Immunoregulatory molecules secreted by *Trichuris muris*

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**Abstract**

*Trichuris*, whipworm nematode infections are prevalent in humans, domestic livestock and mammals. All share an epithelial dwelling niche and similar life cycle with the chronic infections that follow implying that immune evasion mechanisms are operating. Nematode excretory secretory (ES) products have been shown to be a rich source of immunomodulatory molecules for many species. The *Trichuris muris* model is a natural parasite of mice and has been used extensively to study host–parasite interactions and provides a tractable platform for investigation of the immunoregulatory capacity of whipworm ES. The present review details progress in identification of the composition of *T. muris* ES, immunomodulatory components and their potential mechanisms of action. The adult *T. muris* secretome is dominated by one protein with modulatory capacity although remains to be completely characterized. In addition, the secretome contains multiple other proteins and small molecules that have immunomodulatory potential, certainly by comparison to other *Trichuris* species. Moreover, *T. muris*-derived exosomes/exosome-like vesicles contain both protein and multiple miRNAs providing an alternate delivery process for molecules with the potential to modulate host immunity.

**Introduction**

**The mouse model of human disease**

*Trichuris* is a genus comprising intestinal nematodes including the human whipworm parasite, *Trichuris trichiura*, that is currently believed to infect approximately 465 million people worldwide causing considerable morbidity and economic hardship (Hotez et al., 2014; Pullan et al., 2014). It was estimated to be responsible for the loss of 0.64 million disability adjusted life years from 1990 to 2010 and was ranked 10th in the neglected tropical disease rankings (Hotez et al., 2014) with long lived, chronic infections the norm. It is mostly children who suffer pathology with *Trichuris* dysentery syndrome, growth retardation, cognitive problems (Lee and Wakelin, 1983). Low dose and trickle infections lead to chronicity without external stimuli (Smits et al., 2014; Pullan et al., 2010; Alcantara-Neves et al., 2017) although it is important to recognize that these infections cause a considerable collective damage to endemic communities (Cooper and Bundy, 1988).

Our laboratory and many others have used the murine parasite *Trichuris muris*, a natural parasite of mice which is closely related to the human parasite to study host worm interactions (Cliffe and Grencis, 2004). In this scenario, many variables such as infection levels, host genetics and nutrition, can be controlled. A single low dose infection or more realistically repeated low level i.e. trickle infections (Bancroft et al., 2001; Glover et al., 2019) of *T. muris* can be used to reflect the infection levels and dynamics seen in the wild. Parasites reach patency around 33 days after infection and adult worms survive for extended periods with some adults dying from senescence from 100 days onwards.

The majority of inbred laboratory mouse strains expel a high dose infection, whereas immune-deficient mouse strains will take high dose infections through to chronicity showing that host protective immunity can operate (Cliffe and Grencis, 2004; Klementowicz et al., 2012; Grencis et al., 2014). Moreover, transient immunosuppression given to immunocompetent strains of mice during the second week of infection will also allow progression to patency (Lee and Wakelin, 1983). Low dose and trickle infections lead to chronicity without external immunosuppression and all without obvious negative effects on the health of the host. Taken together with the observations that chronic infection is associated with qualitative and quantitative differences in the immune response generated when compared to resistant mice suggest that the parasite has immunomodulatory activity (Klementowicz et al., 2012;
Grencis et al., 2014; Colombo and Grencis, 2020. This is further emphasized by blocking the regulatory cytokine, interleukin (IL)-10 during the chronic phases of infection, which leads to excessive intestinal pathology sometimes with fatal consequences (Grencis et al., 2014). An earlier study examined the effect of abbreviated infections or chronic infection upon the capacity of the host to clear a subsequent infection. These data strongly indicated that exposure to later stages of the infection prior to a challenge was less effective at inducing protection and thus potentially immunomodulatory (Else et al., 1989). Research from our laboratory using models of chemical contact hypersensitivity that depend upon type 1 or type 2 cytokine responses showed that a chronic T. muris infection modulated challenge responses to the sensitizers in the ear, a remote non-mucosal site distant from the caecum where the parasite resides (Grencis et al., 2014). It was also apparent that modulation operated only against type I sensitization and not type 2 sensitization influencing local cytokine responses and ear pathology. This depression in sensitization was associated with a reduction in movement of class-II positive cutaneous dendritic cells from the skin and elevated IL-10 levels in the ear draining lymph node (Grencis et al., 2014). This would suggest that the immunoregulation induced by T. muris infection operating systemically reflects that generated during chronic T. muris intestinal infection, i.e., a regulated Th1 response. It has also been shown that a chronic T. muris infection can modulate the brain inflammatory response in stroke (Denes et al., 2010). Systemic effects of chronic T. muris infection were subsequently observed by Chenery et al. (2016) where haematopoietic responses in the bone marrow were influenced by intestinal infection driven cytokines. Broadhurst et al. (2010) showed that T. trichiura infection could induce remission in an individual with ulcerative colitis and associated increase in IL-22+ CD4 cells and decrease in IL-17+ CD4 cells supporting an immunomodulatory role for human whipworm infection. How T. muris or T. trichiura achieves such immunomodulation remains to be completely defined but, by analogy with other parasitic nema-todes (Hewitson et al., 2009) is likely to involve the excretions and secretions (ES) of the worm especially from the later larval and adult stages of the parasite.

Here, we aim to review immunomodulation by Trichuris infection with a focus on known and potential immunomodulatory proteins in T. muris ES, regulatory RNAs and exosome-like vesicles (ELVs).

**Excretory secretory (ES) proteins and other molecules**

The ES of multicellular parasites has been the source of target molecules for disruption of the host/parasite interaction, the hunt for vaccine candidates and a potent source of immunomodu-latory proteins. A study in 2018 (Eichenberger et al., 2018) provided a detailed characterization of T. muris adult ES (day 35 post infection), the T. muris secretome. Using two biological replicates 148 proteins were confidently identified corresponding to 34.1% of the total predicted secreted proteins from the T. muris genome as based on the presence of a signal peptide. GO pfam analysis suggested a dominance of trypsin peptidases, thio-redoxin-like and tetracopeptide repeat molecules and in terms of putative molecular function, a dominance of protein binding, metal ion binding and nucleic acid binding molecules. Proteases are highly represented and a number of serine proteases and whey acidic protein (WAP) domain proteins were also identified. Of particular interest were the SCP/TAPS or CAP-domain proteins which although have been shown to be abundant in other soil transmitted helminths had not been well characterized in clade I nematodes and have been suggested to play multiple roles including potential immunomodulatory function (Cantacessi and Gasser, 2012). Substantial research on the ES of Trichuris suis has also been undertaken that has shown multiple and varied immunomodulatory activities in vitro for total ES and fractions of T. suis ES. This included the suppression of proinflammatory cytokine secretion by activated bone marrow-derived macrophages (BMDMs) and bone marrow-derived dendritic cells, the up-regulation of nitric oxide and arginase and the induction of IL-10 from BMDMs. ES was also shown to suppress the proliferation of CD4+ T cells in vitro. Three proteins from ES were identified for further study in recombinant form (a triosephosphate isomerase MW 27 kDa, a nucleoside diphosphate kinase MW 26 kDa and a small nuclear ribonucleoprotein MW 8 kDa) which exhibited modulatory effects on BMDM in vitro. Readers are referred to Leroux et al. (2018) for more details. A study by Laan et al. (2017) demonstrated that T. suis ES contained compounds that downregulated inflammatory cytokine production from LPS stimulated human dendritic cells. The active component was identified as prostaglandin E2 (PG-2), the secretion of which was found to be cyclooxygenase independent. A study by Bancroft and Grencis (unpublished) showed that T. muris also secreted a similar molecule, putatively PG-2, from the L3 stage onwards. Addition of aspirin and indomethacin affected worm motility suggesting that T. suis secretion of PG-2 was similar to that seen in the studies with T. suis (Laan et al., 2017). Rhoads et al. (2000) suggested a chymotrypsin/elastase inhibitor secreted by T. suis may act as an immunomodulatory mediator. Adult T. muris was also found to have a potential orthologue of l-dopachrome-methyl ester tautomerase (i.e. macrophage inhibition factor, MIF) although little is known as to any possible immunomodulatory activity (Pennock et al., 1998). A study by Santos et al. (2013) also detected a homologue of MIF in T. trichiura extracts using a proteomic approach. This study also identified fructose phosphate aldolase and heat shock protein 70 as potential immunomodulatory mediators. Whether any of these molecules play a role in vivo, remains to be determined.

The ES of T. muris is unusual as although it secretes a mixture of proteins throughout its life in adults 90%+ of total secreted protein is a single poly-cysteine and histidine tagged protein termed p43 (Bancroft et al., 2019). This protein was shown to have a novel sequence and protein structure but following sub-structure analysis using ProBis (Konc and Janezic, 2012) revealed domains with high homology to TSP-1 repeats and IL-13 Rα-2. It did not show high levels of homology with the cytokine interferon (IFN)-γ which had been suggested from previous studies (Grencis and Entwistle, 1997). Structurally p43 is a compact molecule containing 36 cysteines all disulphide bonded, implying a very stable conformation consistent with the harsh environment into which it is secreted, the caecum and proximal colon. It also contains a natural poly-histidine tail which has the potential to bind a number of different divalent metal cations. The observation that domains of the molecule shared homology with IL-13 Rα-2, a receptor which binds with extremely high affinity to IL-13 neutralizing IL-13 function (Lupardus et al., 2010; Karmele et al., 2019) raised the possibility that p43 may share a similar function. This would potentially benefit the parasite as IL-13 is known to be critical to expulsion of T. muris (Bancroft et al., 1998). Indeed, p43 was shown to bind to IL-13 and also glycosaminoglycans (GAGs) putatively through the TSP-1 repeat region with nanomolar affinities. Moreover, IL-13-driven immune responses could be downregulated by p43 both in vitro and in vivo. p43 is expressed in all larval stages of T. muris (Bancroft et al., 2019) with the largest quantity of secreted p43 from adult parasites consistent with high levels of production of a poly-cysteine and histidine-tailed protein by other species of whipworm e.g. T. suis (Leroux et al., 2018). Chronic infection by whipworms is associated with extensive remodelling of the extracellular matrix
Metabolomic profiling has also been carried out on *T. muris* egg/L1 extracts and adult ES. A study comparing the metabolomes and lipidomes of *T. muris* eggs and *Nippostrongylus brasiliensis* L3 using liquid chromatography-mass spectrometry (LC-MS) showed that many of the major polar metabolites identified have potential anti-inflammatory properties whereas the lipidomic analysis revealed a high level of triglycerides. There was little overlap between these parasites which is perhaps not surprising as they ultimately occupy different niches and have markedly different life cycles (Yeshi et al., 2020). Wangchuk et al. (2019) used targeted gas chromatography-mass spectrometry and LC-MS of adult ES and identified known polar and non-polar metabolites together with fatty acids including short chain fatty acids (SCFAs). A number of these compounds have been shown to have anti-inflammatory activity in a variety of assays although their activity in *vivo* is unknown. SCFAs that are known to be produced by a number of commensal bacteria and have immunomodulatory effects were also identified although their origin is still a matter of debate. Propionate was the major SCFA detected from *T. muris* although butyrate was also found (Wangchuk et al., 2019). Certain butyrate, which is known to influence colonocyte growth and T regulatory cells, is unlikely to be *T. muris* derived as this species does not contain the genes required to synthesize butyrate or any butyrate transporter orthologues (Foth et al., 2014). The source may be related to the fact that *T. muris* has its own intestinal microbiota (White et al., 2018).

**Exosomes and exosome-like vesicles**

In 2014, Buck et al. (2014) showed that exosomes secreted by *Heligmosomoides polygyrus*, which reside in the murine small intestine could transfer small RNAs and nematode proteins to mammalian cells to modulate innate immunity. Tritten et al. (2017) isolated ELVs/particles the size of exosomes from adult *T. muris* over an 18–48 h period of *in vitro* culture and from them identified 14 *T. muris* miRNA candidates with high confidence and 73 putative *Trichuris* proteins. Eichenberger et al. (2018) identified 364 *T. muris* proteins in ELVs from adult worms, the most common being trypsin domain-protein, sperm-coating protein extracellular proteins, a poly-cysteine and histidine-tailed protein, a glyceraldehyde-3-phosphate dehydrogenase and a TB2/DP1HVA22 domain containing protein. A *T. muris* tetraspanin supportive of ELV formation was also identified. Only 13.7% of the identified *T. muris* proteins had a transmembrane domain and 33% had a signal peptide. Of the 56 miRNAs identified, 34 had homologues to other nematodes and 22 were novel. Shears et al. (2018a) identified 125 proteins from adult *T. muris* ELVs and again confirmed a number of potential *T. muris*-derived exosome associated proteins many of which did not contain a classical signal peptide. Moreover, the ELVs were able to induce a degree of protection when used for immunization without adjuvant.

Eichenberger et al. (2018) showed that *T. muris* ELVs could be internalized by mouse colonic organoids raising the possibility of the ELVs communicating with intestinal epithelial cells. Duque-Correa et al. (2020) developed a novel caecaloid model micro-injected with *T. muris* adult-derived ELVs which enabled study of the host pathogen interaction *in vitro*. Their study argues that the caecum is a unique and different niche to the colon and so provides additional and novel data to that provided in Eichenberger et al. (2018). RNA-seq data showed a significant downregulation of viral response associated genes by caecal IECs. A downregulation of IFN-stimulated genes suggested that a direct effect on the caecal epithelium may at least partly explain the anti-inflammatory effects of whipworm infection. This was
the first demonstration of an involvement of type I IFNs in *T. muris* infection and Duque-Correra postulated that this immunosuppression may allow ELV cargo entry to the host immune system. White et al. (2020) compared small RNAs in ELVs from *T. muris* and Heligmosomoides bakeri and found they were quite distinct from each other and concluded that this did not depend on their method of preparation but reflected their different intestinal niches and different functions within their hosts. Layton et al. (2020) reviewed regulatory RNAs: miRNAs, siRNAs and piRNAs and advocated that miRNAs played a wider role than mere regulation of transcription of the host but also were involved in communication between different taxonomic kingdoms including microbiota.

**Genomic analysis of potential novel immunomodulatory genes**

In 2011 the draft genome of *Trichinella spiralis* was published (Mitrev et al., 2011) providing valuable information on a clade I parasitic nematode for the first time. Furthermore, in 2014, the publication of the genome of three whipworm species: *T. muris*, *T. trichiura* (Foth et al., 2014) and *T. suis* (Jex et al., 2014) expanded the available information on clade I nematodes and the potential identification of many genes that could encode immunomodulatory molecules. Collectively, these papers are a wealth of information for studies on host–parasite interactions in whipworm infection. The *T. spiralis* study compared parasitic and non-parasitic genes by evaluating the *T. spiralis* and *Caenorhabditis elegans* genome and also focused on potential vaccine candidates from secreted proteins. The *T. suis* study provided transcriptomic data from different life cycle stages together with the description of small non-coding RNAs. Multiple parasitic genes and miRNAs were identified as having potential immunomodulatory activity and the reader is referred to Jex et al. (2014) for more details. *Trichuris suis* has been used in trials to treat humans for a range of clinical conditions, most notably inflammatory bowel disease (IBD) (Summers et al., 2005), see Hayes and Grencis (2021) supporting an immunomodulatory role for *T. suis* infection.

The *T. muris* reference genome (Foth et al., 2014) was the first study to show the pairwise genomes of a major human STH and its murine counterpart. It described both sex and life cycle stage specific genes. It also contained detailed RNA-seq data on host gene expression in chronic low dose *T. muris* infection when immune-mediated regulation is believed to operate. *Trichuris muris* in the mouse has an unusual niche in common with *Trichuris spp.* with an anterior end forming syncytial tunnels within the intestinal epithelium and occupying between 1000 and 2000 epithelial cells. The large posterior end of the worm hangs free within the caecal lumen. Whipworms also possess atypical structures for nematodes, the bacillary band and the stichosome (Sheffield, 1963) which together occupy the bulk of the anterior half of later larval stages and adult parasites. The stichosome is believed to perform a secretory function associated with digestion whereby stichocytes are ducted into the worm intestine (Lee and Wright, 1978). The bacillary band has been suggested to be involved in both absorption of nutrients and secretion (Tilney et al., 2005; Hansen et al., 2016; Lopes-Torres et al., 2020). Looking at the patterns of parasite gene expression in the anterior region of adult *T. muris*, Foth et al. (2014) identified a dominance of chymotrypsin A-like serine proteases and protease inhibitors. These inhibitors had high similarity to a mammalian secretory leucocyte peptidase inhibitor (SLPI). They were encoded for by 63 genes, which is far higher than in any other nematode species so far examined and a high percentage of these genes suggested they encoded secreted proteins based on the presence of a signal peptide. It was suggested that these proteins had potential immunomodulatory function, degrading intestinal mucins which provide a physical barrier to parasite entry into epithelial cells (Foth et al., 2014). Certainly, *T. muris* ES contains serine proteases that can degrade the intestinal mucin Muc2 (Hasnain et al., 2012). The SLPI-like proteins mostly contained a WAP domain and the *T. muris* genome contained 44
genes which encoded between 1 and 9 WAP domains (Foth et al., 2014). WAP domains of unknown function in mammalian SLPs and eelans have immunomodulatory, anti-inflammatory and anti-microbial properties (Williams et al., 2006; Scott et al., 2011; Wilkinson et al., 2011). They are produced by epithelial cells and have a role in modulating inflammation and wound healing. The direct role of T. muris-produced WAP proteins has not been assessed but they have been postulated to play a role in regulating mucosal immunity (Wilkinson et al., 2011).

Interestingly, one WAP protein from T. muris has been used in vaccine studies with some efficacy (Briggs et al., 2018). The reference genome for T. muris provided a basis for comparison with the human pathogen T. trichiura highlighting a number of important similarities supporting a commonality of potential function in human infections (Foth et al., 2014).

**Concluding remarks**

*Trichuris muris* has been used to inform on the complex interaction between the host, parasite and the rich microbial ecosystem that forms part of its niche. It has also provided valuable information on the host immune system *per se*. Low level infection with *T. muris* has been shown, in immunocompetent mice to result in changes in host pathology and a remodelling of the epithelial matrix. However, considering this parasite breaches the epithelial barrier exposing the host to the caecal microbiota and potential pathogens, *T. muris* must regulate its environmental niche as other many helminths do. But, it is also evident that the parasite exerts effects beyond its niche. Indeed, infection has been shown to affect the host at sites far away as the brain implying a systemic regulation. The ES is at the host–parasite interface and is a rich source of molecules and EVs which have been suggested to have immunoregulatory activity. Publication of the genome of *T. muris* and closely related species has also given scope to uncover previously undiscovered immunoregulatory molecules. What is unusual about *T. muris* (and by extrapolation about other *Trichuris* spp. and clade I nematodes (Giorello et al., 2017)), is the dominance of ES from adult parasites quantitatively in changes in host pathology and a remodelling of the epithelial matrix. It has also provided valuable information on the complex inter-

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