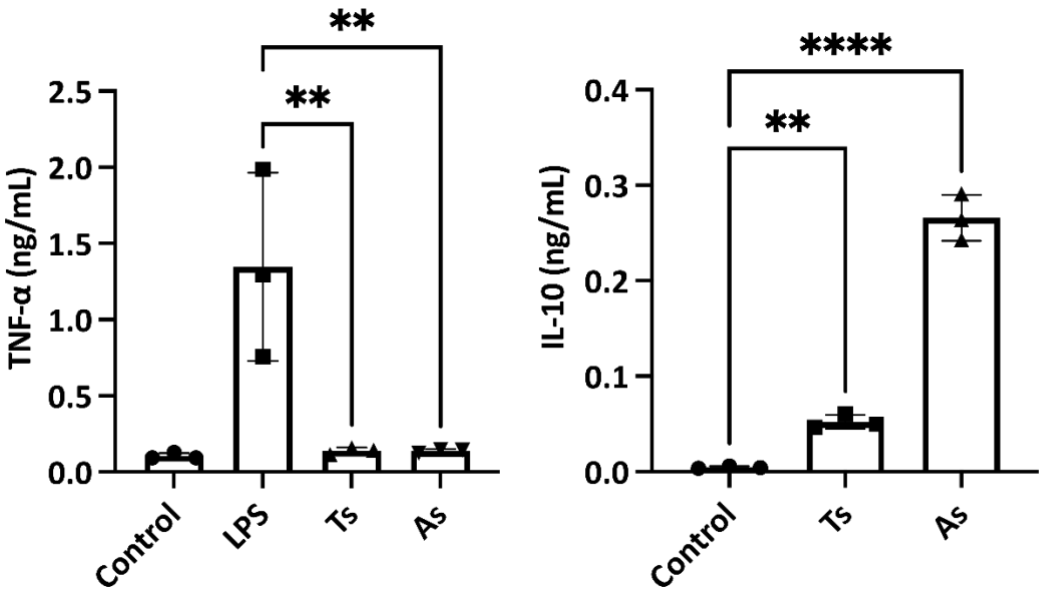


Figure 10. A. suum ESP induce a Type 2- like response in PBMC monocytes.
Left, AsESP suppresses LPS-dependent TNF-α secretion to the same degree as T. suis ESP (positive control). Right, In an LPS-independent manner, AsESP stimulates the secretion of IL-10. Statistics were calculated using one-way ANOVA, where groups are compared to the LPS positive control. ** p < 0.01, **** p < 0.0001.

After observing the immunostimulatory potential of *A. suum* ESP, such that it suppressed the LPS-dependent secretion of TNF-α, we decided to characterize the response further. Figure 10 wishes to show that AsESP suppresses the secretion of TNFα in LPS-stimulated monocytes to levels which are comparable to the parasite we were previously studying extensively, *T. suis.* Moreover, to characterize the response *in vitro,* we decided to test the effects on IL-10, an anti-inflammatory cytokine often seen in Type 2 responses.

DISCUSSION

Excreted and secreted products have been studied for their interest in being immunogenic. Here we describe the specific components of the ESP of different parasites which have proven to be immunomodulatory. The study of immunomodulatory effects of helminth-derived proteins reveals that there is specific immunomodulatory activity that can be attributed to different proteins in the ESP. Crude samples demonstrate immunomodulatory activity, and specific proteins were identified through a systematic approach to module BMDM, BMDC and PBMC monocytes. Specifically, NKD, TPI and G6PI reduced LPS-dependent TNFα secretion in these myeloid derived primary cells. These data suggest a molecular target within myeloid derived cells, or an

enzymatic modification of substrates used by the myeloid derived cells, by the ESP. Future studies can aim to understand the mechanism of action of *T. suis* recombinant proteins and better understand the cellular pathways that are affected by ESP. Mechanistic experiments were conducted by Dr. Louis-Philippe Leroux, whereby cell signalling pathways were studied in BMDM to determine the molecular effects of ESP treatment in LPS-stimulated cells (23). Specifically, these studies went on to show that C/EBPβ was induced by crude ESP as well as recombinant proteins, which is an important factor in macrophage differentiation and, therefore, specialized macrophage function. Moreover, treatment with these products and recombinants led to the phosphorylation of STAT3, independent of LPS stimulation. Finally, expression of NFIL3, involved in STAT3 signalling as well as a suppressor of *Il12b* gene for the inflammatory cytokine IL-12, was induced by ESP and recombinants. These molecules are all important upstream regulators of anti-inflammatory processes, suggesting direct modulation of inflammatory pathways by these helminth-derived proteins. These studies pave the way for the increasing understanding of dysregulated inflammation in autoinflammatory diseases such as IBD.

Experiments that study the ESP of *H. diminuta* demonstrate that helminth proteins are of interest for their ability to downregulate inflammatory cytokines can predominantly be found in the soluble ESP. Notably, the soluble and the insoluble fractions make up the crude fraction, which can be shown to contain different proteins on gel electrophoresis (Figure 4). It was also demonstrated that the level of immunomodulatory activity varies between these fractions (Figure 9) and it appears that the degree of TNF-α modulation of the crude fraction falls between that of the other two fractions. That is, while TNF-α was secreted at higher levels in the crude than cells treated with LPS alone, it was suppressed in the soluble protein treatments and induced to an even greater degree than the crude in the exosome treatments. Therefore, we can conclude that the sum of the components of crude ESP can be separated into the soluble and insoluble parts in order to increase the specific activity of each. This study demonstrates that *H. diminuta* proteins can be separated into the soluble and insoluble components to increase the specific activity of the anti-inflammatory mediators and decrease the inflammatory mediators. While crude *H. diminuta* ESP appeared to stimulate production of TNF-α in PBMC when subsequently stimulated with LPS, when it was separated into soluble and insoluble components, the soluble protein was able to achieve downregulation of TNF-α secretion. However, the exosome fraction demonstrated an even greater induction of TNF-α secretion when stimulated with LPS, suggesting that the specific

activity of inflammatory mediators was increased in this fraction. These data show that there are specific compounds involved in anti-inflammatory activity, and for *H. diminuta,* the compound of interest are likely most abundant in the soluble ESP, while there may be some antagonistic compounds in the exosome fraction of the ESP. Taken together, these data support the hypothesis that the components of ESP include both inflammatory and anti-inflammatory mediators that work together to orchestrate a balanced state whereby the immune system tolerates infection.

Chapter 4: Helminth-derived nonpolar molecules modulate inflammation *in vitro* and *in vivo*

ABSTRACT

Studies including ours have thoroughly investigated the ESP proteins of parasites for their functions and targets. Protein studies have allowed for the characterization of a large component of parasite excretomes and allowed for mapping of biological pathways involving these proteins. Following this, we became interested in studying the metabolites produced by parasitic helminths, with the hypothesis that the immunomodulation seen in helminth infection is mediated in part by small molecules and that specific compounds can be identified. This work will study the immune mechanisms of modulation evoked by small molecules found in the helminth-conditioned medium. ESP were collected as described in the previous sections of this thesis, and rather than selecting large molecules, small molecules were selected using the flow-through from the 3 kDa MWCO separation of crude extracts. Helminth-conditioned media from *Trichuris suis, Ascaris suum, Heligmosomoides polygyrus bakeri* and *Dirofilaria immitis* were purified using the methods described. Specifically, we used manual chromatography to purify fractions from the crude medium, to increasingly purify fractions. Purified fractions were tested along the way for bioactivity with the BMDM assay described in previous sections, and secreted cytokine modulation was assessed by ELISA analysis of the supernatants. A crude fraction obtained from manual chromatography, which we named AsNP25, was escalated to murine experiments due to its potency *in vitro*. Mice given DSS to induce colitis were subsequently treated with AsNP25 via intraperitoneal injections. Upon necropsy, mice were found to have improved colitis on gross pathology as well as histological and RT-PCR analysis of gut cytokines. Specifically, histopathology scores were approximately 50% improved compared to diseased mice and TNFα mRNA was not different from baseline in healthy controls and was significantly improved from diseased mice. AsNP25 was then further purified through HPLC where fractions were collected, tested for bioactivity in BMDM, and select fractions with bioactivity were advanced to MS and structural analyses. Various NMR experiments were utilized for structural analyses to identify moieties and narrow down a compound within the immunomodulatory isolates. Structural analyses provided a shortlist of possible structures from the purified, immunomodulatory fraction. The NMR analyses showed evidence of structural isomers of the identified molecule, likely attributable to numerous chiral carbons.

INTRODUCTION

Biologic proteins have been found to be useful for the purpose of proof-of-concept studies as well as building the knowledge bank around biological processes of a specific or model organism and pathways. Namely, our previous work looked at the immunomodulatory potential of helminth-secreted proteins from *T. suis* larval and adult stages (23), with the aim of unveiling their role in the host-parasite interaction as well as to aid in investigating mechanisms of inflammation in inflammatory bowel disease. However, proteins have come to possess less of a therapeutic role due to their immunogenicity (67). There are several methods which have been developed to make biologics or protein-based therapies less immunogenic through modification of their biochemical nature, however, this adverse outcome can be avoided through developing small molecule therapeutics. With this, we decided to turn our attention to the small molecules found in the helminth-conditioned medium and to investigate their role in immunomodulation. By investigating small molecules, or metabolites, we envisioned an arm of the response that could be deployed therapeutically with a lower risk of immunogenicity than larger molecules such as proteins. Small molecules may still evoke an immune response via a process known as haptenization, which is, by definition, a process that occurs in small molecules (<1kDa). However, there are molecules that are more likely to undergo haptenization based on their chemical properties. Small molecules which are more likely to undergo haptenization are notably those with reactive groups, electrophilic groups, or groups that form covalent bonds with nucleophilic components of proteins (68). Therefore, we more heavily investigated nonpolar molecules found in helminth-conditioned medium such that they would have a low risk of haptenization.

Studies have elucidated different metabolites found to be secreted by helminths that may modulate host immune responses. One group identified succinate, a previously known Type 2 immune response driver, in the ESP of *Nippostrongylus braziliensis*. Specifically, this response triggered pathways involved in downstream ILC2 cell activation (69). Other groups took a similar approach, studying the most abundant metabolites and fatty acids to characterize the dominant components of the metabolite excretome (43). Conversely, rather than investigating very specific molecules, some groups have looked at the total metabolic profile of helminths, used metabolomics to organize it, and utilized these analyses to map out pathways of metabolism (70). These studies provide a foundation for a bottom-up approach and help future studies investigate key pathways

of the host-parasite interaction, search for molecules of therapeutic potential, as well as identify potential drug targets. Our study presented in this work looks at the small molecules extracted from the crude helminth-conditioned medium and studies their immunomodulatory potential in bone-marrow derived macrophages.

METHODOLOGY

Proliferation and differentiation of AsNP25-treated BMDM precursors

Bone marrow was isolated from C57BL/6 mice, male or female, and total cells were cultured overnight in 15% LCCM in non-TC-treated plates. Next day, non-adherent cells are collected and counted, and plated in 24-well plates at $5x10^5$ cells/well in complete BMDM medium supplemented with 30% LCCM, with or without AsNP25. Supplemented AsNP25 was performed in quadruplicate and concentrations were tested in a dose-response using 0.5, 1.5, 5, 15, 50 µg/mL. Cells were cultured for 7 days, exchanging the medium on day 3 to remove non-adherent cells and taking a cell count using a Cytation 5 Cell Imaging Multimode Reader (BioTek Instruments) by gating for BMDM size. Cells were cultured for a total of 7 days, after which they were stimulated overnight with LPS 10 ng/mL for 18-20 h. Next day, cell supernatants were collected and assayed for secreted cytokines using ELISA. Adherent cells were fed fresh medium supplemented with 10% Alamar Blue to assess viability.

Reactive oxygen species production in AsNP25-treated BMDM

BMDM that were differentiated as described above were seeded at 2 x 10^5 cells/well in black-walled 96 well plates and treated with the same protocol described in the "Cell culture treatment and stimulation" section of the methods for 4 h or left untreated. Fresh medium was then added with or without LPS (10 ng/mL final) to BMDM cultures, and they were incubated for 18-20 h. H_2O_2 was a positive control for ROS production, and antimycin A, an electron transport chain (ETC) uncoupler. Forty-five minutes before the end of the 24 h incubation, 100 µM of 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was added to the cultures. At the end of the 18 h culture, supernatants were removed, and fresh medium was added. Fluorescence was allowed to develop and was read after 12 and 24 hours at an excitation wavelength of 495 nm and emission at 527 nm.

DSS colitis optimization

The model was adapted from Chassing et al. (71) and dose concentrations and length of administration were modified. Six to eight-week-old male mice housed at the Macdonald Campus SARU Animal Facility were administered 3, 3.5 or 4% Dextran Sulfate Sodium (DSS) or normal water for 5, 6 or 7 days. On the respective last day of DSS administration, the mice were returned to normal drinking water and given intraperitoneal (IP) injections of PBS to simulate treatments. Mice were weighed each day and assessed for weight loss exceeding 20% of their original body mass, as this would result in them having to be euthanized according to the ethics protocol. On the last experimental day mice were euthanized using 5% isoflurane and CO_2 based on the number of cages and mice, and according to the approved Darwin protocol (#8066). Following euthanasia, colons and spleens were removed and stored at -80°C for mRNA, protein, and MPO studies and in 10% formalin at room temperature for histology studies.

Mouse treatment experiments

Six to eight-week-old mice housed in cages of 5 at the Macdonald Campus SARU Animal Facility were administered 3% Dextran Sulfate Sodium (DSS) or normal water for 5 days. On day 6, DSS was replaced with regular water and intraperitoneal treatments of AsNP25 were commenced, one per day for 3 days. On day 8 mice were euthanized according to the approved protocol and necropsy was performed. The large colons and spleens of all mice were collected, and the colon was divided into 3 sections for further testing by RT-PCR and ddPPCR, histology, and myeloperoxidase (MPO) activity.

Histology

Colons were divided in three equal sections during the necropsy for consistency, voided, and the first section immediately stored in 10% formalin. Samples were placed in micromesh chambers (Cat#. 23-038-118). The tissue processor (Cat#. 12612613) program was set as depicted in Table 1.

Table 1. Tissue processor program steps

Solvent	Concentration	Time
Formalin	10%	1 min
EtOH	70%	2 h
EtOH	80%	1 h 30 min
EtOH	90%	2 h 10 min
EtOH	95%	1 h
Formalin	10%	1 min
EtOH	100%	3 h
Neoclear	-	2 h
Neoclear	-	1 h 30 min
Paraffin	-	2 h
Paraffin	-	1 h 30 min

Following fixation, samples were embedded using the embedding centre. Samples were embedded upright to use the intestine strops method. Slicing was performed using a microtome in 6 µm and 4µm thick slices. Multiple sections of each sample were made and placed in the hot-water bath, placed on slides and allowed to dry and stained with hematoxylin and eosin (Cat#. ab245880).

High performance liquid chromatography (HPLC) fractionation of metabolites

AsNP25 was resuspended in tissue culture grade water as described above. HPLC solutions used were 0.1% formic acid in nylon-filtered water (solvent A) and 0.1% formic acid in 100% acetonitrile (solvent B). A Beckman Ultrasphere reversed phase ODS (4.6 x 250 mm) column was used for high-performance liquid chromatography reversed phase analysis. A blank, as well as the samples AsNP25 and AsNP50 were injected, and the fractions were collected at a rate of 1 tube/min (and thus, 1 mL per tube). A total of 2.25 mg of each sample was injected and the fractions were collected. The UV/Vis detector was set to 210 nm and 280 nm. A SpeedVac concentrator was used to evaporate the fractions to complete dryness, and they were resuspended in 50 µl tissue culture grade water.

RESULTS

T. suis nonpolar metabolites modulate LPS-induced TNF-α secretion in murine BMDM

To assess the general phenomena of helminth low molecular weight compounds we extracted both the nonpolar and polar metabolites present in the helminth conditioned medium. To obtain these data, the helminth-conditioned medium was processed as described in the methods to obtain the soluble ESP, and the small molecule fraction was obtained using a 3 kDa MWCO filter. Crude metabolites were further purified using column chromatography as described in the methods, yielding biochemically different fractions. Fractions were first concentrated on a reverse phase column, selecting for molecules with nonpolar moieties (NP), and subsequently eluted in a stepwise gradient. Immediately after, the flowthrough was concentrated on an SPE column to select for molecules with polar moieties (P), and again elute in a stepwise gradient. Six fractions were obtained from this purification: C18-RP column and the polar SPE column were each eluted with 25%, 50% and 100% acetonitrile (ACN) supplemented with 0.1% formic acid. These fractions represent nonpolar and polar metabolites, respectively. Henceforth, these fractions will be referred to as NP25, NP50, NP100, P25, P50, and P100.

Work done previously by Dr. Louis-Philippe Leroux in Professor Jardim's laboratory demonstrated immunomodulatory activity of nonpolar *T. suis* crude metabolites whereby cytokine levels were altered in metabolite-treated cultures, which led to the interest to further explore these metabolites as well as those from other parasites. BMDM cultures were treated with ESP metabolites extracted from different life stages for 4 h after which the medium was substituted for fresh with or without LPS. The next day, supernatants were analysed using ELISA for the secretion of TNF-α. It was observed that crude metabolites as well as nonpolar fractions of metabolites suppressed LPS-induced TNF-α secretion. Through previous work, we determined that *T. suis* crude ES suppresses TNF-α (23). We can observe that nonpolar fractions from *T. suis* metabolites suppress LPS-induced TNF-α secretion. The magnitude of this suppression varies depending on the fraction (Figure 11). Similar purifications of *A. suum*, *D. immitis*, *H. diminuta*, and *H. polygyrus bakeri* metabolites were performed for this work, detailed in the methods, to study the immunomodulatory ability of different parasites in our system.

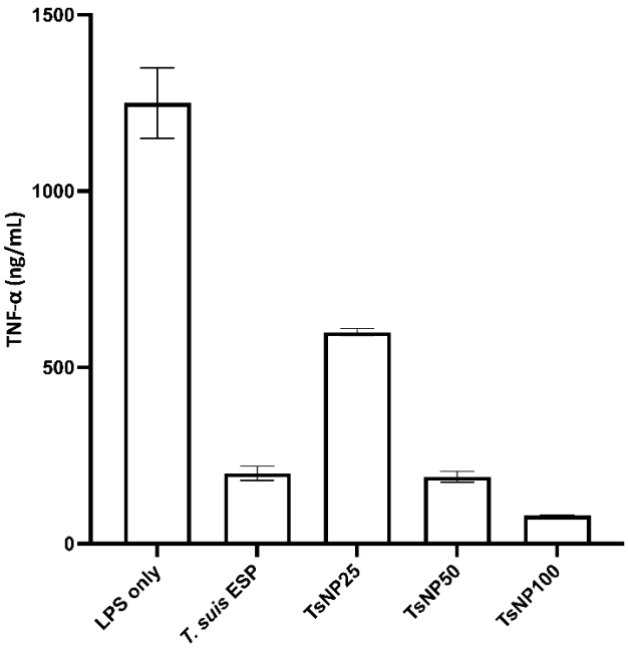


Figure 11. Suppression of LPS-induced TNF-α secretion by T. suis metabolites. [Adapted from the data from experiments performed by Dr. Louis-Philippe Leroux, 2014].

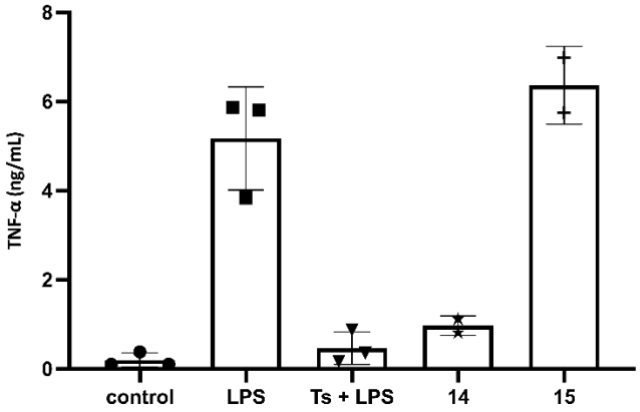


Figure 12. Specific HPLC-fractionated T. suis metabolites suppress LPS-induced TNF-α secretion. Fractions 14 and 15 denote two subsequent eluates from HPLC. Statistics were calculated using one-way ANOVA, where groups are compared to the LPS positive control. **** p < 0.0001, *** p < 0.001.

Pooled fractionated *T. suis* metabolites which were found to be immunomodulatory *in vitro* were fractionated using reverse-phase HPLC. Fractions were collected and subjected to the same *in vitro* treatment assay [data not shown]. From this, a fraction was isolated which was found to suppress LPS-induced TNF-α secretion in BMDM (Figure 12).

Helminth-derived excreted and secreted metabolites are produced in varying amounts across species

Table 2 wishes to provide details and representative values of the mass of material isolated in each of these purification steps. One can observe that *T. suis* yields the most product of total metabolites in both the nonpolar and polar purification techniques. *A. suum*, although a much larger worm, yields less secreted metabolites than *T. suis*, although more than *H. polygyrus bakeri* or *D. immitis*.

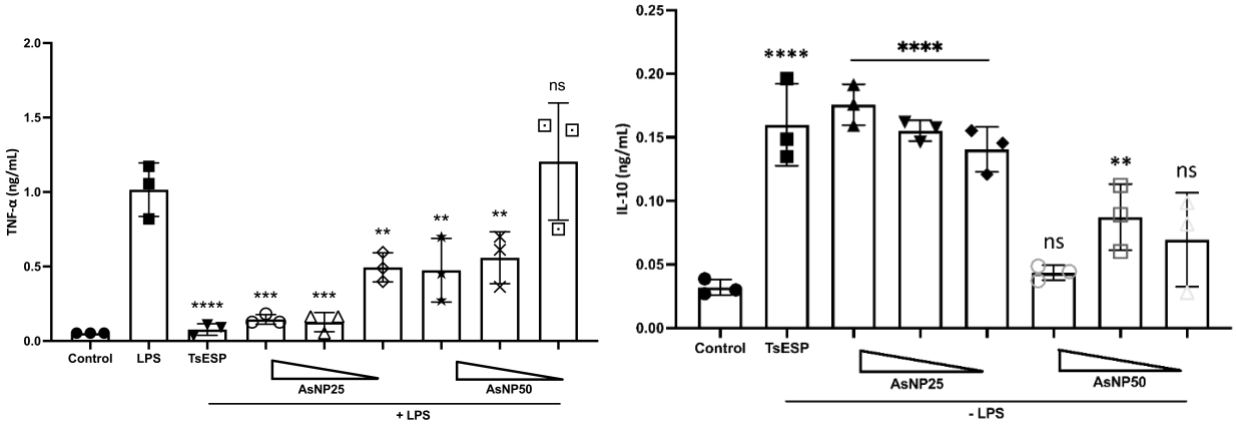
Table 2. List of parasite fractions that were a focus of the study and their yields obtained using the purification methods described.

	Ts	**As**	**Hpb**	**Di**
Initial volume of media*	**2 L**	**1-1.5 L**	**60 mL**	**25 mL**
Culture concentration	**20 worms/mL**	**16 worms/L**	**1000 worms/mL**	**250/mL**
NP25	**20 mg**	**~3-5 mg**	**~1mg**	**~1 mg**
NP50		**~1-2 mg**	**<200 µg**	**~500 µg**
NP 100		**<200 µg**	**<200 µg**	**<200 µg**
P25	**30 mg**	**~10 mg**	**~10 mg**	**~10 mg**
P50		**~2-5 mg**	**~2-5 mg**	**~2-5 mg**
P100		**~1 mg**	**~1 mg**	**~1 mg**

* Initial volume is before any loss that occurs from filter sterilizing the media with a 0.2 µm filter

Helminth-derived ESP modulate the immune response of primary monocytes and BMDM in a dose-dependent manner

A.



B.

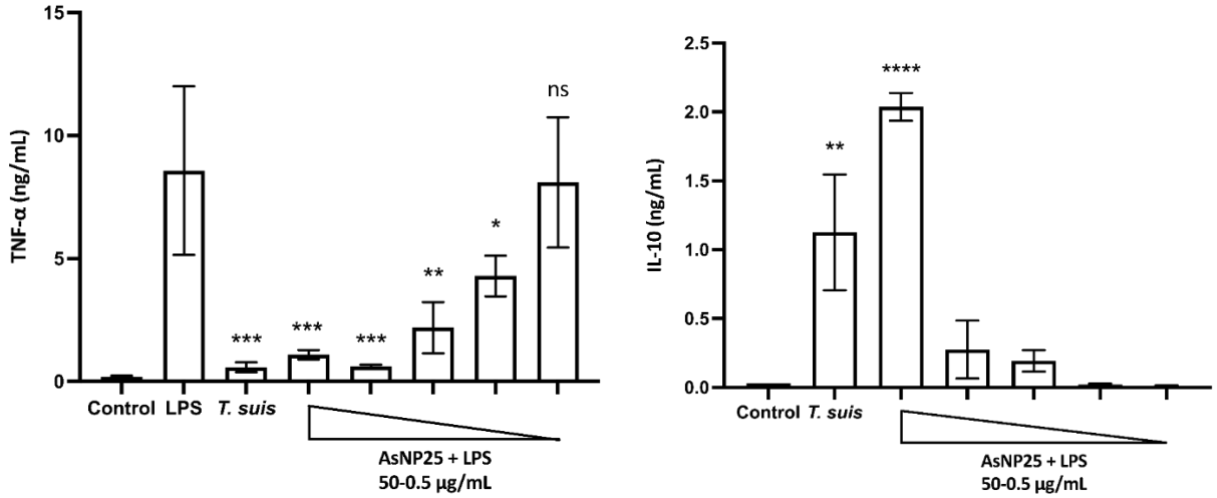


Figure 13. A. suum crude metabolite fractions modulate cytokine secretion in PBMC monocytes in a dose-dependent manner.
A. AsNP25 and AsNP50 modulation of LPS-induced TNF-α secretion (top panel) and induction of IL-10 (bottom panel). B. AsNP25 modulates TNF-α (left) and IL-10 (right) in PBMC monocytes over a broad range of concentrations (50-0.5 µg/mL). **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05.

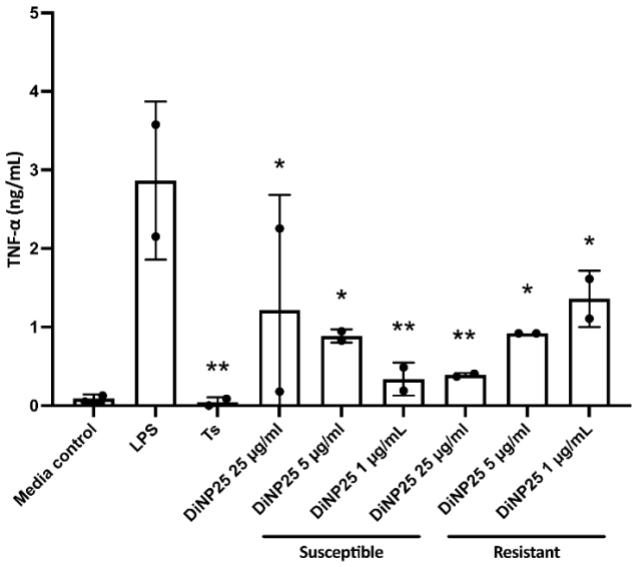


Figure 14. D. immitis crude metabolite fractions from susceptible and resistant strains modulate TNF-α secretion by BMDM stimulated with LPS. ** p < 0.01, * p < 0.05.

Molecules enriched for on a C18-RP column were eluted with 25%, 50% and 100% acetonitrile with 0.1% formic acid (AsNP25, AsNP50 and AsNP100, respectively). A pure culture of PBMC monocytes was obtained, and cells were plated and allowed to rest overnight. Stable primary cultures were treated with the indicated concentrations of AsNP25 and AsNP50 (Figure 13A) for 4 h, following which medium was exchanged and cells were stimulated with or without LPS for 18-20 h. Secretion of the cytokines TNF-α and IL-10 were assessed by ELISA. Fractions found to have the most activity were the AsNP25 and AsNP50. While AsNP50 had comparable bioactivity, AsNP25 had activity at the lowest concentrations (Figure 13A). AsNP50 showed suppression of TNF-α at concentrations of 50 and 25 µg/mL as well as an increase in IL-10, while AsNP25 showed significant reduction in TNF-α and induction of IL-10 at all tested concentrations. Thus, AsNP25 was assessed for its range of activity in a repeat assay, testing doses 50, 15, 5, 1.5 and 0.5 µg/mL (Figure 13B). Testing these concentrations revealed that concentrations as low as 1.5 µg/mL resulted in statistically significant reduction in secretion of inflammatory cytokines. IL-10 was only induced significantly at higher concentrations of treatment, although a dose-effect can be observed.

D. immitis suppresses LPS-induces TNF-α secretion by BMDM

We sought to study other parasitic helminths to assess their ability to modulate immune responses; we hypothesized that an immunomodulatory response may be conserved among helminths. Crude fractions of *Dirofilaria immitis* metabolites were prepared from conditioned medium following the same protocol as with *A. suum*. BMDM treatments were performed using *D. immitis* 25% ACN-eluted NP fractions (DiNP25) and stimulated with or without LPS overnight. Supernatants were collected and assayed for TNF-α secretion by ELISA. We found that DiNP25 from susceptible parasites (sDiNP25) and resistant parasites (rDiNP25) suppress LPS-induced TNF-α secretion in BMDM (Figure 14). sDiNP25 was more potent in suppressing TNF-α than rDiNP25. That is, 25 ug/mL of rDiNP25 was necessary to suppress LPS-induces TNF-α to comparable levels as 1 ug/mL of sDiNP25. It appears that higher concentrations of crude DiNP25 did not necessarily result in stronger anti-inflammatory responses, as evidenced by the inverse dose-dependent suppression of TNF-α. In other words, higher concentration of DiNP25 was insufficient to reduce the secretion of TNF-α.

To assure that lower TNF-α secretion could not be explained by cellular toxicity or cell death, Alamar Blue was added to fresh medium following harvesting of the supernatants post-stimulation. Cells all had similar viability compared to control (Figure 15). There was one well treated with DiNP25 25 µg/ml which had low TNF-α secretion as well as low fluorescence, indicating that the well was likely an outlier, as other replicates did not exhibit the same phenotype.

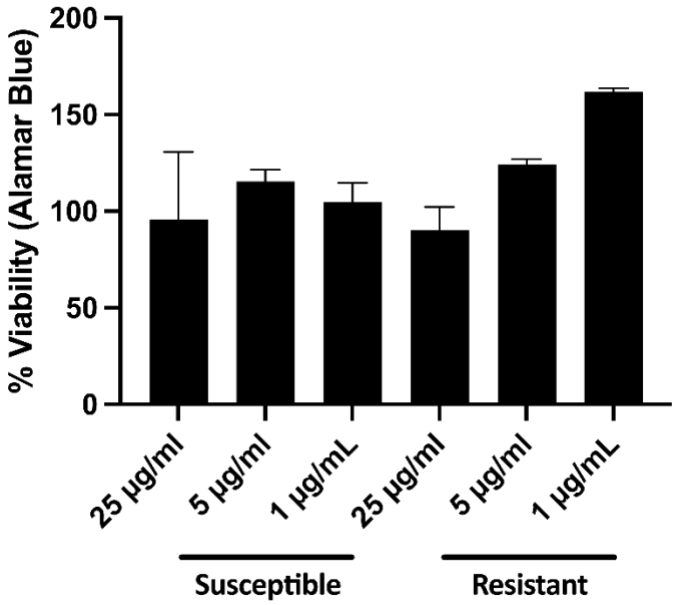


Figure 15. Alamar Blue fluorescence of BMDM treated with D. immitis metabolites. (Percent is compared to LPS control)

AsNP25 modulates cytokine secretion in BMDM stimulated with LPS

Fresh BMDM cultures were treated with 25 µg/mL of AsNP25 for 4 h, then exchanged for fresh media with or without LPS to stimulate overnight for 18-20 h. A concentration of 25 µg/mL AsNP25 was previously found to be the optimal concentration in PBMC monocytes with near 100% suppression of TNF-α (Figure 13). Similarly, as was seen in PBMC monocytes, secretion of TNF-α was decreased in BMDM treated with AsNP25, while IL-10 was induced (Figure 16). From this we conclude that this was the optimal concentration in both PBMC monocytes as well as BMDM.

Of note, purification yields of fractions are on the order of µg-mg and are measured using a scale. They may be inaccurate and are meant as an estimate to resuspend the pellet and have a relative concentration. Because of this inevitable range of error, the measured concentration is inconsistent from batch-to-batch. Moreover, as the parasites might vary in their population as well (size of worms, exact age, concentration of worm culture, health of worms, male-to-female ratio, etc.). Therefore, the optimal concentration and amount were determined for each batch by performing a wide-ranging dose-response treatment following the dilution pattern: 100 – 30 – 10 – 3 – 1 – 0.3 … etc.

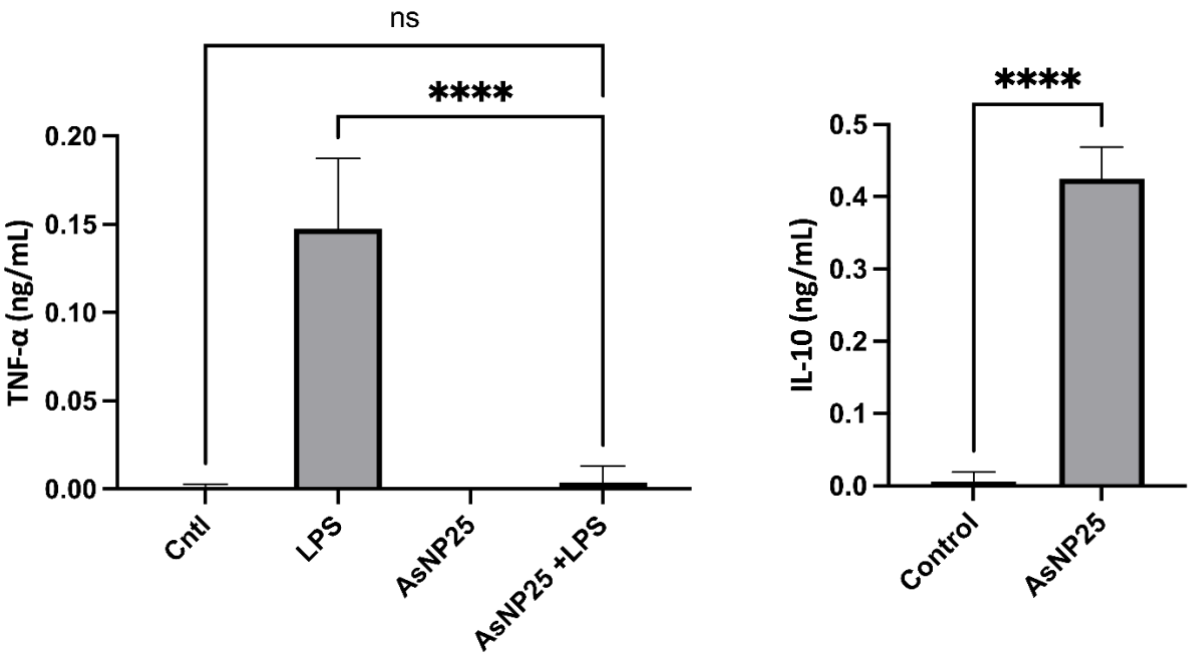


Figure 16. A. suum metabolite crude fractions modulate cytokine secretion in PBMC in a dose-dependent manner. **** p < 0.0001.

Crude helminth-derived ESP suppresses the differentiation and proliferation of BMDM and alters their metabolism

Cells were isolated from the femurs, tibia, hips, and humorous bones of mice as per the protocol described in the methods, and cells were allowed to rest for 1 day in complete DMEM supplemented with 15% L929-cell conditioned medium (LCCM). Next day, non-adherent cells are transferred to 24-well plates for differentiation to BMDM in BMDM medium (complete DMEM supplemented with 30% LCCM), supplemented with AsNP25 (either 50, 15, 5, 1.5 or 0.5 µg/mL). On day 3, fresh BMDM medium is added, non-adherent cells get washed away and re-supplemented with AsNP25 at the respective concentration. With only precursor cells remaining attached, a cell count was taken using a Cytation 5 machine. Cell counts revealed a dose-dependent reduction in treatment wells supplemented with AsNP25 (Figure 17).

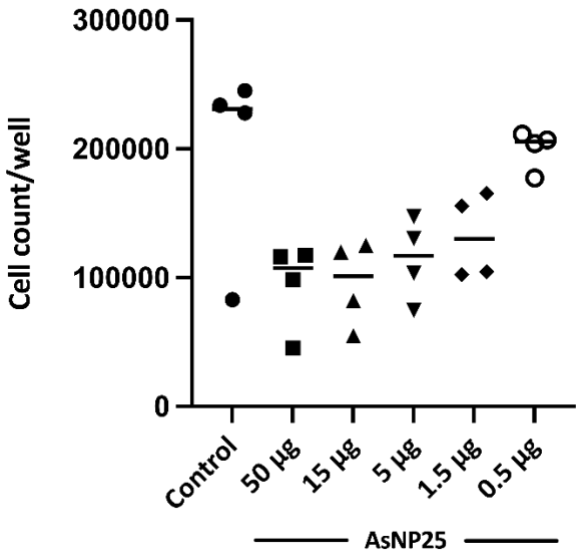


Figure 17. Proliferation of BMDM is suppressed in a dose-dependent manner when differentiated in medium supplemented with AsNP25.
Cell counts were achieved through optimization of the Cytation 5 program for the experimental parameters and by gating for adherent BMDM and cell counts/well were recorded.

BMDM were cultured with or without 50 µg/mL of AsNP25 in quadruplicates throughout the normal differentiation process described in the methods; and on day 7, BMDM culture medium containing LCCM was replaced for fresh DMEM with or without 10 ng/mL LPS for 18-20 h (dividing the quadruplicate cultures into duplicates of stimulated and unstimulated cells). Next day, Alamar Blue reagent was added, and fluorescence readings were taken every 1 h for 4 hours (Figure 18). Fluorescence increased proportionally in all samples, and they did not plateau over time, signifying that no wells had saturated fluorescence. Unstimulated and stimulated cells within the same treatment group were replicated during differentiation, and therefore had no difference in cell count or viability. As seen in Figure 18 and Figure 19, LPS increases the Alamar Blue fluorescence compared to the media control, which, since the cell count is the same, can be attributed to increased metabolism when stimulated with LPS. Of interest, when differentiated in the presence of AsNP25, the addition of LPS did not induce such changes in the fluorescence as it did in the LPS only. This suggests that the metabolism-related changes seen in the LPS vs control are aberrated when AsNP25 is added and that components of AsNP25 may be responsible for modulating the metabolism. Alamar Blue is widely used for assessing the viability of cell cultures or toxicity of treatments indirectly through the reducing intracellular environment (72). Here, knowing from previous assays that AsNP25 does not confer any toxicity and knowing that cells

were cultured identically, an explanation for changes in fluorescence is that the cells had altered metabolism following treatment with AsNP25. Resazurin, the intermediate electron acceptor in the Alamar Blue assay, is not specific to one reductase nor is it specific for mitochondrial activity.

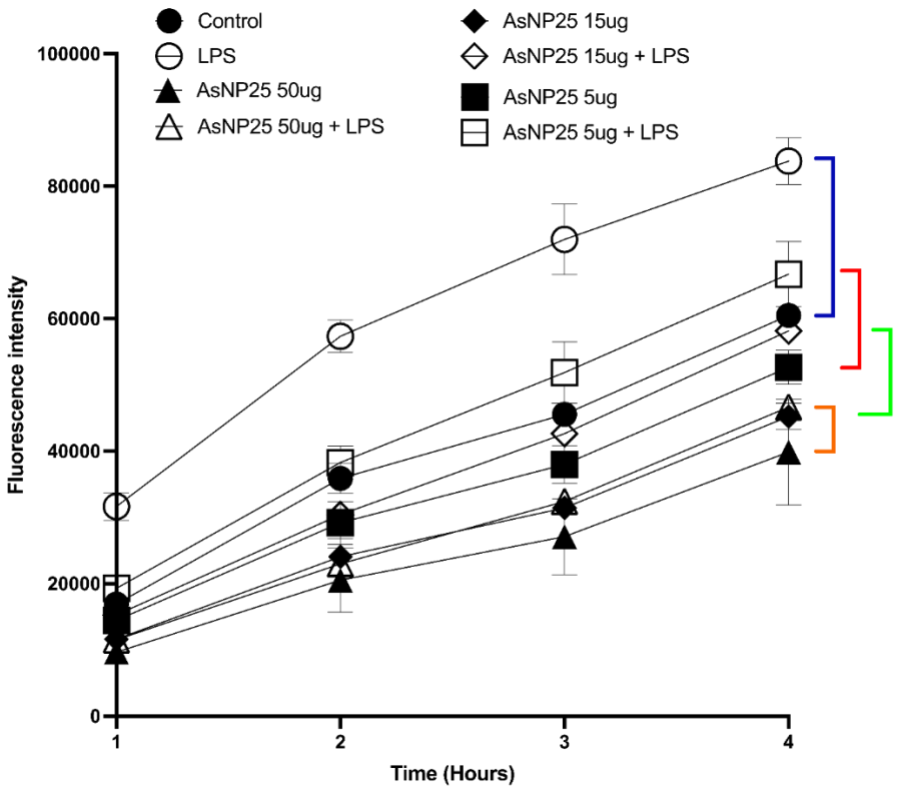


Figure 18. Alamar Blue fluorescence intensity of day 8 differentiation cultures.
Post LPS-stimulation, hourly measurements were taken. Pairs denote groups which were stimulated or not with LPS.

BMDM treated with AsNP25 produce ROS in a dose-dependent manner

To further understand the effects of AsNP25 on metabolism, H2DCFDA intracellular fluorescent assay was used to determine if the metabolism changes occur at the level of the electron transport chain (ETC). With Alamar Blue, neither a specific enzyme nor an organelle can be targeted when considering metabolic changes, as it is not specific. H2DCFDA is a fluorescent dye that measures hydroxyl, peroxyl and other reactive oxygen species, in that fluorescence depends on the oxidation of this compound with ROS produced in the cell. Cells that were treated with AsNP25 displayed an increased in fluorescence, indicating that they produced more ROS during their incubation (Figure 20). Antimycin A (AMA), an electron transport chain uncoupler, was used as a negative control for ROS activity derived from the ETC, by blocking complex III (73).

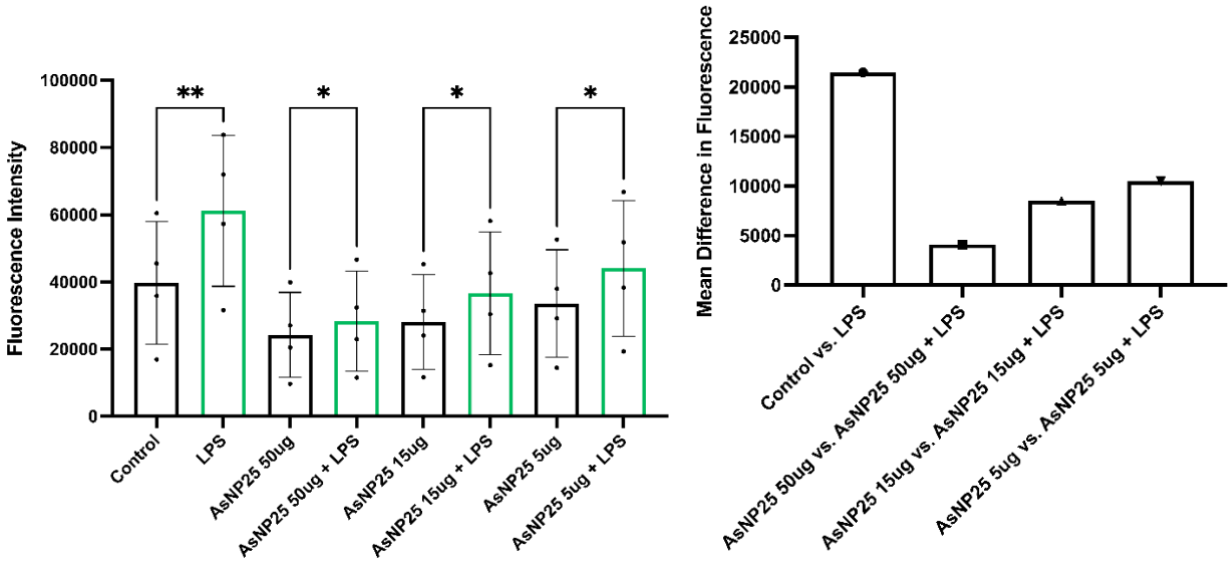


Figure 19. Alamar Blue fluorescence intensity of day 8 differentiation cultures, post LPS-stimulation. Left: Fluorescence intensity averages per treatment. Statistics calculated by One-way ANOVA. Right: Mean difference in fluorescence intensity calculated by ANOVA. ** p < 0.01, * p < 0.05.

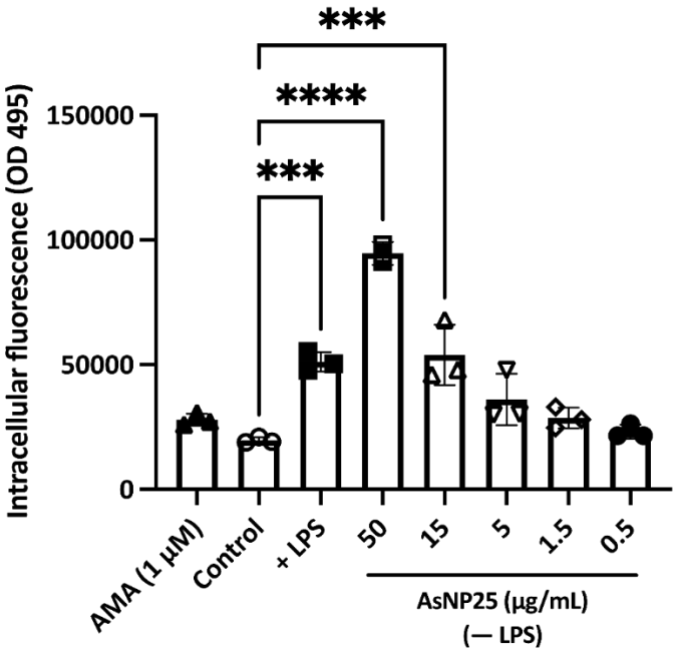


Figure 20. Metabolism of AsNP25 treated BMDM was further investigated using H2DCFDA to assay the production of ROS. **** p < 0.0001, *** p < 0.001.

DSS-colitis protocol optimization

It was necessary to determine, prior to commencing treatment experiments using the DSS colitis model, the optimal DSS colitis protocol which we would thereafter follow. The DSS colitis

mouse model, although widely used in IBD research, is found to have several issues including variability in site of injury along the colon and cecum, environmental variability, as well as strain variability (74). Thus, to reduce both environmental and strain variability, we tested different concentrations and duration of DSS administration in C57BL/6NCrl mice, which would be the strain that we proceed with moving forward. BALB/c mice were found not to develop consistent or reproducible colitis [data not shown]. Mice were given either regular water or DSS (3% for 5, 6, or 7 days or 3.5 or 4% for 5 days). On day 5 DSS was replaced with regular water. Mice which lost 20% or more of their initial body weight were euthanized according to protocol and were considered in the results to have deceased (indicated by early ending lines in Figure 21). Note, black and fuchsia lines were terminated on day 8 as planned.

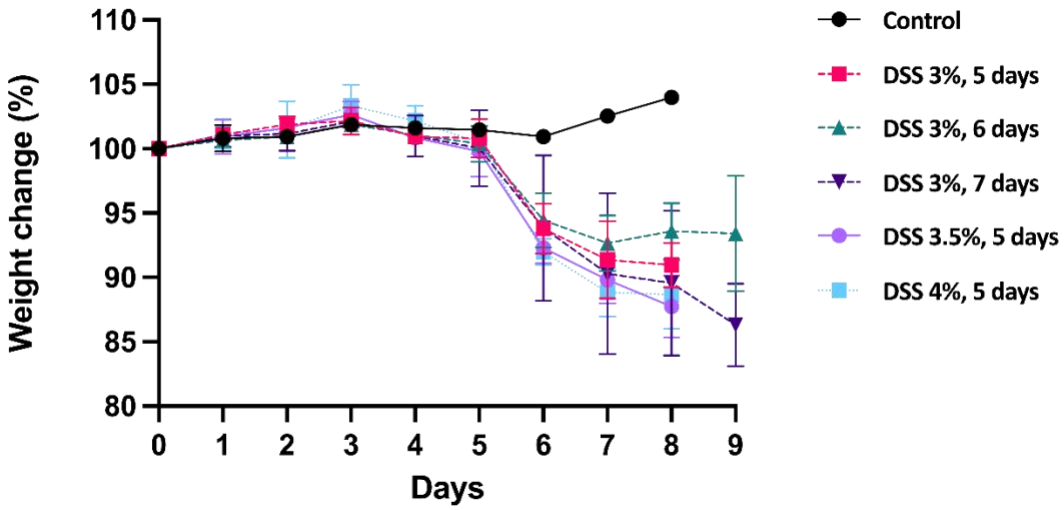


Figure 21. Mouse weight change (%) over time for optimization of DSS colitis model.

Mouse weights were recorded once daily before IP injections, when applicable. Black solid line represents the control group, dashed lines represent 3% DSS, solid coloured lines 3.5%, and dotted lines 4%.

We found that mice that received more than 3% DSS (i.e., 3.5% (violet, solid) and 4% (blue, dotted) lost more than 20% of their weight before the perceived end date of the experiment (Figure 21). The group receiving 3% DSS for 7 days was terminated after 6 days of DSS, due to having lost more than 20% of their initial weight. The 3% for 5 days group was terminated on day

5 as planned, and mice had lost less than 15% of their weight (as well as in all 3 of the 3% DSS groups) by the day 8. Moreover, groups which received 3% DSS for 6 days (green, dashed) had subjects losing variable amounts of weight by day 9, none of which fell below 20%. Control group subjects maintained consistent increase in their weight throughout the experiment (black, solid). Since 3% for 5 days and 6 days both resulted in an acceptable amount of weight lost, the better protocol is to proceed with the 5-day duration from an ethical standpoint, to have mice suffering from induced colitis for the least amount of time as possible.

We then assessed the colon lengths of these mice on their respective necropsy days (Figure 22). There was a dose-response trend observed. There was no significant difference between groups, however, all groups had significantly shorter colons ($p < 0.05$) than the control and the 3.5% DSS group had $p < 0.01$. However, the groups with 3% for 7 days, 3.5% and 4% were subjected to euthanasia earlier than planned due to weight loss reaching 20% of their initial weight, as indicated in Figure 21. Thus, these data taken together allow us to we conclude that mice housed at the Macdonald Campus SARU Animal Facility have optimally induced colitis using a dosage of 3% DSS for 5 days.

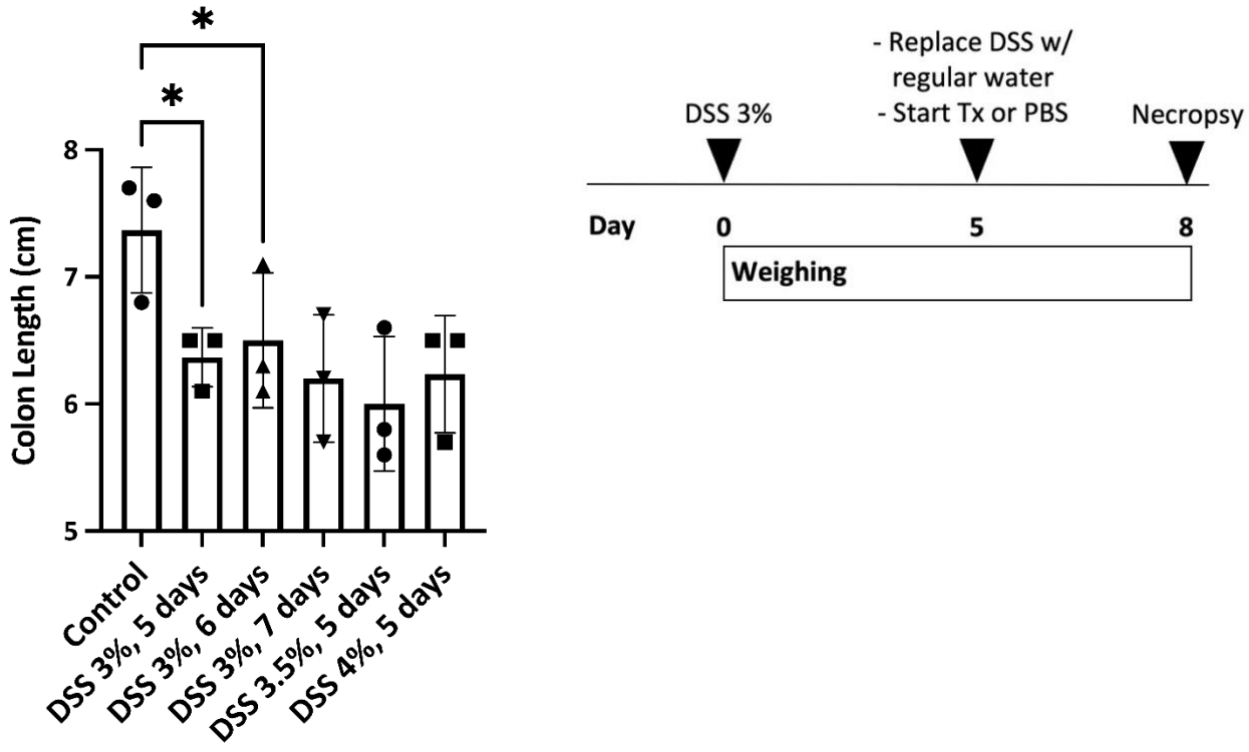


Figure 22. Mouse experiment optimization necropsy results.
Left: Mouse colon lengths on respective necropsy day for optimization of DSS colitis model. Right: Schedule for mouse experiments. Statistics calculated by One-way ANOVA.

Bioactive metabolite fraction improves disease in DSS colitis

Colitis was induced, or not, in C57BL/6NCrl mice according to the approved protocol detailed in the methods section. Briefly, mice were given 3% DSS or regular water for 5 days, after which all mice were returned to regular water and were injected intraperitoneally (IP) with AsNP25, either 25 or 50 µg per mouse, per day for 3 days or PBS for the control. On day 8, mice were euthanized according to protocol. Mice were weighed every day before injections (Figure 23). Colons were measured, divided in 3 sections, and labelled accordingly, and spleens were collected. We found that colitic mice which were given AsNP25 intraperitoneally had significantly longer colon lengths than colitic mice that were given PBS. Mice that were given DSS or regular water with AsNP25 did not have colons that were significantly shorter in length compared to healthy controls (Figure 24). Mouse colons were assessed for gross pathology (Figure 25). This revealed that colitic mice which received AsNP25 treatments had significantly reduced gross

pathology than those who received PBS, indicating that mice benefit macroscopically and possibly clinically from the bioactivity of the treatment.

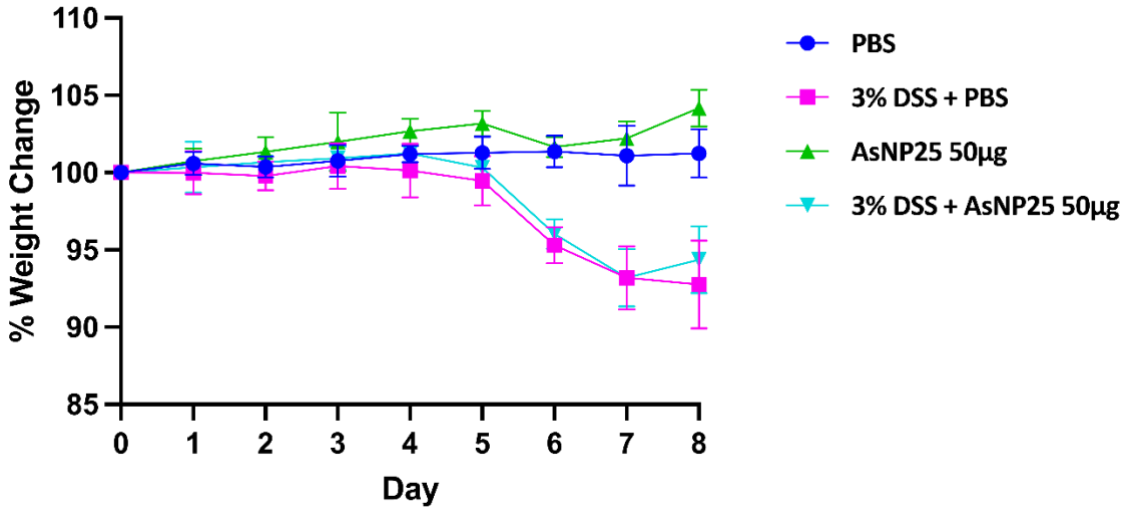


Figure 23. Mouse weight changes over time given 3% DSS followed by 3 days of IP AsNP25.

Histology was performed using the paraffin technique as described in the methods. As seen in Figure 26, with representative images of the colon histology sections stained with hematoxylin and eosin to visualize (H&E), histological phenotype is improved in colitic mice that were given AsNP25. Histopathology scoring was performed with a well-characterized scoring system by a blinded researcher (75–77). Scoring criteria includes loss of architecture, cellular infiltration, goblet cell depletion, ulceration, edema, muscle thickening, and crypt abscess. A higher score indicates greater pathology. Figure 27 shows the results of the histopathology scoring, where there was a marked improvement for colitic mice treated with AsNP25 compared to diseased mice. Treated mice retain a higher histopathology score than control mice and healthy mice given AsNP25.

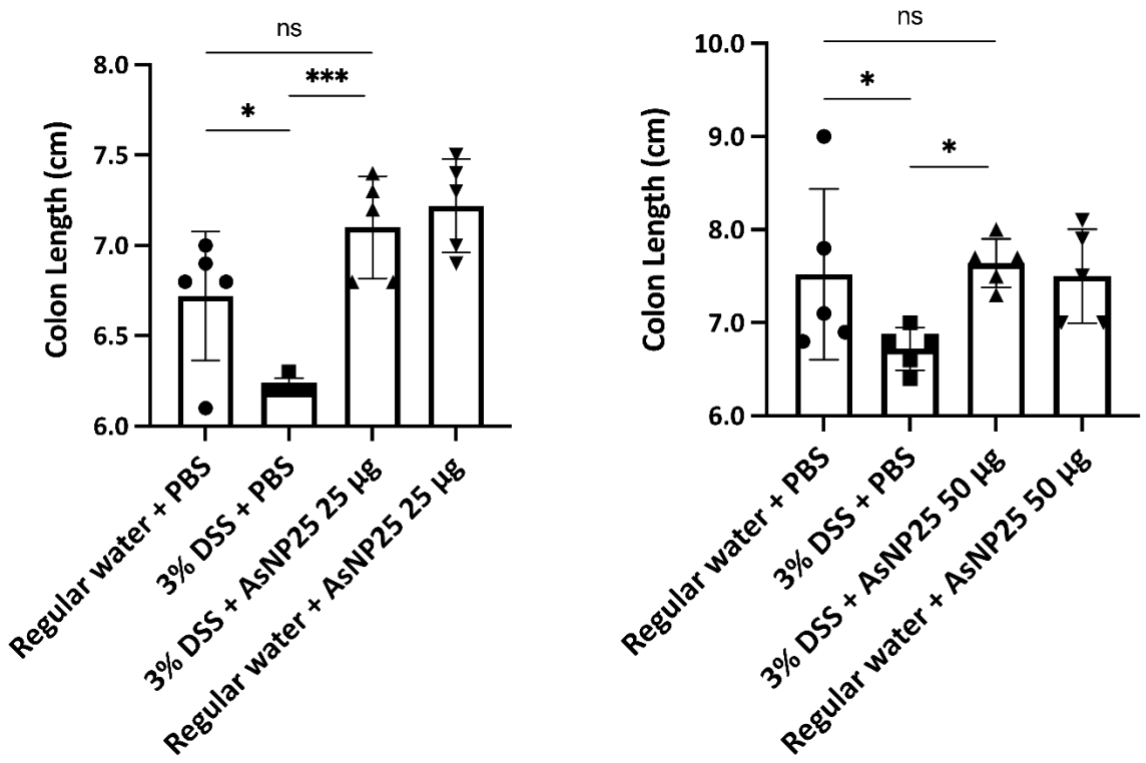


Figure 24. Mouse colon lengths measured during necropsy.
Following Mice were given AsNP25 intraperitoneally (25 µg, left; 50 µg, right) each day for three days.
Statistics calculated by One-way ANOVA. *** $p < 0.001$, * $p < 0.05$.

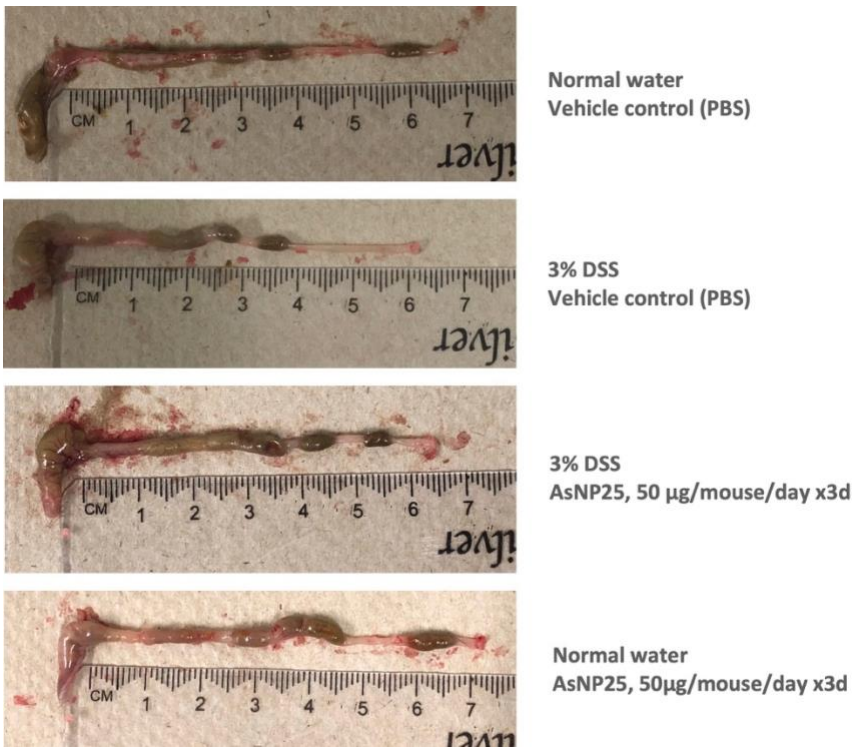


Figure 25. Representative images of post-cecum colons of mice treated with or without AsNP25.

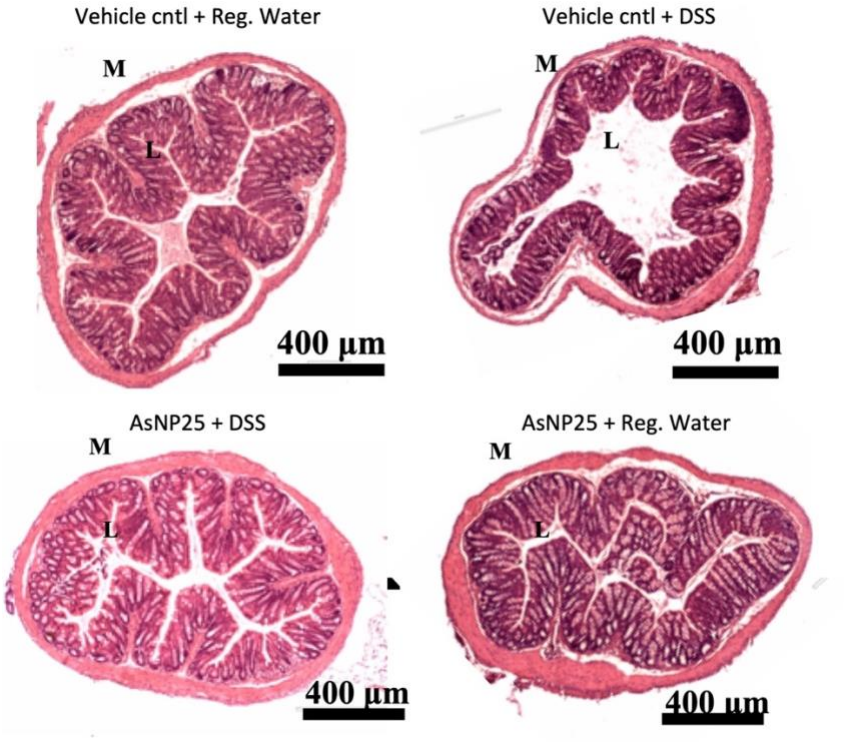


Figure 26. Images of histological sections stained of mouse colons treated with or without AsNP25. Top left, Regular water + PBS; top right, 3% DSS + PBS; bottom left, 3% DSS + AsNP25; bottom right, regular water + AsNP25. (H&E)

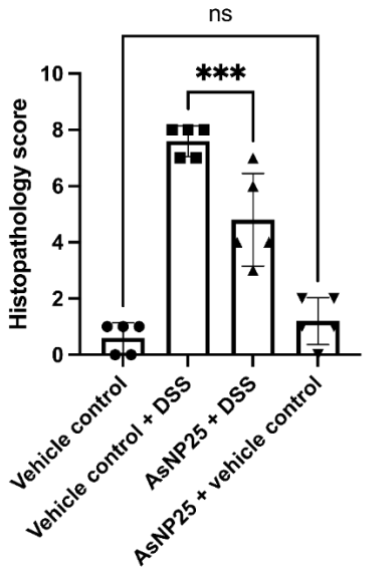


*Figure 27. Histopathological score of mice treated with or without AsNP25. Histopathology was assessed using a described scoring system by two independent researchers (75–77). Statistics calculated by One-way ANOVA. *** $p < 0.001$.*

HPLC separation of bioactive metabolite fractions

Reverse phase separation of AsNP25 using HPLC yields several peaks of varying amplitudes in 210 nm (red line) and 280 nm (blue line), which are not present in the blank tracing (green) (Figure 28).

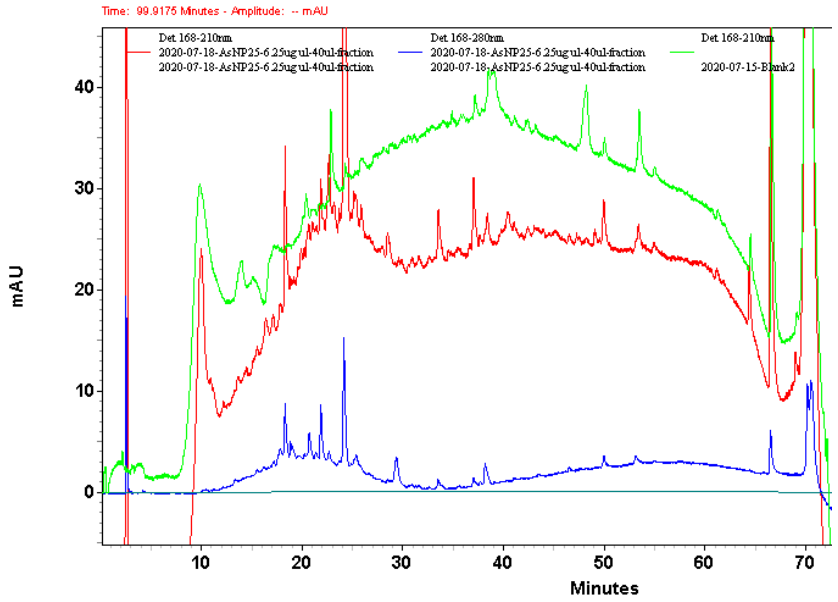


Figure 28. UV/Vis tracing of AsNP25 at 210 nm (red) and 280 nm (blue), and a blank run at 210 nm (green).

Nonpolar metabolites from helminth conditioned medium have immunomodulatory compounds common to multiple parasites

In the previous experiments, we demonstrated the bioactivity and colitis-reversing potential of AsNP25 crude metabolite fraction. However, the material from AsNP25 was insufficient to fractionate and use in subsequent experiments including those that would help us determine a molecular structure associated with the bioactivity. Since a similar immunomodulatory phenotype was observed using *D. immitis* crude metabolites *in vitro*, we proceeded to analyse those samples for their components. Reverse phase HPLC-MS/MS was used to fractionate the pooled crude, nonpolar DiNP25, DiNP50 and DiNP100 into ~80 fractions. Following separation, fractions evaporated and resuspended in methanol to dissolve, then once evaporated in HPLC grade water. The absolute mass of the product in each of the fractions was too low to be determined using a scale. Therefore, the volume of water which they were suspended in was recorded and a relative concentration was noted, including a series of dilutions. These

fractions were subsequently tested in the same treatment assays as previously depicted in BMDM. Multiple dilutions of the fractions were tested before finding that an approximately 100-fold dilution from the initial dilution was the optimal concentration to elicit a suppression of LPS-induced TNF-α secretion in fractions 53-64 (Figure 31).

Mass spec analysis of the *T. suis* purified immunomodulatory fraction (bioactivity depicted in Figure 12) shows multiple peaks including a predominant molecule of a mass of approximately 687.215[M+H] (Figure 29). These multiple peaks at different retention times represent a molecule of the same molecular weight and with the same molecular formula eluting at increasing concentrations of the acetonitrile gradient. The same molecule can be detected in crude AsNP25, but in much lower abundance. Similarly, a signal for the same mass can be detected in *D. immitis* nonpolar metabolites as well as *A. suum* (Figure 30) and *H. polygyrus bakeri*. *A. suum* crude fraction was insufficient to purify the molecule in the same manner as *T. suis*, therefore, this figure shows the chromatogram pattern for the molecule in the crude fraction.

In immunomodulatory fractions 62-65 a signal for 687.215[M+H] was also detected, as was in the *T. suis* immunostimulatory fraction. This fraction corresponded with the fractions that were determined to be immunostimulatory *in vitro* (Figure 31), which were between fractions 52-64.

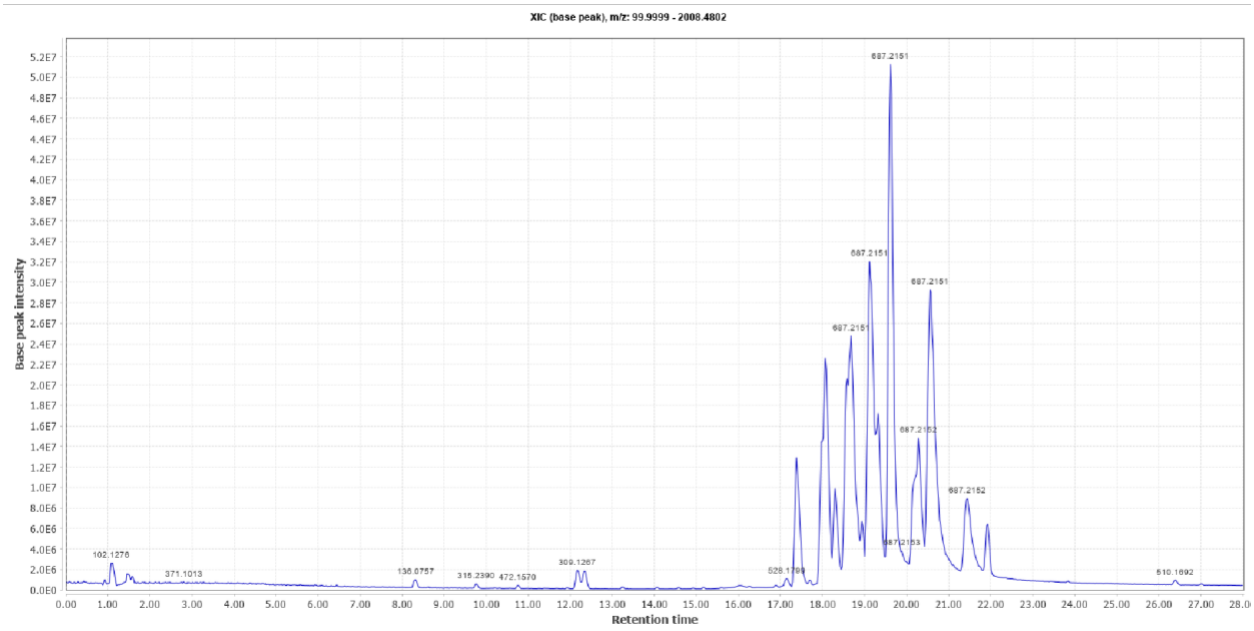


Figure 29. Total ion count chromatogram for immunomodulatory fraction of T. suis.

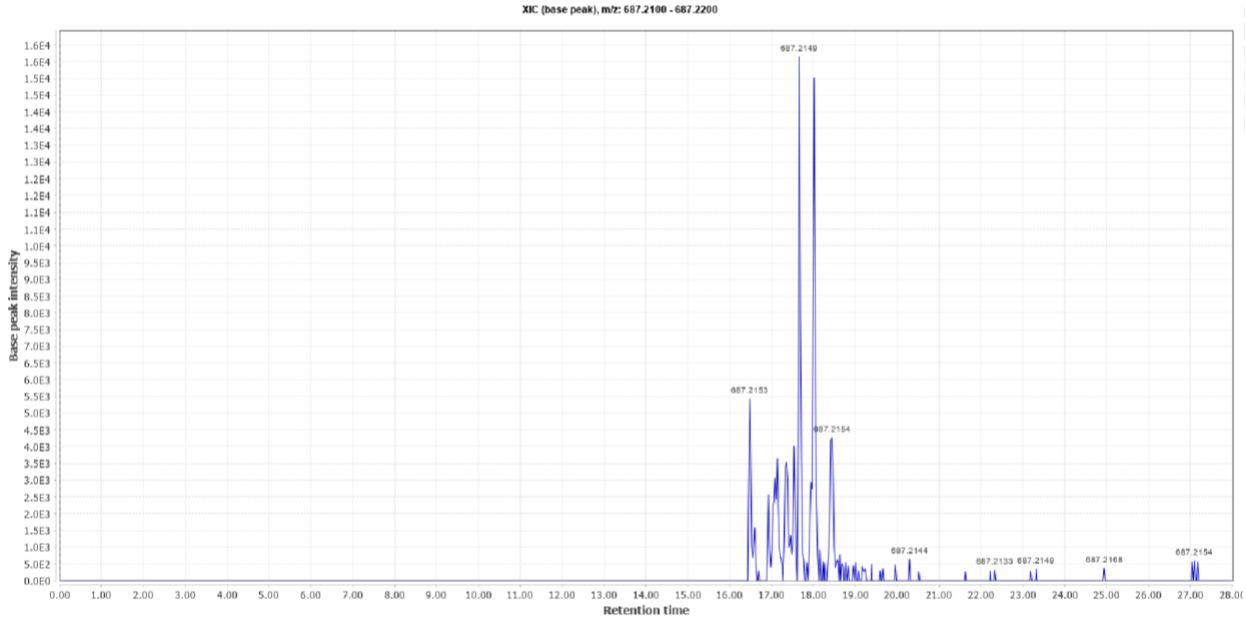


Figure 30. Total ion count chromatogram for AsNP25 gated for 687.215[M+H] ion only.
Crude AsNP25 was analysed for the presence of 687.215[M+H] mass, as a purer fraction could not be obtained.

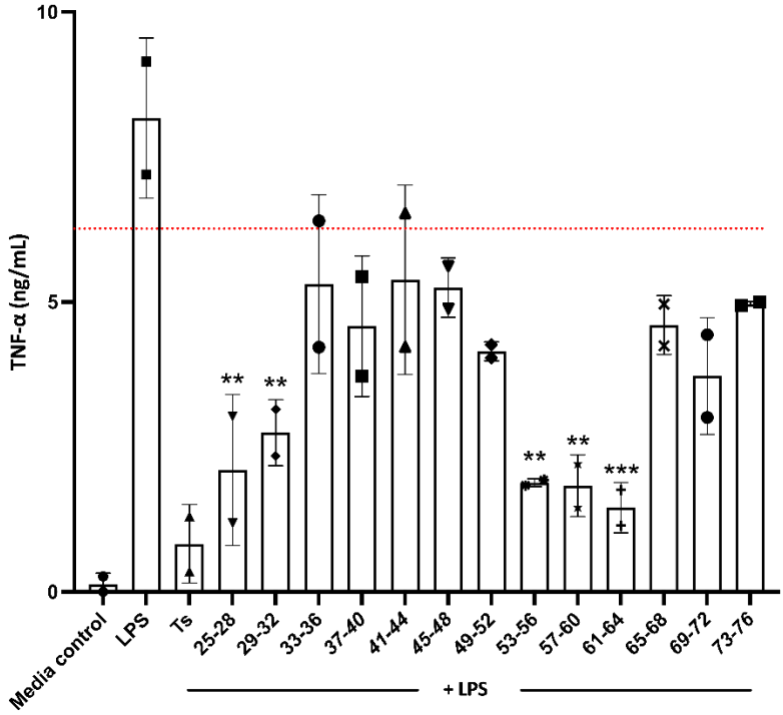


Figure 31. Secretion of TNF-α by murine BMDM treated with D. immitis UPLC fractions subsequently stimulated with LPS.
Statistics calculated by One-way ANOVA.

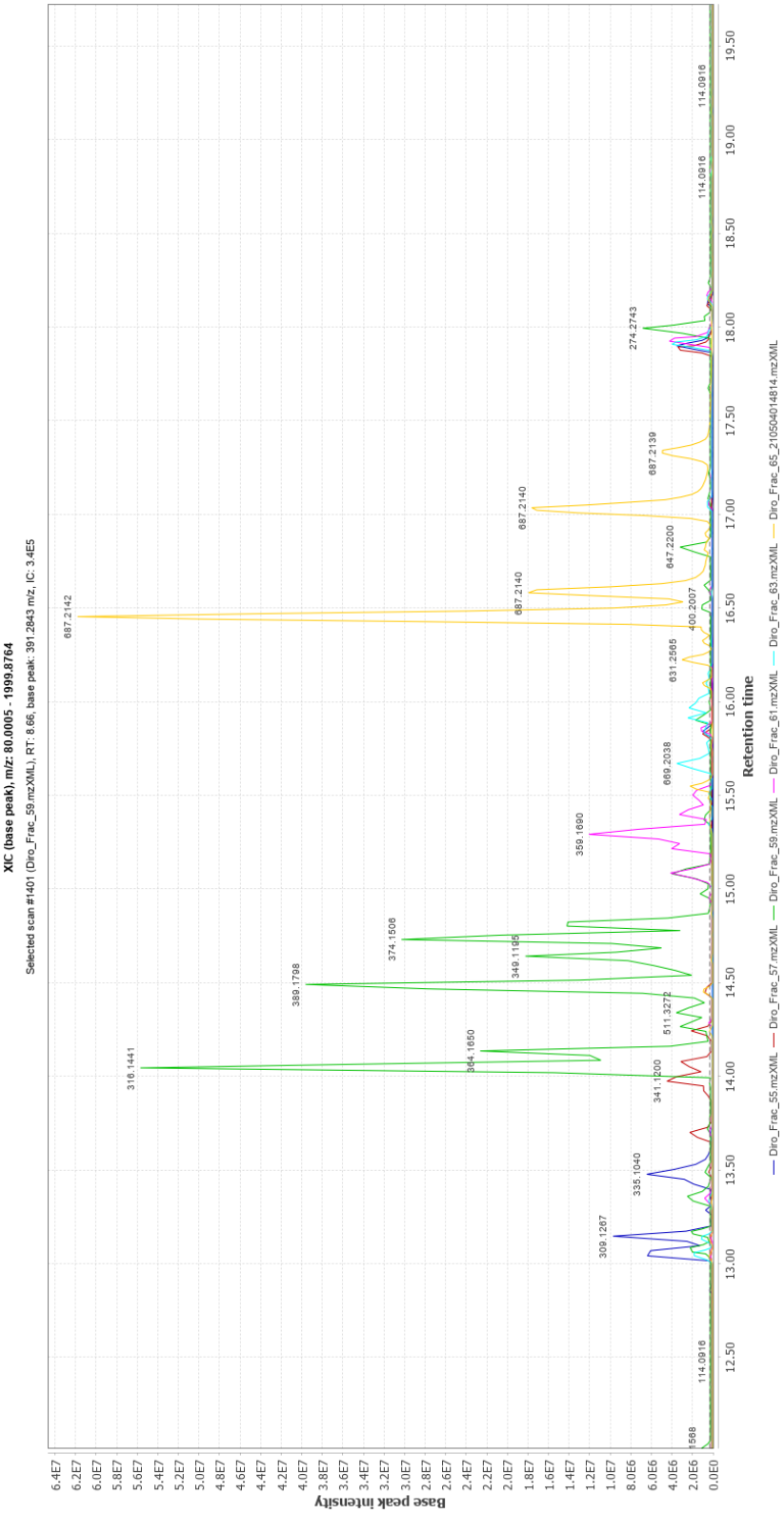


Figure 32. Total ion count chromatogram for D. immitis fractions 59-65.

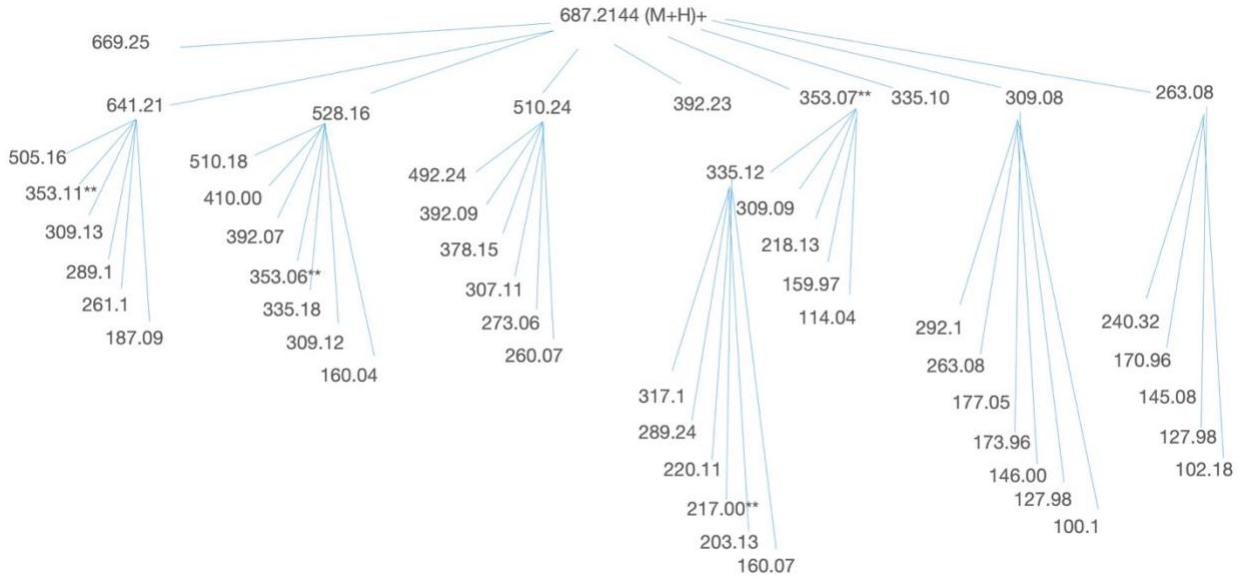


Figure 33. Fragmentation tree of 687.215[M+H] helminth-conditioned medium-derived molecule

Mass spectrometry yielded the isolated molecule of mass 687.215[M+H], and under electrospray ionization, this molecule breaks down into these various components. MS/MS/MS provided the masses seen in Figure 33, which represent molecules that can arise from the parent molecule, 687.215 [M+H] with the molecular formula $C_{32}H_{38}N_4O_9S_2$.

Table 3. Activity and predominant molecule in bioactive fractions classified by parasite.

Parasite ESP	309.12	510.169	528.180	669.205	687.215
T. suis fraction 14	++++	+	++	++	++++
D. immitis fractions 62-65	+++	+	++	++	++++
H. diminuta	Np	np	np	np	np
H. polygyrus NP25	++++	np	np	np	±
H. polygyrus NP50	++++	np	++	++	++++
D. immitis NP crude	++++	np	np	+	+++
D. immitis NP25	++++	np	np	np	+
D. immitis NP50	++++	np	np	++	++++

Fragmentation pattern of a bioactive molecule from the HCM was identified

The abundant molecule in the bioactive fractions of the HDM has a MW of 686.2144 amu, and the breakdown products are exemplified in the fragmentation tree. The other masses in this tree are also found in abundance in the crude samples from different helminths, and some appear to be present in the bioactive fractionated samples from *D. immitis* nonpolar metabolites, specifically, fractions 62-65 (Figure 31). Table 3 demonstrates the masses of the molecules that arise as fragments in tandem MS of the parent molecule, 687.215[M+H], and it wishes to provide a summary of the abundance of these molecules in the metabolite fractions.

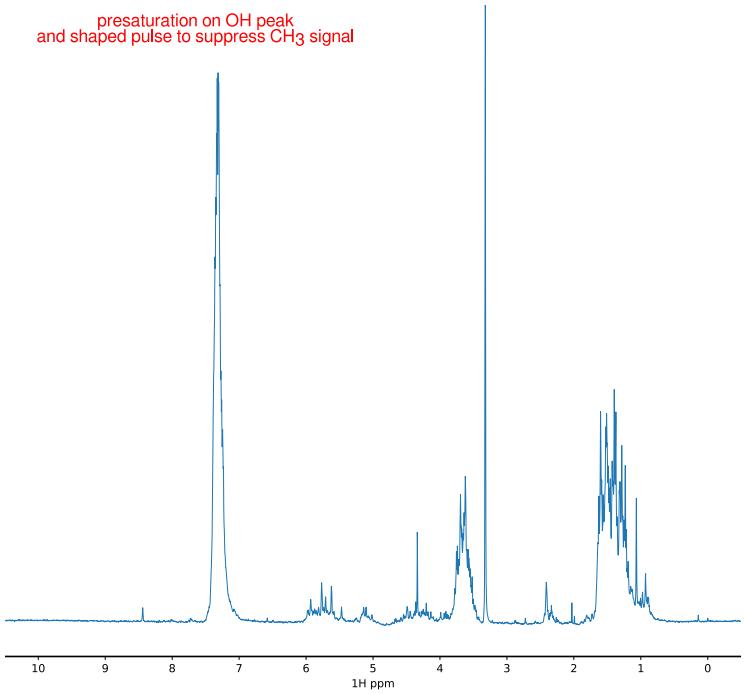


Figure 34. 1D proton NMR (^{1}H-^{1}H)
One-dimensional proton NMR plot of purified fraction of the bioactive compound with the MW of 687.215[M+H] with OH and CH_3 solvent suppression.

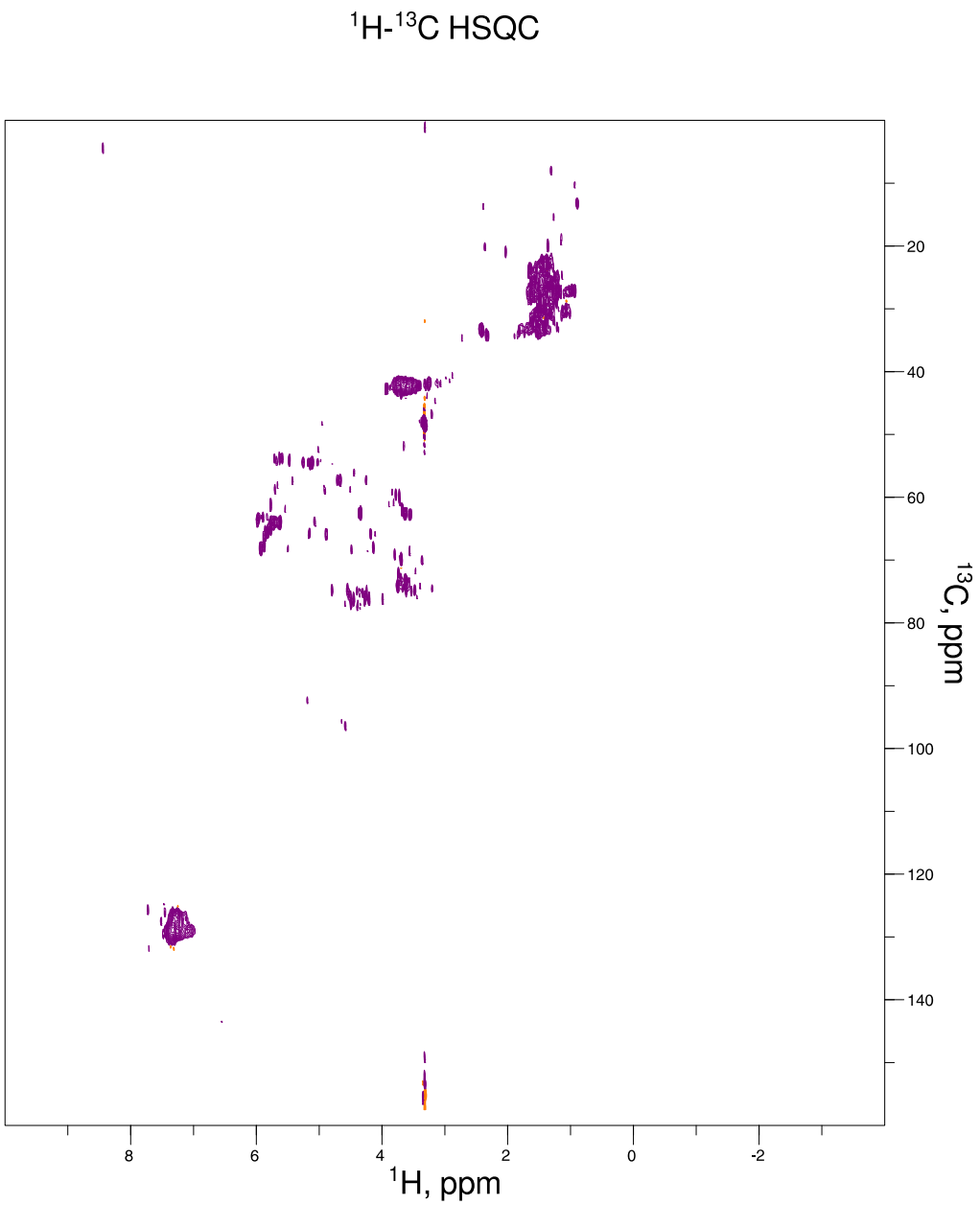


Figure 35. 2D NMR (HSQC) plot of the bioactive compound with the MW of 687.215[M+H].

In an effort to understand the structure of the molecule, we were able to isolate sufficient amounts from the *H. polygyrus bakeri* and the *D. immitis* to perform an initial experiment using 1D NMR (DQF-COSY) and 2D NMR (HSQC) (Figure 35). This image shows the diversity of proton signals. However, due to the complexity of the chemical environments additional experiments that include ^{13}C NMR are critical to be able to decipher the structure of the 686 species.

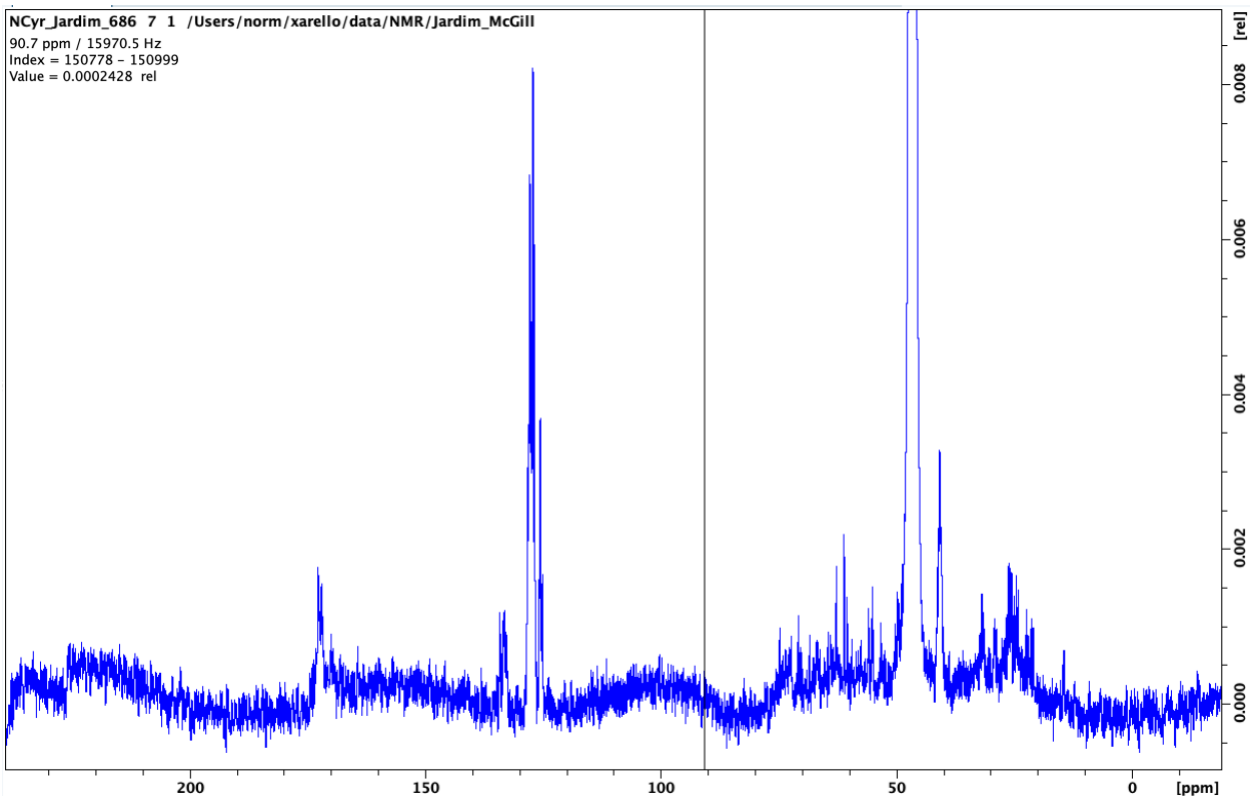


Figure 36. ^{13}C NMR analysis of the bioactive compound with the MW of 687.215[M+H].

Putative structure of compound isolated from immunomodulatory fractions

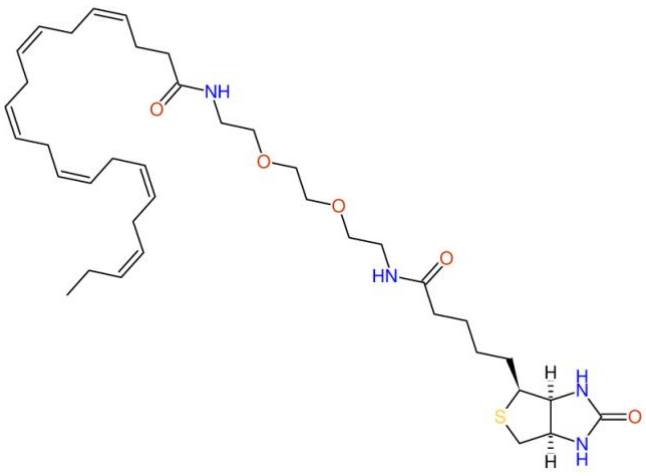


Figure 37. Predicted best structure of compound of 687.215 amu isolated from helminth-conditioned medium based on NMR detailed analysis

There was one overall best compound predicted by the software, and additionally, a total of 84 putative structures from the software. The condition that was given for the output of these structures was the molecular weight of 686.215 amu, however, there were no compounds in the output that match the molecular formula. There appears to be a discrepancy between the ^{13}C NMR data, and the molecular formula obtained through tandem MS. The ^{13}C structures and analysis are noisy, therefore, to have a reliable structure from this analysis additional purification is required in addition to larger amounts of the isolated fraction. The other top structures from the analysis can be found in the Appendix B (Figure 38).

The overall results of NMR predictions can be found at the following address: https://c13nmr.at/overall_result/61e17b70933611ec8a1a901b0efced59.html.

DISCUSSION

Parasitic immunomodulatory factors have, for a long time, been of interest for the study of immune dysregulation. Most studies to date have focused on helminth derived proteins. Our studies incorporate both the role of proteins as well as the role of small molecules in this host parasite interaction. Our methods describe a stepwise approach to dissecting the contents of ESP for their experimentation. The methodology begins by separating based on size followed by separation of proteins based on size and charge and separation of metabolites based on polarity. Ultimately, the aim is to increase the specific activity of individual compounds to isolate and study them in an immunostimulatory context.

These studies commenced with the study of the small molecules in HCM of *T. suis*, and the studies were piloted by Dr. Louis-Philippe Leroux. Initial studies revealed anti-inflammatory activity in the nonpolar isolated fractions, which led to subsequent purification using preparatory HPLC. The immunomodulatory fraction from these preparations, fraction 15, showed >90% suppression of LPS-induced TNFα secretion in BMDM (Figure 12). Limited material from *T. suis* parasites prompted the investigation of other parasitic helminths for interest of their nonpolar metabolites, which included *A. suum*, *Hpb*, and *D. immitis*. Here we show the studies surrounding *A. suum* and *D. immitis* HCM, while the laboratories of Profs Mary Stevenson and Fernando Lopes have a focus on the study of *Hpb* HCM.

Ascaris suum conditioned media contained nonpolar metabolites which were suppressive of LPS-induced TNF-α and which independently induced IL-10 secretion. Specifically, nonpolar metabolites which eluted in the 25 and 50% ACN reversed phase column chromatography fractions suppressed TNF-α in PBMC monocytes stimulated with LPS and induced IL-10 independently of LPS (Figure 13). Low amounts of metabolites were required for immuno-modulatory activity; between 1-50 µg/mL consistently displayed cytokine modulation.

D. immitis nonpolar metabolite treatment of BMDM results in a dose-dependent modulation of LPS-induced TNF-α secretion. Crude sDiNP25 suppressed TNF-α secretion in LPS-stimulated BMDM at lower concentrations than did rDiNP25, suggesting a higher concentration of the immunomodulatory molecule of interest in sDiNP25. However, this cannot be concluded absolutely as the relatively low yield of these fractions makes it impossible to be certain of a reliable mass using a scale. Nonetheless, regardless of the absolute concentration, it appears that the molecule responsible for the immunomodulation is active in an optimal range of concentrations, whereby below and above this range the suppression of TNF-α is abrogated. As seen in Figure 14, with increasing concentrations the immunomodulation is weaker. This is reminiscent of a hormesis response to drug (78).

Further studies were conducted to better understand the mechanism of immunosuppression and pathways that may be affected by helminth-derived metabolites. One way in which we investigated these mechanisms is by studying alterations in cell metabolism. Alamar Blue reagent is used to determine cell viability through mitochondrial activity. Alamar Blue, or resazurin, can be reduced by several different enzymes in the cell which normally contribute to the reducing environment of the cell. These enzymes are located both in the cytosol and mitochondrial membrane, and therefore reduction activity in an Alamar Blue assay is not only indicative of mitochondrial dysfunction, but overall cellular dysfunction (79). While in the past we used Alamar Blue to rule out cytotoxic effects of helminth-derived products in our experiments where cytokine modulation was observed, here we noticed an interesting pattern in how ESP were affecting cell metabolism. Here we show that Alamar Blue fluorescence was dampened in BMDM treated with *A. suum*-derived metabolites, suggesting a remodeling of their metabolic pathways. This avenue was further explored using H2CDFDA, which conversely demonstrated that treatment with metabolites was directly correlated with increased ROS production in the cell. Taken together, we

can understand that metabolic changes measured by Alamar Blue fluorescence are modulated by helminth-derived metabolite independent of ROS, and that other metabolism avenues need to be explored to understand their effects of immune cells.

Following multiple *in vitro* experiments demonstrating the immunostimulatory potential of nonpolar metabolites, we proceeded to study the effect these metabolites have in a murine model of colitis. We found that AsNP25 demonstrated significant reduction in colitis in our model, as well as reduced molecular evidence of colitis. Specifically, colon lengths, gross pathology, histopathology and colonic TNF-α transcription were significantly improved in AsNP25-treated mice compared to diseased mice. Notably, mouse weights were not affected by treatment, negatively or positively. Colon lengths were significantly improved in mice treated with 25 µg/mouse/day ($p < 0.001$) and 50 g/mouse/day ($p < 0.01$). Histopathology score is significantly improved in treated mice ($p < 0.001$); however, it is still significantly higher than control mice, evidenced by residual microscopic evidence of disease. This may be able to be addressed by adjusting the dose to a higher one than 50 µg/mouse/day, which is the highest dose that was tested, or by administering treatment at different points in the DSS experiment. However, AsNP25 material is limited, and we are primarily interested in isolating a bioactive compound. Therefore, future experiments will be performed using purified compound with the purpose of demonstrating that a single bioactive compound was responsible for the improvement of colitis seen in these experiments as this would be more biologically relevant than optimizing treatment of a crude fraction.

Thus, these data taken together suggest that helminth-derived metabolite fraction AsNP25 contains a bioactive molecule(s) that is responsible for significantly improving pathology in DSS-colitis without causing any unwanted effects in non-colitis mice as no toxicity was observed on gross analysis, clinically (weight/adverse events), or on histopathology.

To further investigate the etiology of the immunomodulatory responses seen in nonpolar metabolites, *D. immitis* nonpolar crude metabolites were fractionated to increase their specific activity and proceed to narrow down a molecule. Specific fractions of DiNP metabolites revealed significant reduction in LPS-dependent TNF-α secretion in BMDM. These fractions were bioactive at an approximately 100-fold dilution from the HPLC derived-fractions, indicating a relatively potent molecule. These fractions were advanced to MS analysis, where a mass of

687.215[M+H] was identified in fraction number 62-65. As depicted in Figure 29 and in Figure 30, the outputs of these analyses are exceedingly similar in *T. suis* and *D. immitis.* The conservation of this molecule across species strongly suggests a helminth-mediated response to host inflammation having a specific cellular target that is paramount to the survival of the parasite.

The production of this fraction was expedited, and structural analyses were initiated. A combination of NMR analyses revealed a list of putative structures that may correspond to the bioactive molecule. The next steps would include increased the production of the molecule even more to increase the resolution of these analysis and improve the confidence of the results of the structural analysis.

Summary and Conclusions

SUMMARY

Inflammatory bowel disease is increasing in incidence globally with no known cure. The current incidence in Canada is higher than any other country and continues to rise in Canada and globally. Current treatments for IBD vary in their efficacy from patient to patient and often have undesired side effects, and there is a need for novel therapeutics to address dysregulated inflammation at the source.

Intestinal parasites are complex organisms which have co-evolved to survive and balance the immune response in the gut. They have specific mechanisms to combat the host immune system, and their defences can be studied to better understand this host-parasite interaction as a means of better understanding the dysregulated inflammation that occurs in chronic diseases. This work demonstrates how, evolutionarily, parasites have developed molecular means of communication with host immune cells that modulate the secretion of immune regulators and subsequently affect the host response. These immune regulators come in different forms including, but not limited to, proteins, small molecules, exosomes, and others described by other groups and those not yet studied. This work demonstrates both novel proteins found in the excretome of helminths which are found to be immunomodulatory in addition to small molecules found in the excreted and secreted products which demonstrate immunostimulatory potential. This work also demonstrates how these parasite-derived immune modulators can be exploited for the purposes of therapeutics for dysregulated inflammation in chronic diseases.

CONCLUSIONS

Parasites hold the unique ability to survive within a host and evade their host's immune response. Host-parasite interactions are important to understand both from the point of view of understanding infection dynamics as well as understanding the dynamics of a host's immune system. This work describes various molecular sources of immune modulation by parasites via their ESP. We show numerous proteins found in *T. suis* ESP which change the cytokine expression in BMDM, BMDC and PBMC monocytes. Future work can study these proteins for their mechanism of action in modulating immune cells, to help better understand their molecular targets.

The data presented in this work reveal the potential in parasitic helminths as therapeutics for autoinflammatory diseases. This work demonstrates that there are multiple potential candidates for anti-inflammatory compounds in helminth ESP, and that they take the form of proteins, metabolites, and potentially other molecules not studied by us. The data presented here also suggests that the different immunomodulators may contribute to the response in different ways and that they may have different targets in the host immune system. This is strengthened by data produced by collaborators which show bioactivity of the compounds in different biological systems or cells, such as epithelial cells and human intestinal organoids [data not shown]. Here, we describe several recombinant *T. suis* proteins which show immunomodulatory activity in murine BMDM as well as PBMC monocytes.

While helminth-derived proteins have been described and studied in the literature for their immune modulation abilities, this is the first work, to our knowledge, which has described specific parasite-derived, immunomodulatory metabolites. This work describes a methodological purification and subsequent identification of a metabolite which is conserved across at least four parasites: *T. suis*, *A. suum*, *H. polygyrus bakeri*, and *D. immitis.* The mass, molecular formula, and, and some moieties of the 686.215 amu (NP686) molecule are described in this work. The molecular structure of the metabolite is to be determined, and experiments for the resolution of the structure are underway.

There is still ample landscape regarding the investigation of the 687.215[M+H] molecule found in the HCM as well as other immunostimulatory proteins from the various parasites. These studies include, but are not limited to, confirmatory studies that this molecule can be produced *in*

vitro, and that the same bioactivity be recapitulated. Future directions include synthesizing the molecule found in the helminth-conditioned medium to specifically study it and discern its molecular role. Moreover, if a bioactive molecule can be produced, future work would proceed with the investigation of its effects *in vivo* to characterize its potential in anti-inflammatory activity for the purpose of therapeutic use.

Appendix A

Table 4. Composition of helminth culture media

Trichuris suis, pH 7.2
DMEM
4.5 g/mL glucose
L-glutamine
Sodium bicarbonate
250 U/mL Penicillin G
250 µg/mL Streptomycin
0.625 µg/mL Amphotericin B
400 µg/mL Chloramphenicol

Heligmosomoides polygyrus bakeri, Dirofilaria immitis, Ascaris suum
RPMI 1640 Glutamax
Gentamycin (80 mg/mL)
Polymyxin B
Glucose
Penicillin G-Streptomycin (500 U/mL, 500 µg/mL, respectively)
Gentamycin (80 µg/mL)

Table 5. Components of cell culture media used

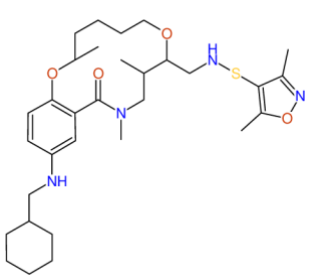
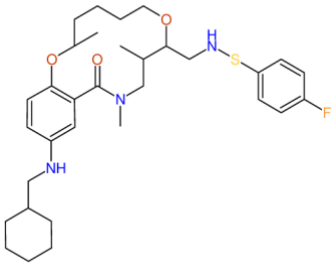
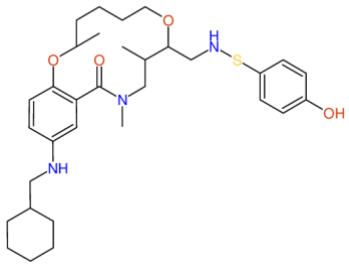
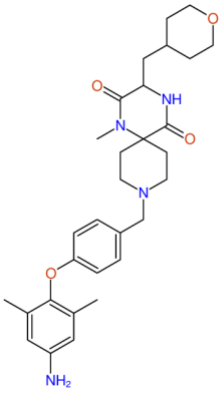
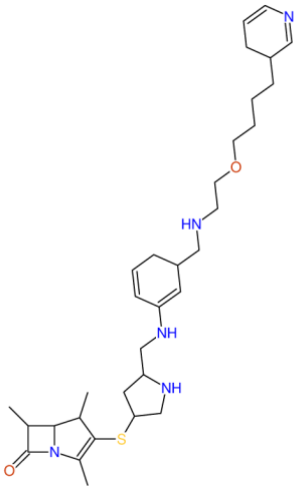
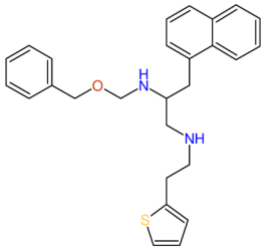
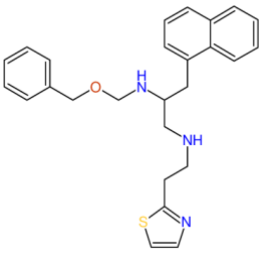
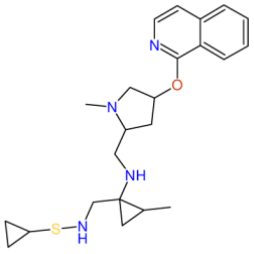
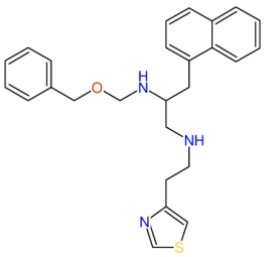
DMEM	
Components	*Concentration (mg/L)*
Amino acids	
Glycine	30.0
L-arginine hydrochloride	84.0
L-cystine 2HCl	63.0
L-glutamine	584.0
L-histidine hydrochloride-H20	42.0
L-isoleucine	105.0
L-leucine	105.0
L-lysine hydrochloride	146.0
L-methionine	30.0
L-phenylalanine	66.0
L-serine	42.0
L-threonine	95.0
L-tryptophan	16.0
L-tyrosine disodium salt dihydrate	104.0
L-valine	94.0
Vitamins	
Choline chloride	4.0
D-calcium pantothenate	4.0
Folic acid	4.0
Niacinamide	4.0

Pyridoxine hydrochloride	4.0
Riboflavin	0.4
Thiamine hydrochlorisw	4.0
i-inositol	7.2
Inorganic salts	
Calcium chloride (anhyd.)	200.0
Ferric nitrate	0.1
Magnesium sulfate	97.67
Potassium chloride	400
Sodium bicarbonate	3700.0
Sodium chloride	6400.0
Sodium phosphate monobasic	125.0
Other components	
D-glucose (dextrose)	4500
Phenol red	15.0
RPMI 1640 Glutamax	
Amino acids	
Glycine	10.0
L-alanyl-glutamine	446.0
L-arginine	200.0
L-asparagine	50.0
L-aspartic acid	20.0
L-cystine	50.0
L-glutamic acid	20.0

L-histidine	15.0
L-hydroxyproline	20.0
L-isoleucine	50.0
L-leucine	50.0
L-lysine hydrochlorise	40.0
L-methionine	15.0
L-phenylalanine	15.0
L-proline	20.0
L-serine	30.0
L-threonine	20.0
L-tryptophan	5.0
L-tyrosine	20.0
L-valine	20.0
Vitamins	
Biotin	0.2
Choline chloride	3.0
D-calcium pantothenate	0.25
Folic acid	1.0
Niacinimide	1.0
Para-aminobenzoic acid	1.0
Pyridoxine hydrochloride	1.0
Riboflavin	0.2
Thiamine hydrochloride	1.0
Vitamin B12	0.005

i-inositol	35.0
Inorganic salts	
Calcium nitrate	100.0
Magnesium sulfate	100.0
Potassium chloride	400.0
Sodium bicarbonate	2000.0
Sodium chloride	6000.0
Sodium phosphate dibasic (anhyd,)	800.0
Other components	
D-glucose (dextrose)	2000.0
Glutathionine (reduced)	1.0
Phenol red	5.0

Appendix B

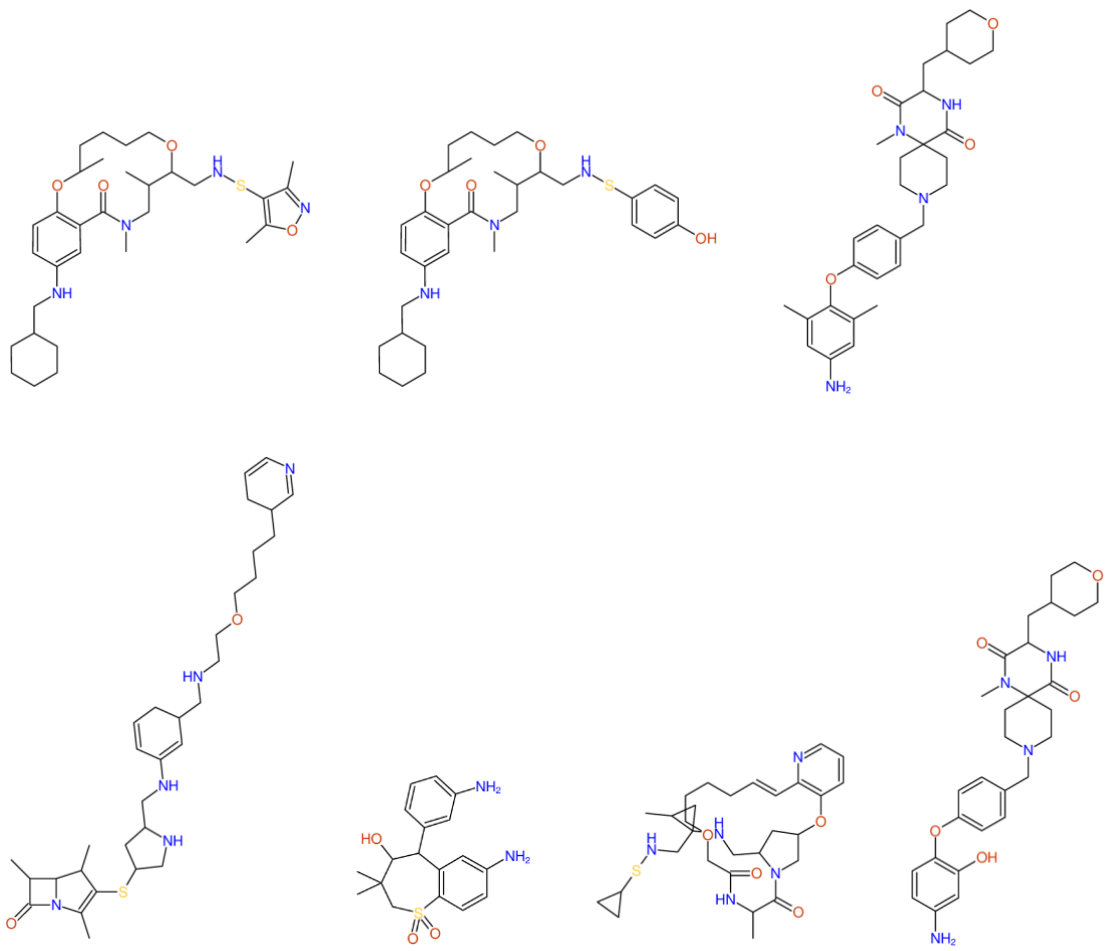


Figure 38. Best putative structures for the 686.215 amu compound found in helminth-conditioned medium provided by NMR analyses of D. immitis and Hpb pure fractions

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