

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Therapeutic potential of larval excretory/secretory proteins of the pig whipworm *Trichuris suis* in allergic disease

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To cite this article: Ebner F, Hepworth MR, Rausch S, Janek K, Niewianda A, Kühl A, Henklein P, Lucius R, Hamelmann E, Hartmann S. Therapeutic potential of larval excretory/secretory proteins of the pig whipworm *Trichuris suis* in allergic disease. *Allergy* 2014; **69**: 1489–1497.

Keywords

asthma; excretory/secretory proteins; helminth therapy; immunomodulation; *Trichuris suis*.

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Accepted for publication 26 July 2014

DOI:10.1111/all.12496

Edited by: Hans-Uwe Simon

Abstract

Background: Gastrointestinal nematodes are currently being evaluated as a novel therapeutic in the treatment of chronic human inflammatory disorders, due to their unique ability to induce immunoregulatory pathways in their hosts. In particular, administration of ova from the pig whipworm *Trichuris suis* (*T. suis*; TSO) has been proposed for the treatment of allergic, inflammatory and autoimmune disorders. Despite these advances, the biological pathways through which TSO therapy modulates the host immune system in the context of human disease remain undefined.

Methods: We characterized the dominant proteins present in the excretory/secretory (E/S) products of first-stage (L1) *T. suis* larvae (Ts E/S) using LC-MS/MS analysis and examined the immunosuppressive properties of whole larval Ts E/S *in vitro* and in a murine model of allergic airway disease.

Results: Administration of larval Ts E/S proteins *in vivo* during the allergen sensitization phase was sufficient to suppress airway hyperreactivity, bronchiolar inflammatory infiltrate and allergen-specific IgE production. Three proteins in larval Ts E/S were unambiguously identified. The immunomodulatory function of larval Ts E/S was found to be partially dependent on the immunoregulatory cytokine IL-10.

Conclusions: Taken together, these data demonstrate that the released proteins of larval *T. suis* have significant immunomodulatory capacities and efficiently dampen allergic airway hyperreactivity. Thus, the therapeutic potential of defined larval E/S proteins should be exploited for the treatment of human allergic disorders.

The increased prevalence of allergic, inflammatory and autoimmune diseases in the Western world has been correlated with a coinciding loss of infections, especially with helminths, a consequence of increased hygiene and reduced parasite exposure (1, 2). In support of this hypothesis, treatment of patients in helminth endemic countries with antihelminthic drugs has been shown to lead to exacerbated allergic disease (3, 4). Conversely, infection of allergic patients with helminths has been shown to ameliorate disease symptoms (5). Experimental evidence derived from murine helminth infection models has identified multiple regulatory and immunomodulatory mechanisms through which helminths modulate

host immune responses to bystander antigens and regulate ongoing concurrent allergic and inflammatory diseases. In particular, helminth-derived molecules potentially elicit regulatory T-cell (6, 7) and B-cell responses (8, 9), modulate dendritic cells (DCs) (10–12), elicit suppressive macrophage subsets (13, 14) and modulate the function of innate effector cells, including mast cells (15).

The helminth *Trichuris suis* (*T. suis*) naturally infects the caecum and colon of pigs, although it has been suggested that *T. suis* may also transiently establish in the large intestine of humans (16). In recent years, administration of *T. suis* ova (TSO) has been proposed as a novel and safe

clinical therapeutic for chronic human allergic and inflammatory diseases (17, 18). TSO administration to patients suffering from Crohn's disease and ulcerative colitis resulted in a significant reduction of clinical symptoms of disease and the onset of remission in many patients (19, 20). In recent preliminary phase I clinical trials, TSO administration has further been suggested to have immunomodulatory and therapeutic potential in patients with multiple sclerosis (21–23), although larger clinical trials are required to confirm its efficacy in other diseases. In contrast, TSO therapy failed to improve clinical symptoms or to significantly reduce allergen-specific immune responses during a double-blind, clinical trial in allergic rhinitis patients (24). This study raised important issues regarding the design of clinical trials using TSO and the spectrum of diseases for which therapy may be beneficial (17, 25), thus provoking the need for basic research studies aimed at interrogating the biological activities of *T. suis* excretory/secretory (E/S) proteins to develop refined helminth-based therapeutics and to inform future clinical trials.

The potential success of therapeutic helminth application is likely to be hindered by problems with patient compliance, due to the current necessity for live helminth administration. Indeed, TSO therapy has been reported to induce significant gastrointestinal symptoms, including diarrhoea, during the initial phases of therapy (24, 26). Moreover, it currently remains unclear whether *T. suis* is able to establish and mature in human hosts or whether the worm only transiently infects the host intestine (16, 27). However, the likelihood that *T. suis* may only establish transiently in human hosts is supported by the observation that multiple applications of TSO are required for therapeutic efficacy than single-treatment protocols in clinical trials with IBD patients (19, 20). Therefore, an increased understanding of the helminth-derived molecules that act to modulate the host immune system is necessary to develop

novel helminth-based immunomodulatory molecules that negate the requirement for live infection.

Further, although TSO therapy appears safe for use in humans, the mechanisms through which *T. suis* may act to modulate the host immune system in contrasting disease settings remain unknown. In this study, we aimed to assess the immunomodulatory potential of E/S proteins derived from the first-stage larvae of *T. suis* (Ts E/S) – thus, mirroring the predicted situation in patients undergoing transient exposure to larval stages during application of TSO. Here, we demonstrate the potent immunomodulatory capacity of Ts E/S in both *in vitro* systems and an *in vivo* murine model of allergic disease and identify three dominant novel proteins present in Ts E/S. These findings may very well have implications for the development of future helminth protein-based therapies by removing the need for exposure to live parasites.

Materials and methods

All methods and experimental procedures used in this study are described in detail in Supporting Information, including mice, production of *Trichuris suis* larval (L1) excretory/secretory supernatants, LC-MS/MS analysis of Ts E/S and data processing, allergic airway hyperreactivity, histology and immunohistochemistry, ELISA and flow cytometry. All animals were maintained at the Institute for Molecular Parasitology at the Humboldt University, Berlin, in accordance with the national authorities and approval of ethical revision (G0144/10).

Results

Larval Ts E/S has immunomodulatory activity *in vitro*

The first larval stage (L1) of *Trichuris suis* is likely to be the most clinically relevant stage of the life cycle in the context

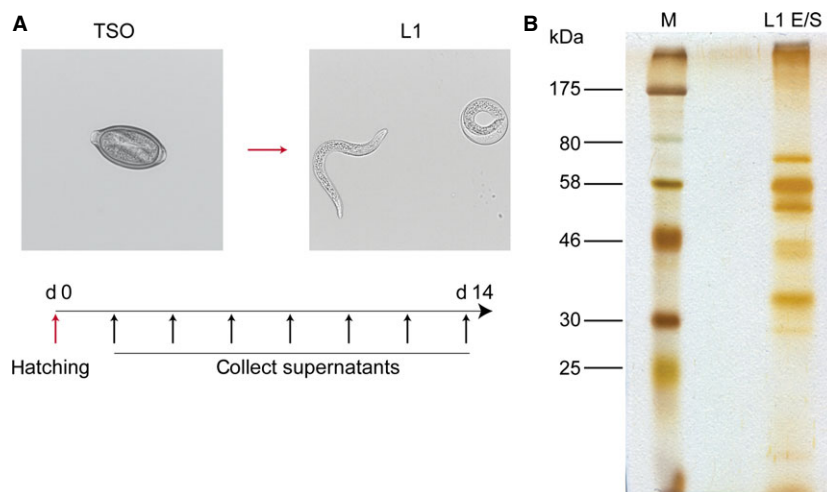


Figure 1 Generation and characterization of L1 *Trichuris suis* excretory/secretory (Ts E/S). (A) Viable *Trichuris suis* ova (TSO) were hatched and L1 cultured *in vitro* for 2 weeks and supernatants collected every 2 days. (B) Collected Ts E/S was TCA-precipitated sepa-

rated by gel electrophoresis and silver stained to visualize dominant protein bands. All data are representative of five or more independent experiments.

of therapeutic application in humans, as efficacious treatment in patients has been shown to be dependent upon repeated applications of high doses of embryonated TSO, whereas no sexually mature adult stages were found in TSO-treated patients, suggesting that *T. suis* establishment is transient in the human host. Helminths modulate their host's immune system largely by release of E/S products. We therefore first generated E/S products derived from the supernatants of L1 *T. suis* kept in culture (Fig. 1A). To initially characterize the protein composition of Ts E/S, we performed 1D gel electrophoresis and silver staining (Fig. 1B). Using this approach, we could visualize at least seven distinct Ts E/S protein bands. To determine the immunomodulatory capability of whole larval Ts E/S *in vitro*, splenocytes from TcR-restricted DO11.10 mice were cultured for 2 days with cognate antigen (OVA) in the presence or absence of different amounts of

concentrated Ts E/S, or with heat-inactivated Ts E/S (HI) (Fig. 2A). Splenocyte proliferation in response to OVA was not significantly altered in the presence of Ts E/S (Fig. 2A). In contrast, addition of Ts E/S significantly suppressed OVA-specific IFN- γ and IL-4 production that was partially dose dependent and was abrogated by heat inactivation of Ts E/S (HI) (Fig. 2B). To further determine the immunomodulatory capacity of larval Ts E/S, we determined its ability to alter activation of DCs by TLR ligands *in vitro* (Fig. 2C). Bone marrow-derived DCs stimulated with the TLR-9 agonist CpG upregulated expression of the costimulatory molecule CD86 and MHC class II in comparison with unstimulated controls and produced high levels of IL-6 and IL-12/23p40 (Fig. 2C). In contrast, DCs pretreated for 4 h with Ts E/S prior to CpG stimulation failed to upregulate expression of CD86 and MHC class II and showed significantly suppressed

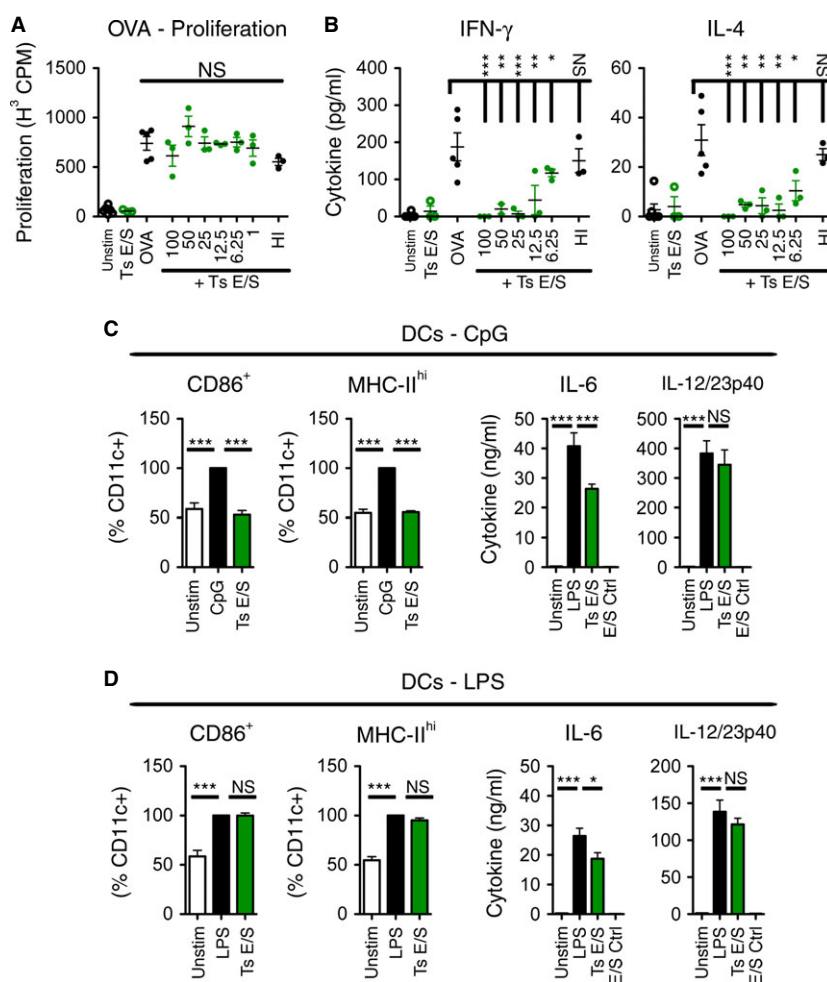


Figure 2 *Trichuris suis* excretory/secretory (Ts E/S) demonstrates immunosuppressive activity *in vitro*. DO11.10 splenocytes were cultured with ovalbumin (OVA) plus/minus varying amounts (μ l) of a Ts E/S stock solution (15 μ g/ml) or 100 μ l heat-inactivated Ts E/S (HI) and (A) proliferation measured by H³-thymidine incorporation and (B) production of IFN- γ and IL-4 measured in culture supernatants via ELISA. Bone-marrow-derived dendritic cells were stimu-

lated with (C) CpG (1 μ M) or (D) LPS (100 ng/ml) following pretreatment with or without Ts E/S and assessed for surface marker expression (CD86, MHCII) or cytokine production (IL-6, IL-12/23p40). Data are mean $\pm$ SEM and are representative of $n = 3$ independent experiments with a minimum of three replicates per treatment group. Statistical significance * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

production of IL-6, but not of IL-12/23p40 (Fig. 2C). *Trichuris suis* excretory/secretory was unable to significantly modulate DC maturation in response to the TLR-4 agonist LPS, although a mild but not significant decrease in IL-6 levels was consistently seen (Fig. 2D). Taken together, these data demonstrate that larval Ts E/S proteins possess immunomodulatory activity *in vitro*.

Larval Ts E/S suppresses the onset of OVA-specific allergic airway inflammation

To date, there is very little experimental evidence for the efficacy of *T. suis*-based therapies in murine disease models. Thus,

we aimed to determine whether administration of larval Ts E/S was sufficient to modulate disease in a murine model of Th2-driven allergic airway inflammation. Compared to OVA-sensitized and challenged controls, mice that received larval Ts E/S treatment during the sensitization period displayed significantly reduced airway hyperreactivity following OVA airway challenges (Fig. 3A; area under the curve analysis – PBS 0.13; OVA 5.8; Ts E/S 1.6), suggesting larval Ts E/S reduced airway inflammation. In line with the reduced airway hyperreactivity in larval Ts E/S-treated mice, the infiltration of inflammatory cells into the bronchoalveolar lavage fluid (BAL) was also found to be dramatically reduced, mainly attributed to significantly decreased numbers of lymphocytes and eosinophils

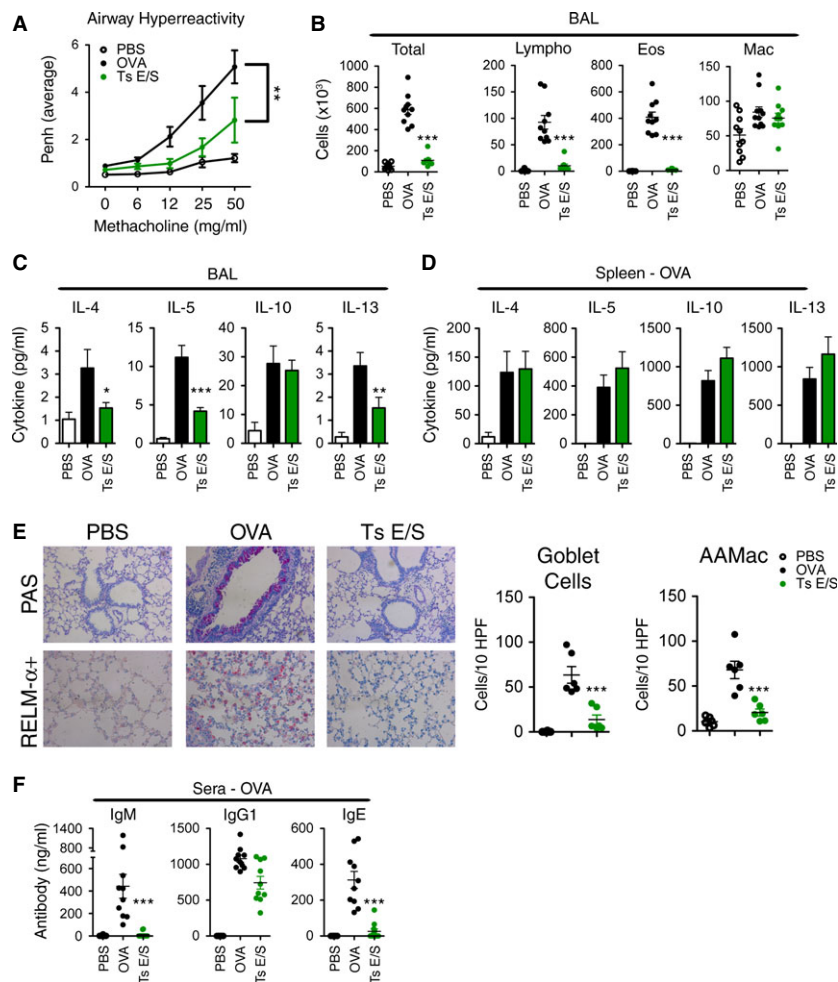


Figure 3 *Trichuris suis* excretory/secretory (Ts E/S) reduces allergic airway hyperreactivity and type 2 inflammation *in vivo*. Mice were sensitized with PBS and ovalbumin (OVA) and challenged intranasally with OVA to induce allergic airway disease. In addition, some mice were treated with Ts E/S during sensitization with OVA (Ts E/S). (A) airway hyperreactivity (AHR) was assessed in response to inhalation of increasing doses of methacholine. (B) Immune cellular infiltration and (C) cytokine levels in the bronchiolar lavage fluid (BAL). (D) Cytokine production from ovalbumin (OVA)-

restimulated splenocytes. (E) Lung histopathology following staining with periodic acid-Schiff's reagent (PAS) or anti-RELM- α monoclonal antibody and quantification of PAS+ goblet cells and RELM- α + alternatively activated macrophages (AAMac) per 10 high-powered fields (HPF). (F) OVA-specific serum antibody titres. Data are mean $\pm$ SEM and are representative of $n = 3-5$ independent experiments with a minimum of three mice per treatment group. Statistical significance * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

(Fig. 3B). Furthermore, control mice displayed increased levels of the Th2 cytokines IL-4, IL-5 and IL-13 in the BAL, and these cytokines were suppressed in mice treated with larval Ts E/S during sensitization (Fig. 3C). In contrast, no significant effect of larval Ts E/S treatment was detected on OVA-specific responses in the spleen (Fig. 3D), suggesting the effects of larval Ts E/S may be restricted to allergen-specific responses generated locally in the lung and draining lymph nodes upon airway challenge. In line with reduced local Th2 responses and cellular infiltration into the lung, larval Ts E/S-treated mice exhibited significantly fewer mucus-secreting bronchiolar goblet cells as well as reduced numbers of RELM- α^+ alternatively activated macrophages in the lung tissues in comparison with OVA control mice (Fig. 3E). Furthermore, larval Ts E/S treatment resulted in significantly reduced OVA-specific IgM and IgE levels (Fig. 3F) and a trend towards reduced OVA-specific IgG1 (Fig. 3F). Taken together, these data suggest larval Ts E/S treatment during allergen sensitization is sufficient to modulate allergic airway disease and Th2-associated inflammation in the lung.

Larval Ts E/S treatment elicits specific Th2 cytokine and antibody responses

Larval Ts E/S treatment during the priming phase of OVA-specific sensitization resulted in a significant reduction in disease-associated inflammation, local cytokine production and OVA-specific antibody production. However, intraperitoneal

host exposure to helminths and helminth-derived products is also likely to elicit potent immune responses against the parasite itself. Thus, we aimed to address whether larval Ts E/S administration elicited a Ts E/S-specific host immune response. Restimulation of splenocytes from Ts E/S-treated animals with larval Ts E/S alone led to the antigen-specific production of IL-4, IL-5 and IL-13 and a significant induction of IL-10 in larval Ts E/S-treated mice (Fig. 4A), whereas negligible cytokine production was detected following restimulation of spleen cells from mice sensitized with PBS or OVA that had not received larval Ts E/S treatment (Fig. 4A). In line with a Th2-skewed immune response to Ts E/S, we were unable to detect specific IFN- γ or IL-17A production in Ts E/S-restimulated culture supernatants (Fig. 4A). In addition, larval Ts E/S treatment resulted in specific antibody production as demonstrated by elevated Ts E/S-specific IgM, IgG1 and IgE titres, which could not be detected in control groups (Fig. 4B). These results suggest that in addition to modulating responses to bystander antigens in the lung (i.e. the model allergen OVA), Ts E/S treatment also elicited a systemic host Th2 response and production of antibodies directed against itself.

Larval Ts E/S suppression of airway inflammation is partially mediated through IL-10

Immunomodulatory helminth-derived E/S products have previously been shown to ameliorate inflammation via the

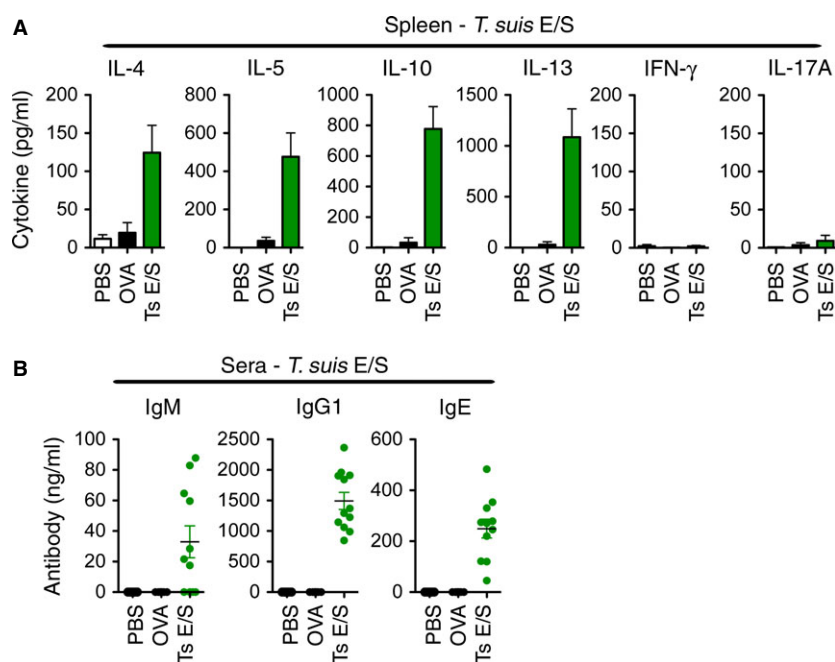


Figure 4 *Trichuris suis* excretory/secretory (Ts E/S) administration elicits an E/S-specific host immune response. Mice were sensitized with PBS and ovalbumin (OVA) and challenged intranasally with OVA to induce allergic airway disease. In addition, some mice were treated with Ts E/S during sensitization with OVA (Ts E/S). (A)

splenocytes were restimulated *ex vivo* with Ts E/S and cytokines measured in the culture supernatant via ELISA. (B) Ts E/S-specific serum antibody titres. Data are mean $\pm$ SEM and are representative of pooled data from $n = 2$ –3 independent experiments. Statistical significance * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

induction of regulatory immune cell types, particularly $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg). Excretory/secretory products derived from the murine helminth *Heligmosomoides polygyrus bakeri* (Hp) have been reported to contain TGF- β -like activity and to directly induce *de novo* Treg differentiation *in vitro* and *in vivo* (6). To determine whether larval Ts E/S has a similar capacity to induce Treg differentiation, we cultured whole splenocytes with larval Ts E/S or adult Hp E/S in the presence or absence of the potent T-cell-stimulating mitogen, ConA (Fig. 5A). In contrast to adult Hp E/S, larval Ts E/S was unable to induce FoxP3 expression in stimulated $CD4^+$ T cells *in vitro* (Fig. 5A). Furthermore, suppression of allergic airway inflammation was not associated with increased frequencies of Tregs in the spleen, lung-draining lymph node (PBLN) (Fig. 5B) or lung parenchyma (Fig. 5C). Helminths commonly elicit a so-called modified Th2 response in their host, associated with the production of high levels of IL-10, which acts to facilitate the persistence of the helminth infection while also suppressing immune responses to unrelated bystander antigens (13, 14). Although no significant increase in local IL-10 production in

the lungs or systemic OVA-specific IL-10 production by spleen cells was detected in larval Ts E/S-treated sensitized and challenged mice (Fig. 3), we did detect a significant induction of Ts E/S-specific IL-10 production in the spleen (Fig. 4). Thus, to further interrogate the mechanism through which larval Ts E/S suppressed allergic airway inflammation, we blocked IL-10 *in vivo* via administration of anti-IL-10 receptor (α IL-10R)-neutralizing antibody (Fig. 5D) prior to intranasal allergen challenge. Neutralization of IL-10R significantly ablated the ability of larval Ts E/S to suppress inflammatory infiltration into the BAL – as indicated by a significant increase in total cell numbers, lymphocyte and eosinophil numbers to levels comparable with OVA-sensitized and challenged controls (Fig. 5E). Levels of IL-13 and IL-5 in the BAL were also increased in the BAL in larval Ts E/S-treated mice administered with α IL-10R antibody when compared to larval Ts E/S-treated mice, although these did not reach statistical significance (Fig. 5E). In contrast, α IL-10R administration failed to restore OVA-specific antibody titres to control levels (Fig. 5F). These data suggest IL-10 is at least partially

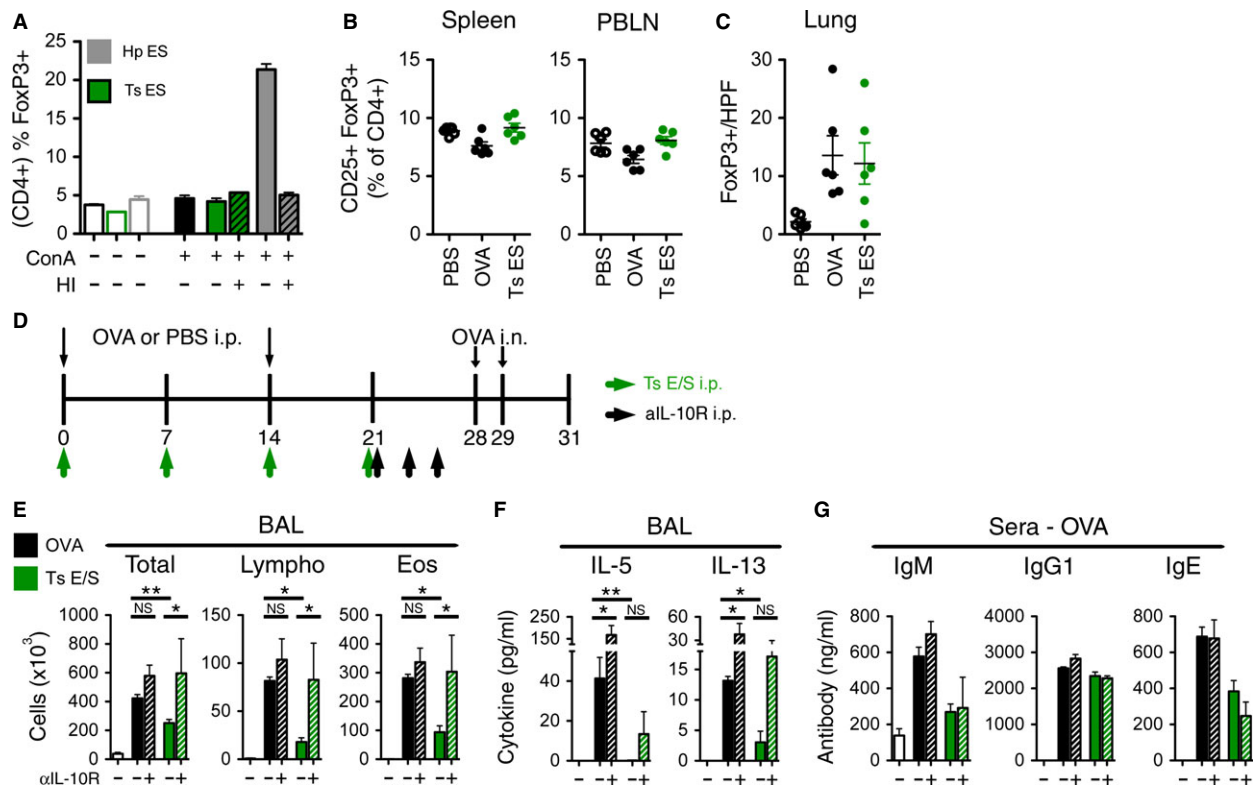


Figure 5 *Trichuris suis* excretory/secretory (Ts E/S) immunomodulation is partially dependent on IL-10. (A) Frequency of FoxP3-expressing T cells following stimulation of $CD4^+$ T cells with ConA $\pm$ Ts E/S or Hp E/S *in vitro*. (B) Frequency of $(CD4^+) CD25^+ FoxP3^+$ regulatory T cells in the spleen and PBLN of allergic mice assessed via flow cytometry or (C) FoxP3+ cells in the lung parenchyma per HPF assessed via immunohistochemistry. (D) Schematic of treatment regimen for anti-IL-10R neutralization of allergic mice.

(E) Immune cell infiltration and (F) cytokine levels in the bronchoalveolar lavage fluid of anti-IL-10R allergic mice treated with or without Ts E/S. (G) Serum antibody titres in allergic mice administered anti-IL-10R-neutralizing antibody and treated with or without Ts E/S. Data are mean $\pm$ SEM and are representative of pooled data from $n = 2-3$ independent experiments. Statistical significance $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Table 1 The up-to-now identified proteins of L1 *Trichuris suis* excretory/secretory (Ts E/S). In-solution tryptic digests of precipitated Ts E/S supernatants were analysed by mass spectrometry and Mascot search tool

Accession*	Protein score†	Sequence‡	[M+H] ⁺ _{cal.}	Peptide score†	P value	Size [Da]	Description	NCBI homologues	GO terms
Tsui7583957	447	¹²² TGYPVFIK ¹³⁰	1023.587	80	3.70E-04	32510	Unknown	–	–
		¹⁶⁹ QLLSNDSPVR ¹⁷⁹	1243.605	96	1.60E-05				
		¹⁸⁰ FREEEEIC _{carb} R ¹⁸⁸	1267.524	48	0.46§				
		²³⁶ LITYAAR ²⁴²	807.492	47	0.65§				
		²⁶² HSFLHGQEVWTVTR ²⁷⁵	1696.834	129	7.30E-09				
		²⁸¹ IYYREPVL ²⁸⁸	1052.552	48	0.85§				
Tsui7304731	83	¹⁹⁵ KSEC _{carb} ILSVR ²⁰⁴	1190.735	48	0.31§	40146	Unknown	–	Serine-type endopeptidase activity; proteolysis
		²⁰⁹ _{pyr} QQPYLIEVEPK ²¹⁹	1326.685	34	23§				
Tsui7234544	128	⁸⁶ LC _{carb} TGHVSC _{carb}	1890.791	64	0.01§	15737	Unknown	Unknown protein [Trichinella spiralis]	–
		DDAQVC _{carb} IR ¹⁰¹							
		¹¹⁴ C _{carb} HVGALC _{carb}	1299.572	64	0.016§				
		AAGQR ¹²⁵							

*HelmDB.

†Mascot.

‡Modified amino acids, C_{carb}: carbamidomethylated cysteine, pyrQ: pyroglutamate.

§Identity was confirmed by comparison of the MS/MS spectrum with the fragmentation pattern of a synthetic reference peptide. Table 1 combines data generated in two independent experiments.

responsible for the suppression of local airway inflammation by larval Ts E/S, although other IL-10-independent mechanisms may also contribute to the suppression of the broader allergen-specific immune response, particularly the generation of allergen-specific antibodies.

Identification of novel Ts E/S proteins

As Ts E/S was found to contain significant immunomodulatory activity both *in vitro* and *in vivo*, we aimed to identify the potential individual Ts E/S proteins responsible for the immunomodulatory activity. Excretory/secretory was precipitated and digested by trypsin. The resulting peptides were analysed via mass spectrometry to generate Ts E/S-derived peptide sequences. Database searches using NCBI, NemBase4 and HelmDB identified three novel *T. suis* E/S proteins, ranging between 15.7 kDa and 40 kDa, with homology to proteins with unknown function and identity (Table 1). All proteins were identified based on HelmDB (Sequence IDs: Tsui7583957, Tsui7234544 and Tsui7304731). Homology and gene ontology searches with the full sequence information revealed homology of Tsui7234544 to an unknown protein from *Trichinella spiralis*, whereas Tsui7304731 yielded gene ontology (GO) term matches for serine-type endopeptidase activity/proteolysis. Thus, we successfully isolated and characterized L1 E/S proteins and identified three of the L1 E/S proteins potentially reflecting the dominant proteins produced by first-stage larval *T. suis*. Further future studies to recombinantly express these proteins and interrogate their individual immunomodulatory activity are required in order to develop refined helminth-derived therapeutics.

Discussion

Gastrointestinal helminths have coevolved within mammalian hosts and as a result have developed elegant strategies to modulate their host's immune system in order to survive and persist. The associative correlation between loss of helminth infections in the Western world and increases in allergic disease provokes the hypothesis that inappropriate immune activation to harmless allergens may be in part due to the loss of coevolved helminth-induced immunomodulatory pathways. This hypothesis has led to an increased interest in developing novel therapeutics aimed at exploiting the inherent immunomodulatory capacity of helminth parasites to treat patients suffering from inflammatory, allergic and autoimmune diseases. In particular, early clinical trials in IBD patients with application of eggs from the pig whipworm TSO resulted in remission of disease in a number of patients (19, 20), suggesting nematodes with unrelated definitive hosts may have beneficial immunomodulatory effects in human patients. In addition, although initial studies suggest TSO treatment may also have efficacy in patients with multiple sclerosis (21, 23), other trials in allergic rhinitis patients have to date been unsuccessful (24); however, further investigations into the timing and length of helminth application may yet yield positive results (17, 28).

In this study, we demonstrate that E/S proteins derived from the larval stage of Ts E/S have potent immunomodulatory effects using both *in vitro* and *in vivo* murine model systems of allergic disease. In particular, Ts E/S acted *in vitro* to modulate activation of professional antigen-presenting cells and to reduce antigen-specific CD4⁺ T-cell cytokine production. Similarly, administration of Ts E/S

during sensitization to a model allergen resulted in a reduction of CD4⁺ Th2 responses and allergen-specific antibody responses (including IgE), as well as innate immune responses downstream of the Th2 cytokines IL-5 and IL-13 such as eosinophilia, alternative activation of macrophages and goblet cells hyperplasia. Taken together, these data raise the possibility that Ts E/S may act to interfere with T-cell priming, possibly through immunomodulation of professional antigen-presenting cells. Indeed, one recent study demonstrated that Ts E/S products act to suppress maturation of human DCs through recognition of Ts E/S glycans through C-type lectin receptors (29).

Excretory/secretory products from adult-stage *T. suis* have previously been shown to have enzymatic activities with homology to other known immunomodulators from related parasitic nematodes (30–33). In addition, the genome and transcriptome of *Trichuris suis* and its murine counterpart *T. muris* were recently elegantly defined (34, 35). Intriguingly, the *T. suis* genome predicted over 600 individual E/S proteins across all life cycle stages, many of which contain serine protease activity (34). In this study, we generated and visualized E/S supernatants from L1-stage *T. suis* and identified and characterized three novel proteins of yet unknown function, although one protein (Tsui7304731) had predicted serine-peptidase activity, in agreement with the findings of Jex et al. (34). Notably, we did not detect any of the three identified L1 proteins in adult male or female Ts E/S by LC-MS/MS (data not shown), suggesting the protein identity of larval E/S may be distinct from that of adult worm E/S. Indeed, Jex et al. (34) were able to demonstrate significant differences in the mRNA transcriptome of L1/L2 larval-stage worms in comparison with adult stages, suggesting expression of E/S proteins may be tightly regulated during maturation. Furthermore, stage-specific gene expression may explain why we were only able to identify and sequence a small fraction of the 600 plus predicted *T. suis* E/S proteins in L1-stage E/S supernatants.

Given that efficacious TSO treatment of IBD patients was found to require multiple administrations of high doses of TSO and that to date, no chronic adult-stage *T. suis* infections have been reported in human hosts, it is possible that the E/S products produced by first-stage larvae are critical for the immunomodulatory effects of TSO therapy in human disease. Jex et al. (34) hypothesized that *T. suis* may exert its immunomodulatory effects through the secretion of a range of E/S proteins, identified via the analysis of the *T. suis* genome and transcriptome, including serine proteases, cystatins, calreticulin and cytokine homologues. In agreement with this hypothesis, we have extensively shown that cystatin and calreticulin from related helminth parasites have potent immunomodulatory function and act via the induction of regulatory cytokines, such as IL-10 (13, 14, 36). Here we demonstrate the immunomodulatory

effects of L1 Ts E/S are at least partially dependent upon IL-10, suggesting similar modulatory proteins may be conserved in *T. suis* E/S. In contrast, larval-stage E/S does not appear to directly modulate the immune system through TGF- β homologue activity, as suggested by Jex and colleagues, as L1 Ts E/S was unable to induce *de novo* regulatory T cells as previously demonstrated for *H. polygyrus* E/S (6, 34). Thus, further characterization and interrogation of the immunomodulatory ability of the defined first-stage larval E/S proteins, identified in this study, are required to assess the therapeutic potential of these proteins moving forward and in order to develop novel therapeutics for human disease.

In summary, our data show the immunomodulatory potential of larval Ts E/S products *in vitro* and demonstrate the suppressive effect of larval Ts E/S in a murine model of allergic disease. It is very likely that such studies may lead to the identification of novel nematode-derived immunomodulatory molecules with clinically relevant therapeutic potential and allow for the identification of target pathways in the treatment of allergic and inflammatory disorders.

Acknowledgments

We thank M. Müller and C. Palissa for excellent technical assistance.

Author contributions

MRH, FE, SR and SH conceived the design of the study, performed experiments, analysed the data and wrote the manuscript. AK performed lung histological analysis. KJ, AN and PH performed mass spectrometer experiments and generated reference peptides. RL and EH helped with designing experiments, interpreting the data and writing the manuscript.

Funding

This research was supported by the German Research Foundation (Heisenberg Programme HA2542) to S.H. and by the research cooperation with Coronado Biosciences to S.H.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Material and methods.

References

- Maizels RM, Pearce EJ, Artis D, Yazdanbakhsh M, Wynn TA. Regulation of pathogenesis and immunity in helminth infections. *J Exp Med* 2009;**206**:2059–2066.
- Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE. Helminth parasites—masters of regulation. *Immunol Rev* 2004;**201**:89–116.
- Flohr C, Tuyen LN, Quinnell RJ, Lewis S, Minh TT, Campbell J et al. Reduced helminth burden increases allergen skin sensitization but not clinical allergy: a randomized, double-blind, placebo-controlled trial in Vietnam. *Clin Exp Allergy* 2010;**40**:131–142.
- van den Biggelaar AH, Rodrigues LC, van Ree R, van der Zee JS, Hoeksma-Kruize YC, Souverein JH et al. Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren. *J Infect Dis* 2004;**189**:892–900.
- Feary JR, Venn AJ, Mortimer K, Brown AP, Hooi D, Falcone FH et al. Experimental hookworm infection: a randomized placebo-controlled trial in asthma. *Clin Exp Allergy* 2010;**40**:299–306.
- Grainger JR, Smith KA, Hewitson JP, McSorley HJ, Hargus Y, Filbey KJ et al. Helminth secretions induce *de novo* T cell Foxp3 expression and regulatory function through the TGF- β pathway. *J Exp Med* 2010;**207**:2331–2341.
- Dittrich AM, Erbacher A, Specht S, Diesner F, Krokowski M, Avagyan A et al. Helminth infection with *Litomosoides sigmodontis* induces regulatory T cells and inhibits allergic sensitization, airway inflammation, and hyperreactivity in a murine asthma model. *J Immunol* 2008;**180**:1792–1799.
- Amu S, Saunders SP, Kronenberg M, Mangan NE, Atzberger A, Fallon PG. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J Allergy Clin Immunol* 2010;**125**:1114–1124.
- Wilson MS, Taylor MD, O’Gorman MT, Balic A, Barr TA, Filbey K et al. Helminth-induced CD19 + CD23hi B cells modulate experimental allergic and autoimmune inflammation. *Eur J Immunol* 2010;**40**:1682–1696.
- Massacand JC, Stettler RC, Meier R, Humphreys NE, Grecis RK, Marsland BJ et al. Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. *Proc Natl Acad Sci U S A* 2009;**106**:13968–13973.
- Steinfelder S, Andersen JF, Cannons JL, Feng CG, Joshi M, Dwyer D et al. The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). *J Exp Med* 2009;**206**:1681–1690.
- Segura M, Su Z, Piccirillo C, Stevenson MM. Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression. *Eur J Immunol* 2007;**37**:1887–1904.
- Schnoeller C, Rausch S, Pillai S, Avagyan A, Wittig BM, Loddenkemper C et al. A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. *J Immunol* 2008;**180**:4265–4272.
- Klotz C, Ziegler T, Figueiredo AS, Rausch S, Hepworth MR, Obsivac N et al. A helminth immunomodulator exploits host signaling events to regulate cytokine production in macrophages. *PLoS Pathog* 2011;**7**:e1001248.
- Melendez AJ, Harnett MM, Pushparaj PN, Wong WS, Tay HK, McSharry CP et al. Inhibition of Fc epsilon RI-mediated mast cell responses by ES-62, a product of parasitic filarial nematodes. *Nat Med* 2007;**13**:1375–1381.
- Kradin RL, Badizadegan K, Auluck P, Korzenik J, Lauwers GY. Iatrogenic *Trichuris suis* infection in a patient with Crohn disease. *Arch Pathol Lab Med* 2006;**130**:718–720.
- Hepworth MR, Hamelmann E, Lucius R, Hartmann S. Looking into the future of *Trichuris suis* therapy. *J Allergy Clin Immunol* 2010;**125**:767–768.
- Jouvin MH, Kinet JP. *Trichuris suis* ova: testing a helminth-based therapy as an extension of the hygiene hypothesis. *J Allergy Clin Immunol* 2012;**130**:3–10.
- Summers RW, Elliott DE, Urban JF Jr, Thompson R, Weinstock JV. *Trichuris suis* therapy in Crohn’s disease. *Gut* 2005;**54**:87–90.
- Summers RW, Elliott DE, Urban JF Jr, Thompson RA, Weinstock JV. *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* 2005;**128**:825–832.
- Fleming J, Isaak A, Lee J, Luzzio C, Carriers M, Cook T et al. Probiotic helminth administration in relapsing-remitting multiple sclerosis: a phase I study. *Mult Scler* 2011;**17**:743–754.
- Benzel F, Erdur H, Kohler S, Frentsch M, Thiel A, Harms L et al. Immune monitoring of *Trichuris suis* egg therapy in multiple sclerosis patients. *J Helminthol* 2012;**86**:339–347.
- Rosche B, Wernecke KD, Ohlraun S, Dorr JM, Paul F. *Trichuris suis* ova in relapsing-remitting multiple sclerosis and clinically isolated syndrome (TRIOIMS): study protocol for a randomized controlled trial. *Trials* 2013;**14**:112.
- Bager P, Arnved J, Ronborg S, Wohlfahrt J, Poulsen LK, Westergaard T et al. *Trichuris suis* ova therapy for allergic rhinitis: a randomized, double-blind, placebo-controlled clinical trial. *J Allergy Clin Immunol* 2010;**125**:123–130.
- Hepworth MR, Hartmann S. Worming our way closer to the clinic. *Int J Parasitol Drugs Drug Resist* 2012;**2**:187–190.
- Bager P, Kapel C, Roepstorff A, Thamsborg S, Arnved J, Ronborg S et al. Symptoms after ingestion of pig whipworm *Trichuris suis* eggs in a randomized placebo-controlled double-blind clinical trial. *PLoS ONE* 2011;**6**:e22346.
- Summers RW, Elliott DE, Weinstock JV. Therapeutic colonization with *Trichuris suis*. *Arch Pathol Lab Med* 2006;**130**:1753; author reply 1753–1754.
- Summers RW, Elliott DE, Weinstock JV. *Trichuris suis* might be effective in treating allergic rhinitis. *J Allergy Clin Immunol* 2010;**125**:766–767.
- Klaver EJ, Kuijk LM, Laan LC, Kringel H, van Vliet SJ, Bouma G et al. *Trichuris suis*-induced modulation of human dendritic cell function is glycan-mediated. *Int J Parasitol* 2013;**43**:191–200.
- Rhoads ML, Fetterer RH, Hill DE. *Trichuris suis*: a secretory serine protease inhibitor. *Exp Parasitol* 2000;**94**:1–7.
- Hill DE, Sakanari JA. *Trichuris suis*: thiol protease activity from adult worms. *Exp Parasitol* 1997;**85**:55–62.
- Hill DE, Gamble HR, Rhoads ML, Fetterer RH, Urban JF Jr. *Trichuris suis*: a zinc metalloprotease from culture fluids of adult parasites. *Exp Parasitol* 1993;**77**:170–178.
- Fetterer RH, Hill DE. The occurrence of phenol oxidase activity in female *Trichuris suis*. *J Parasitol* 1993;**79**:155–159.
- Jex AR, Nejsun P, Schwarz EM, Hu L, Young ND, Hall RS et al. Genome and transcriptome of the porcine whipworm *Trichuris suis*. *Nat Genet* 2014;**46**:701–706.
- Foth BJ, Tsai JJ, Reid AJ, Bancroft AJ, Nichol S, Tracey A et al. Whipworm genome and dual-species transcriptome analyses provide molecular insights into an intimate host-parasite interaction. *Nat Genet* 2014;**46**:693–700.
- Rzepecka J, Rausch S, Klotz C, Schnoller C, Kornprobst T, Hagen J et al. Calreticulin from the intestinal nematode *Heligmosomoides polygyrus* is a Th2-skewing protein and interacts with murine scavenger receptor-A. *Mol Immunol* 2009;**46**:1109–1119.

SUPPORTING INFORMATION

MATERIAL AND METHODS

Harvesting of larval (L1) *Trichuris suis* Excretory/Secretory supernatants (Ts E/S)

Purified *Trichuris suis* ova were kindly provided by OVAMED GmbH (Barsbüttel, Germany). 250,000 *T. suis* ova were resuspended in 20ml of 3.6% hypochloride in 0.1N stabilised sulphuric acid (pH 1) and incubated for 45 minutes at 37°C with periodical agitation. *T. suis* ova were repeatedly washed at 800xg for 10 min and resuspended in serum free RPMI-1640 media containing 20mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Hatched *T. suis* first stage larvae were cultured at 45,000 larvae/well in 1ml serum free media. Supernatants were then harvested every 48 hours for 10-14 days and stored at -20°C until further use. Hatched L1 *T. suis* yielded reproducible protein concentrations, at a rate of 7.6 +/- 0.7 pg/larvae/day. To generate Ts E/S protein for gel visualization supernatants were precipitated using 1:5 volumes 100% trichloroacetic acid. The total dried protein pellet was separated on a 12% SDS gel and visualized with the Pierce® Silver Stain Kit following manufacturers instructions (Thermo Scientific). For *in vitro* and *in vivo* use, supernatants were concentrated 20x on concentrators with a molecular weight cut off of 5 kDa (Vivaspin 6; Viva products GmbH) and used as indicated in experimental methods. Final endotoxin levels were detected by *Limulus* amoebocyte lysate test (Cambrex) and consistently found to be ≤0.2 pg/µl.

Tryptic digestion of Ts E/S proteins for LC-MS/MS

TCA-precipitated dried protein pellets of Ts E/S were suspended in 25µl of 0.1M ammonium bicarbonate buffer. For reduction and alkylation of cysteine residues 4µl dithiotreitol (45mM in 0.1M ammonium bicarbonate) were added and the mixture was incubated at 56°C for 45 min. After cooling to room temperature 4µl iodoacetamide solution (100mM in 0.1M ammonium bicarbonate) were applied and the sample was kept in the dark for 30 min and diluted with 95µl of 0.1M ammonium bicarbonate. Trypsin was dissolved in 50mM acetic acid and in-solution digests were performed with 0.1µg trypsin for 16-18h at 37°C. The reaction was stopped with 2.5µl of 20% (v/v) trifluoroacetic acid in water and samples were dried.

LC-MS/MS of Ts E/S and data processing

LC-MS/MS analyses were performed on a 4700 proteomics Analyzer (ABSCIEX, Framingham, MS) off-line coupled with a Dionex UltiMate 3000 RSLC system and Probot fractionation device (Thermo Scientific, Idstein, Germany).

Peptide reconstitution, LC separations and probe spotting were performed as described previously (34). Mass spectra were acquired in a data-dependent mode. MS spectra were recorded in the range of m/z 800–4000 and with the accumulation of 2000 subspectra. MS/MS spectra were measured from the five most intensive precursor ions ($S/N > 30$). 5000–10,000 laser shots were accumulated.

MS and MS/MS peak lists were generated by the “Peak to Mascot” tool of the 4000er Series Explorer v3.6. For MS/MS data analysis MASCOT server (version 2.2, Matrixscience, London, UK) was used. Data base searches were performed using NCBI (31,601,460 protein sequences), Helm database (1288 protein sequences translated from transcriptome data of *T. suis*, www.helmdb.org) and Trichuris EST database (in-house database composed of 12,895 cDNA sequences from *Trichuris muris*, *Trichinella spiralis* und *Trichuris vulpis*, made from www.nematodes.org/nembase4/overview.shtml). The following mass tolerances were set: 80ppm and 0.3Da. Proteins were accepted as identified if at least two MS/MS spectra provided a MASCOT score for identity ($p < 0.01$). MS/MS spectra that did not fulfill these criteria were inspected manually and their identity was verified, when the fragment pattern was comparable with those of synthetic analogs. These reference peptides were synthesized in-house using Fmoc solid phase chemistry as previously described (35).

Animals and Disease Models

Female C57Bl/6J mice and DO11.10 TcR transgenic mice were purchased from Charles River GmbH and female BALB/c were purchased from Harlan Olac Ltd. Allergic airway hyperreactivity was induced as previously described (13). Briefly, 8 week old BALB/c mice were sensitized three times (day 0, 14 and 21) via intraperitoneal injection of 20µg OVA (grade VI, Sigma-Aldrich) or an equal volume of PBS (control) emulsified in 2mg of Alum (Pierce). For Ts E/S treatment, mice received 100µl concentrated Ts E/S supernatant i.p. on day 0, 7, 14 and 21 days post-sensitization. In some experiments mice received 0.5mg anti-IL-10R (clone 1B1, a kind gift from the DRFZ, Berlin) i.p. or Rat IgG (control) on day 21, 23 and 26 post-sensitization. All mice were subsequently challenged via intranasal administration of 50µg OVA in sterile PBS and sacrificed two days later. Airway hyperreactivity was assessed on day 30 via whole-body

plethysmography in unrestrained mice in response to increasing doses of metacholine (MCh, Sigma-Aldrich) as described previously (13, 36). All mice were maintained at the Institute for Molecular Parasitology at the Humboldt University, Berlin in accordance with the national authorities and approval of ethical revision (G0144/10).

Lymphocyte isolation and culture

Lymphocytes from the lung-draining peribroncheolar lymph node (PBLN) and spleen were isolated via manual disruption through a 70µm nylon mesh and cultured in RPMI-1640 media containing 10% fetal calf serum (FCS), 20mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were restimulated with 50µg/ml Ovalbumin, 20µl TsE/S or 1µg αCD3 and αCD28 mAb for 48 hours at 37°C/5% CO₂ for ELISA. Cellular proliferation was analysed via a 20-hour culture in fresh media containing 1µCi methyl-[³H]-thymidine (Amersham Pharmacia) and subsequent detection using a β-counter (Perkin-Elmer). *De novo* regulatory T cell induction assays were performed as previously described by Grainger *et al* (6).

TLR stimulation of dendritic cells

Bone marrow-derived dendritic cells from C57BL/6 mice were stimulated for 24 h with CpG ODN 1668 (1 µM, InvivoGen) or LPS-EK (100 ng/ml, InvivoGen) following pre-treatment (4 h) with or without Ts E/S and assessed for surface expression of MHCII and CD86 by flow cytometry and production of IL-6/IL-12/23p40 by ELISA.

Histology and immunohistochemistry

Lung tissue was degassed and fixed with 3.7% paraformaldehyde and embedded in paraffin and sections were stained with hematoxylin and eosin (H&E) or Periodic Acid Schiff's reagent to visualize goblet cells. In some experiments sections of lung were stained with an antibody against RELM-α (R&D systems) to visualize alternatively activated macrophages. Goblet cells and RELM-α⁺ cells were quantified by counting number of cells in 10 HPF (400x magnification). Bronchoalveolar lavage fluid (BAL) cells were differentiated via centrifugation onto frosted slides and differentiated via H&E staining.

Cytokine and antibody ELISA

Cytokines were determined using Cytometric Bead Assay (CBA, BD Bioscience) as per manufacturers instructions or commercially available ELISA antibody pairs (BD bioscience, eBioscience) following manufacturers instructions. For detection of antigen-specific antibodies ELISA plates were coated with either 50µg/ml OVA or 50µl Ts E/S in bicarbonate buffer at 4°C overnight along with serial dilutions of recombinant IgM, IgG1 or IgE (SantCruz Biotecth).

Flow cytometry

Single cell suspensions were surface stained with fluorescent conjugated antibodies against CD11c, CD86, MHCII, CD3, CD4 and CD25 (eBioscience). Intracellular stains for FoxP3 or isotype controls were performed following a fixation and permeabilisation protocol (eBioscience). Samples were acquired using LSR II (BD Biosciences) and analysed using FlowJo software (Treestar).

Statistics

Experiments were performed with 5 animals per group, unless indicated otherwise and expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism software using Kruskal Wallis analysis for worm burdens and one way ANOVA or two tailed Mann-Whitney test for other analyses. p<0.05 (*), p<0.01 (**) and p<0.001 (***) values were considered to be statistically significant.