**Trichuris suis** secrete products that reduce disease severity in a multiple sclerosis model

Christine Søholm Hansen1†, Henrik Hasselsdam1*†, Idahella Hyldgaard Bacher2, Stig Milan Thamsborg2, Flemming Fryd Johansen1 and Helene Kringel2

1University of Copenhagen, Biotech Research and Innovation Center, Ole Maaloes vej 5, 2200-N Copenhagen, Denmark; 2University of Copenhagen, Faculty of Health, Parasitology and Aquatic Diseases, Dyrlegevej 100, Frederiksberg 1870-C, Denmark

**Abstract**

Multiple sclerosis is a chronic inflammatory central nervous system (CNS) disease, which affects about 1 in 1000 individuals in the western world. It has been suggested that this relatively high prevalence is linked to a high level of hygiene, i.e. a reduced exposure to various microorganisms, including parasites. Parasites are known to employ different immunomodulatory and anti-inflammatory strategies, which enable them to evade destruction by the immune system. We have investigated the immunomodulation by the swine whipworm, *Trichuris suis*, by measuring the impact of oral administration of *T. suis* ova as well as of intraperitoneal administration of *T. suis* excretory/secretory products on the development and progression of experimental autoimmune encephalomyelitis – an animal model that shares clinical and pathological characteristics with multiple sclerosis. Intraperitoneal administration of excretory/secretory products before disease onset, resulted in a significant decrease in disease severity as well as markedly reduced Th1 and TH17 T-cell responses, centrally in the spinal cord as well as in the periphery, i.e. the spleen. Thus, parenteral administration of *T. suis*-derived products results in a skewing of the immune response with a significant impact on disease severity in a CNS inflammatory disease model.

**Keywords**

*Trichuris suis*, multiple sclerosis, immunomodulation, t-cells

**Introduction**

Co-evolution of parasites and their hosts has been driven by the parasites’ aim to survive and reproduce, and the host’s aim to eliminate the invaders. Thus, parasites have developed mechanisms that suppress the host’s immune reaction. The absence of parasite exposure and thus disease pressure in some areas of the world has been correlated to a rise in atopic and autoimmune diseases, and has led to the formulation of the "hygiene hypothesis". This hypothesis suggests that a lack of certain early pathogenic stimuli is causative of the development of atopic or autoimmune diseases, later in life (Strachan 1989; Milo and Kahana 2010; Rook 2012). Fleming and Cook (2006) have reported a reverse relationship between the global presence of the human whipworm, *Trichuris trichiura*, and the prevalence of Multiple Sclerosis (MS) (Fleming and Cook 2006). Similarly, patients suffering from MS that had become naturally infected with parasitic worms, such as *T. trichiura*, were found to have fewer disease exacerbations and reduced disease symptoms before parasite elimination (La Flamme et al. 2003; Correale and Farez 2011).

Parasitic worms, or products thereof, have been tested as treatments in experimentally induced models of autoimmune diseases. In an animal model of MS - experimental autoimmune encephalomyelitis (EAE) – disease severity was reduced if treatment was initiated before or around the time of disease induction (La Flamme et al. 2003; Gruden-Movsesijan et al. 2008; Walsh et al. 2009; Gruden-Movsesijan et al. 2010; Wu et al. 2010), whereas treatment in the effector phase of the disease, where T-cells are primed and migrate to the CNS, had no impact on disease severity (Sewell et al. 2003; Zheng et al. 2008).

Both MS and EAE have demyelinating and neurodegenerative pathologies, which are intimately associated with inflammatory cells (T-cells, macrophages, B-cells etc.) in the central nervous system (CNS) (Compston and Coles 2002). Disease
exacerbation of MS has been linked to T_{h1}7 cells infiltrating the CNS (Kebir et al. 2009), and although no specific cytokine of the T_{h1}7 lineage has been found to be key for development of MS, both IL-17 and IL-22 can make the Blood-Brain Barrier (BBB) leaky and facilitate lymphocyte extravasation into the CNS (Kebir et al. 2007). Parasitic stimulation has been shown to suppress the autoimmune pro-inflammatory T-cell expansion, which is believed to be central in the pathology of EAE (Cua et al. 2003). As such, treatment studies in EAE have shown this to be associated with clinical disease amelioration, where both the pro-inflammatory T_{h1} and T_{h17} cells were down-regulated, while the tolerogenic T_{reg} and the humoral T_{h2} cells were found to be up-regulated (Hasseldam et al. 2013).

Most parasites have immunomodulatory properties and many parasitic infections are chronic and potentially harmful, in their natural host. Trichuris suis, the porcine whipworm, is an intestinal helminth of pigs with a direct life cycle (Beer 1973). By using this parasite in non-native and non-permissive hosts, one can avoid chronic infections, while potentially maintaining the beneficial immunomodulatory effects. Crude adult worm homogenate of T. suis, has been shown to suppress EAE development and clinical disease in a murine host, if administered before disease induction (Kuijk et al. 2012). In patients suffering from inflammatory bowel disease (IBD) - another T-cell associated autoimmune disease - multiple administrations of infective T. suis ova showed therapeutic effects (Summers et al. 2005; Summers et al. 2005). Recently, this treatment regimen was copied in a study with 5 MS patients in whom 3 months of treatment with T. suis ova, decreased the number of new demyelinating lesions in the CNS (Fleming et al. 2011). A similar small-scale trial primarily designed to document safety, including 10 MS patients without appropriate controls, found this treatment regimen to be safe, but ineffective (Voldsgaard et al. 2015).

We have conducted a series of experiments with the aim of investigating whether T. suis ova and adult T. suis E/S products, administrated repeatedly and starting immediately after EAE disease induction, have the potential to dampen EAE disease severity. E/S products are molecules that are shed or excreted/secreted (E/S) from the ova, larvae or adult worms, and partially constitute the immune interacting potential of the parasite (Lightowlers and Rickard 1988).

### Materials and Methods

#### EAE induction and scoring

Female Dark Agouti (DA) (Harlan, The Netherlands or the U.S., for the ova and E/S study, respectively) rats, age 5 weeks, were acclimatized for 5 wk in individually ventilated cages (IVC), with free access to food and water.

EAE was induced by subcutaneous injection of 100 μl emulsification consisting of spinal cord homogenate (SCH) in PBS (1:2; v/w) and Complete Freund’s Adjuvant (CFA) (1:1; v/v), at the tail root. All studies were conducted with randomized treatment and control groups, and in accordance with the guidelines of the Danish Animal Experiments Committee (#2012-DY-2934-00001). Animals were scored on a daily basis for clinical disease symptoms (paresis), by an observer who didn’t know whether the rats were given PBS or T. suis, according to the EAE clinical scoring system (0: no clinical symptoms; 1: tail paralysis; 2: mild paralysis in 1 or 2 hind limbs; 3: moderate hind limb paralysis in 1 or 2 limbs; 4: severe paralysis in 1 or 2 hind limbs; 5: paralysis in 1 or 3 hind limbs and paralysis starting in 4 limbs; 6: moribund or dead) devised by the Danish Animal Experiments Inspectorate. When the scores reached 2, animals were provided with food and water-gel at the bottom of the cage. If the score reached 4 or more, or if weight loss was above 20% of initial live weight, the animal was euthanized. The animal that did not develop disease was excluded from the experiment. All clinical scores of all the remaining rats are included in the data analysis (Fig. 1; Table I).

The experimental timeframe lasted from the day of disease debut (clinical score >0), and until the approximate day of the first relapse peak (a unidirectional rise in scores over a few days), which was reached approximately 10-12 days after disease debut. If no relapse occurred, rats were euthanized at the estimated day of the relapse peak. The EAE model is highly sensitive to many environmental and genetic factors, and we did find some variation in disease onset and severity between the studies.

#### Embryonated T. suis ova

Fresh T. suis ova were isolated from feces of pigs and embryonated in vermiculite (90 days at 22 C) and stored in H_2SO_4

### Table I. Clinical disease manifestations in PBS, T. suis ova and T. suis E/S groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Days before disease onset (mean ± SD)</th>
<th>Cumulative score index (mean ± SD)</th>
<th>Score at relapse peak (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8/8</td>
<td>8.23 ± 0.89</td>
<td>1.48 ± 0.95</td>
<td>3.25 ± 0.76</td>
</tr>
<tr>
<td>T. suis ova</td>
<td>8/8</td>
<td>7.63 ± 0.74</td>
<td>1.63 ± 1.88</td>
<td>3.45 ± 0.50</td>
</tr>
<tr>
<td>Control</td>
<td>10/10</td>
<td>10.10 ± 1.85</td>
<td>1.31 ± 0.78</td>
<td>3.45 ± 1.12</td>
</tr>
<tr>
<td>T. suis E/S</td>
<td>9/10</td>
<td>10.78 ± 1.72</td>
<td>0.94** ± 0.81</td>
<td>2.89 ± 1.36</td>
</tr>
</tbody>
</table>

Clinical disease manifestations in (define) PBS, T. suis ova and T. suis define E/S groups. Treatment was initiated at day of immunization (0-dpi), and all animals, but one, developed disease. The cumulative score index is the mean score of the experimental timeframe, while the relapse peak is the mean score at the day of the relapse peak day (or estimated day if no relapse occurred). **p < 0.01
solution (pH 2) at 5 C until use. Embryonated ova were sedimented, counted and diluted in PBS (7,000 ova/500 µl) immediately before use.

**Adult T. suis excretory/secretory products**

Excretory/secretory (E/S) products were obtained from adult T. suis worms, that had been pulled from the mucosa of the caecum and anterior colon of infected pigs and collected into 0.85% NaCl. They were then passed through a series of washing steps before final incubation in RPMI 1640 containing 1% glucose and penicillin (500 U/mL), streptomycin (0.5 mg/mL) and fungizone (1.25 µg/mL) in Petri dishes at 37 C for 3 days. Culture fluid was collected on a daily basis and replaced with fresh medium. All culture fluids were centrifuged (500 g for 5 min) and the supernatants were collected and filtered through a 0.45µm bottle-top filter before concentration by centrifugation on a 10,000 MWCO membrane (Sigma-Aldrich, St. Louis Missouri). The protein concentration of the E/S products were determined with a commercial BCA-assay (Thermo, Rockford, IL), and diluted in PBS to 250µg protein/200µl.

**Experimental design**

In a pilot study on the infectivity of T. suis in DA and Sprague Dawley (SD) rats, the larvae recovery from rats inoculated with embryonated T. suis ova was investigated by microscopic analysis of mucosal scrapings as well as by a modified EDTA method of larvae release, where T. suis larvae were found to hatch and embed in the intestinal mucosa in this non-natural

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**Fig. 1.** Disease severity in control and Trichuris suis treated rats. Scoring of disease activity in T. suis embryonated ova (a) or T. suis adult worm E/S products (b), applied from the day of disease induction (0 days post immunization). Mean + SD. **p < 0.01**
host but infectivity rates (number of larvae recovered from mucosa in per cent of the amount of embryonated eggs inoculated) were low, i.e. less than 5% (data not shown).

To investigate whether T. suis ova affects disease induction, a group of 8 rats were given 7000 embryonated T. suis ova through a stomach tube (inoculation) 3 times a week from the day of EAE induction (0-dpi), until the day of euthanasia. A group of 8 control rats were given 500 µl PBS in the same fashion. This experiment represents 1 of 2 similar experiments.

To overcome the low infectivity of T. suis ova in DA rats, a group of 9 rats were injected intraperitoneally (i.p.) with 200 µl (250 µg) adult T. suis E/S products, every second day from the day of EAE induction (0-dpi). A group of 10 control rats were given 200 µl PBS in the same fashion.

### Tissue analysis

Animals were euthanized by exsanguination under anesthesia (300 µl Hypnorm [fentanyl, 0.315 mg/ml; fluanisone, 10 mg/ml] and 150 µl Midazolam), and perfused with PBS. The spleen and spinal cord were removed and single cell suspensions were made in DMEM through a 70 µm Falcon cell strainer. Suspensions were washed twice in DMEM by centrifugation (400x g, 8 min), and resuspended in DMEM+10% DMSO+20% FCS and frozen to -80°C. Spinal cord lymphocytes were isolated using 30% Percoll in PBS over 70% Percoll and washed twice in DMEM through a 70 µm Falcon cell strainer. Suspensions were washed twice in DMEM by centrifugation (8 min), and resuspended in DMEM+10% DMSO+20% FCS and frozen to -80°C.

Spinal cord lymphocytes were isolated using 30% Percoll in PBS over 70% Percoll in PBS and centrifuged at 400x g, for 20 min. Red blood cells (RBC) were lysed in RBC Lysis Buffer (Biolegend) for 5 min on ice. Cells (10^7 per ml) were stimulated for 6 hr with Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml)+ Ionomycin (1 µg/ml) in RPMI-1640 w/glutamine+10% FCS+Brefeldin A (1µg/ml)+ Pen-Strep. They were then fixed and permeabilized according to the FoxP3 Fix/Perm protocol (Biolegend). Fluorophore-conjugated antibodies were applied: CD4-FITC (W3/25), IFNγ-PE (DB-1), IL-17A-APC (eBio17B7) and FoxP3-PE (15D).

T-cell subtypes were analyzed in a FACScalibur Flowcytometer (BD Biosciences, San Jose, California). Data were gated for the lymphocyte population based on forward and side scatter and sub-gated for CD4 positive cells and T-helper cell lineage markers (IFNγ, IL-17A, FoxP3). Data was obtained as the percentage of lineage marker positive cells of all CD4 positives, and presented as the treated rats relative to the control animals (PBS). Spinal cord samples containing too few lymphocytes for measurement were excluded from the study.

### Data analysis and statistics

The clinical scores were analyzed with the Mann-Whitney rank comparison of the accumulated scores, and presented as mean±SD. The individual cumulative score index was calculated by the mean of all scores from the day of disease debut until the experimental endpoint. FACS data gating was carried out in FlowJo 7.6 software, and the Mann-Whitney test was used in the statistical analysis. Data are presented as mean±SD. Statistical significance was reached at p-value <0.05.

### Results

#### T. suis embryonated ova have no disease ameliorating effect on EAE

When comparing the clinical scores of the control group and the T. suis ova treated group, there was no significant reduction in clinical disease and no effect on the day of disease debut or on the number of rats developing EAE, within the experimental time frame (Fig. 1a; Table I). Thus, we were unable to demonstrate a therapeutic effect of multiple T. suis ova inoculations in the DA rat EAE model.

#### T. suis excretory/secretory products have a moderate clinical disease ameliorating effect on EAE

Following repeated injections of E/S products from the day of induction (0-dpi), we obtained moderate but consistent lower clinical scores in the treatment group compared to the control group. The difference in clinical scores was statistically significant (p = 0.0091) during the experimental period (Fig. 1b). Except for one animal in the treatment group, all animals developed disease (Table I). This animal was not included in the analyses.

#### T-helper cell responses correlate with disease activity

The T-helper cell responses were investigated with flow cytometry of relevant T-helper cell lineages (T H17: CD4+IL-17A+), (T H1: CD4+IFNγ+) and (T Reg: CD4+FoxP3+). The peripheral T-cell populations were isolated from spleen, whereas the central T cell populations were isolated from spinal cord.

From spleen we obtained 5897 ± 1921 CD3+ T-cells, 2888 ± 790 CD3+CD4+ T-cells, 169 ± 101 CD3+CD4+IL-17+ cells, 280 ± 148 CD3+CD4+IFN-γ+ cells, and 98 ± 39 CD3+CD4+Foxp3+ cells per 25,000 live cells.

From spinal cord we obtained 541 ± 194 CD3+ T-cells, 361 ± 177 CD3+CD4+ T-cells, 80 ± 19 CD3+CD4+IL-17+ cells, 149 ± 69 CD3+CD4+IFN-γ+ cells, and 84 ± 29 CD3+CD4+Foxp3+ cells per 25,000 live cells.

Following T. suis ova treatment, no differences in the T-helper cell response was observed between treatment and control animals (Fig. 2). Lack of significant changes in the number of T-helper cells correlates well with the lack of effects on clinical disease (See Figure 1a).

In contrast to oral administration of live embryonated T. suis ova, i.e. administration of adult T. suis E/S products had a moderate beneficial effect on disease severity (See Figure 1b) which coincides with changes in the T-helper cell
Fig. 2. T-helper cell subtypes in the spleen (a-c) and spinal cord (d-f), after treatment with T. suis ova from day of disease induction. Ratios between CD4+ IL-17A+ (a, d), IFNγ+ (b, e) and FoxP3+ (c, f) cells, between the treatment (n = 7) and the control (n = 7) groups are shown. All statistics are based on the Mann-Whitney test comparing the ranks of each dataset. Data are presented relative to controls; mean +SD. *p < 0.05

Fig. 3. T-helper cell subtypes in the spleen (a-c) and spinal cord (d-f), after treatment with T. suis E/S products from day of disease induction. Ratios between CD4+ IL-17A+ (a, d), IFNγ+ (b, e) and FoxP3+ (c, f) cells, between the treatment (n = 7) and the control (n = 6) groups are shown. All statistics are based on the Mann-Whitney test comparing the ranks of each dataset. Data are presented relative to controls; mean +SD. *p < 0.05
populations (Fig. 3). Following treatment, a 30–40% reduction in T\(_{\text{H}1}\) cells were observed both in spleen (1.1% to 0.7% of all CD4+ cells, a fold difference of 1.6) \((p = 0.0324)\) and spinal cord (15.1% to 10.9%, a fold difference of 1.4) \((p = 0.0465)\) following treatment. T\(_{\text{H}1}\) cell numbers were significantly decreased in the spleen (1.1% to 0.4%, a fold difference of 2.8) \((p = 0.0141)\) and moderately so in the spinal cord (1.0% to 0.7%, a fold difference of 1.4), following E/S treatment. The T\(_{\text{Reg}}\) response was not significantly altered, however a tendency towards an increase in the spleen (from 0.6% to 1.5%; a 2.5-fold increase) following treatment, was observed (Fig. 3).

**Discussion**

Parasite-induced EAE disease amelioration has been demonstrated in several rodent studies using highly pathogenic parasitic worms and protozoa (Hasseldam et al. 2013). Our study found a small, but significant, improvement in clinical disease scores, which was reflected in the T-helper cell profile, following repeated administration of T. suis E/S products, 3 times a week, starting from day of disease induction. Multiple inoculations with T. suis embryonated ova did not have any beneficial effect on the clinical disease progression and did not significantly change any of the analyzed T-helper cell populations. In a pilot study on the infectivity and effects of T. suis embryonated ova in rats, the recovery of T. suis larvae from DA and SD rats was low (data not shown), indicating that few ova hatch in the gastrointestinal tract. Based on this finding the dose size was set to 7,000 T. suis ova 3 times a week. Even at that high a dosage, compared to clinical trials (see below), we did not observe any effects on inflammatory nor clinical parameters. Presumably, the infectivity of T. suis ova in our rats was too low and/or too transient in order to manifest any changes. By administering T. suis E/S products i.p. instead, this low infectivity was surmounted.

Timing of treatment is another paramount issue. A variety of studies have shown disease reductions only if the different helminth species have been administered prior to or at the time of EAE induction. In mice, T. suis SPs, which consist of soluble products (SP) from homogenized adult worms, have been shown to ameliorate disease, when animals were treated 4 weeks prior to disease initiation (Kuijk et al. 2012). Secretory-excretory antigens (SEA) from the pathogenic trematode Schistosoma japonicum, causing chronic infections in humans, pigs, buffaloes and others, reduced disease severity when given at the time of disease induction (Zheng et al. 2008). Infection with the related S. mansoni, showed the same lack of effect when given at later time-points, i.e. during the effector phase where immune cells are primed and infiltrate the CNS and symptoms arise (Owens et al. 2001, Sewell et al. 2003). In accordance with this, unpublished data from our group showed no beneficial effects of embryonated T. suis ova or T. suis E/S products, when given from the day of disease debut (data not shown). Several other studies have likewise shown treatment effects when the helminths are administered prior to disease induction (Hasseldam et al. 2013).

Within the last years, Flemming and co-workers have conducted two small-scale clinical trials investigating whether MS patients could benefit from T. suis ova treatment. Oral administration every second week for 3 (Fleming et al. 2011) or 10 months (Fleming et al. 2014) resulted in reductions in new gadolinium-enhanced lesions, with the latter study showing alterations in the immunological profile as well (Fleming et al. 2014).

The molecular mechanism(s) responsible for the parasite-induced immunomodulation is an area of intense research. Studies have shown that homogenates made from parasites induce an alternative/modulated activation of antigen presenting cells (APCs), holding the potential of skewing the immune response towards the less inflammatory T\(_{\text{H}2}\) (humoral)/T\(_{\text{Reg}}\) (tolerogenic) responses. This has been shown to be the case for T. suis, which, among others, induced an up-regulation of the OX40L ligand on human DCs in vitro, that leads to expansion of tolerogenic T-helper cells and T\(_{\text{H}2}\)-cells (Kuijk et al. 2012). T. suis products contain mannose-type glycans, that can mediate DC alteration through various receptors, like C-type lectin receptors, similar to the pathways of other parasitic species, such as S. mansoni via fucosylated glycans (Klaver et al. 2013). These glycans seem, at least partially, to be responsible for the treatment effects and the associated immune skewing towards an overall down-regulation of T\(_{\text{H}1}\) and T\(_{\text{H}1}\) associated cytokines (IFN\(\gamma\), IL-17A etc.) and up-regulation of T\(_{\text{H}2}\) associated cytokines (IL-10, TGF\(\beta\) etc.). In rats treated with E/S products, we found similar changes in the T cell populations. Following treatment, the percentages of T\(_{\text{H}1}\) and T\(_{\text{H}1}\) cells were down-regulated in both spleen and the spinal cord, even though this was only a trend for the T\(_{\text{H}1}\) cells in the spinal cord. A tendency towards an upregulation of T\(_{\text{Reg}}\) cells, as a consequence of treatment, was furthermore observed. An increase in the T\(_{\text{Reg}}\) population in the spleen has previously been shown in EAE rats treated with Trichinella spiralis, a natural parasite of rats (Gruden-Movesijan et al. 2010).

This rodent model, ultimately a model for T. suis ova treatment of MS patients, allows future studies on the underlying mechanisms of T. suis E/S induced immunomodulation. A focused research will improve the translational perspectives of these compounds, and potentially increase the life quality of MS patients.

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