

EmTIP, a T-Cell Immunomodulatory Protein Secreted by the Tapeworm *Echinococcus multilocularis* Is Important for Early Metacestode Development

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Abstract

Background: Alveolar echinococcosis (AE), caused by the metacestode of the tapeworm *Echinococcus multilocularis*, is a lethal zoonosis associated with host immunomodulation. T helper cells are instrumental to control the disease in the host. Whereas Th1 cells can restrict parasite proliferation, Th2 immune responses are associated with parasite proliferation. Although the early phase of host colonization by *E. multilocularis* is dominated by a potentially parasitocidal Th1 immune response, the molecular basis of this response is unknown.

Principal Findings: We describe EmTIP, an *E. multilocularis* homologue of the human T-cell immunomodulatory protein, TIP. By immunohistochemistry we show EmTIP localization to the intercellular space within parasite larvae. Immunoprecipitation and Western blot experiments revealed the presence of EmTIP in the excretory/secretory (E/S) products of parasite primary cell cultures, representing the early developing metacestode, but not in those of mature metacestode vesicles. Using an *in vitro* T-cell stimulation assay, we found that primary cell E/S products promoted interferon (IFN)- γ release by murine CD4+ T-cells, whereas metacestode E/S products did not. IFN- γ release by T-cells exposed to parasite products was abrogated by an anti-EmTIP antibody. When recombinantly expressed, EmTIP promoted IFN- γ release by CD4+ T-cells *in vitro*. After incubation with anti-EmTIP antibody, primary cells showed an impaired ability to proliferate and to form metacestode vesicles *in vitro*.

Conclusions: We provide for the first time a possible explanation for the early Th1 response observed during *E. multilocularis* infections. Our data indicate that parasite primary cells release a T-cell immunomodulatory protein, EmTIP, capable of promoting IFN- γ release by CD4+ T-cells, which is probably driving or supporting the onset of the early Th1 response during AE. The impairment of primary cell proliferation and the inhibition of metacestode vesicle formation by anti-EmTIP antibodies suggest that this factor fulfills an important role in early *E. multilocularis* development within the intermediate host.

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Introduction

Alveolar echinococcosis (AE), resulting from the formation, establishment and dissemination of the metacestode (MV) larval stage of the fox tapeworm *E. multilocularis* is considered one of the most severe human parasitoses in the world [1,2]. Upon oral ingestion of parasite-derived, infective eggs by intermediate hosts (rodents and, occasionally, humans), the oncosphere larva is activated, hatches, and penetrates the intestinal barrier, usually evoking a Th1-dominated immune response with IFN- γ associated immune effector functions [3]. Within the liver of the intermediate host, the oncosphere then undergoes a metamorphosis toward the bladder-like metacestode larval stage which grows infiltratively, like a malignant tumor, into the surrounding host tissue. During this process, the early Th1 response is gradually replaced by a Th2

response, dominated by interleukin (IL)-5 and IL-10 [4]. AE has a high case-fatality rate and is associated with severe morbidity. The implementation of benzimidazole-based chemotherapy has markedly improved the prognosis of patients [1,2]. However, this treatment only proved to be parasitostatic [5–7], requiring long-term to life-long administration [8]. Currently, AE therapy is modestly satisfactory [1,2]. Alternative targets for therapy are thus desperately needed.

A defining feature of the disease is the modulation of the host immune response by the parasite larvae as reflected by its widely accepted polar character [4,9,10]. Current hypotheses are that a Th1 response is parasitocidal, whereas a Th2 response associates with parasite growth and disease progression [4,9,10]. This general picture is supported by previous studies which compellingly showed that the resistance of murine [11–13] or human

Author Summary

E. multilocularis is a parasitic helminth causing the chronic human disease alveolar echinococcosis. Current disease control measures are very limited resulting in a high case-fatality rate. A transiently dominating Th1 immune response is mounted at the early phase of the infection, potentially limiting parasite proliferation and disease progression. Understanding the molecular basis of this early anti-*Echinococcus* Th1 response would provide valuable information to improve disease control. The authors found that EmTIP, a T-cell immunomodulatory protein homologue, is secreted by the parasite early larva and promotes a Th1 response in host cells. Interestingly, EmTIP binding by antibodies impairs the development of the early parasite larva towards the chronic stage. Altogether the authors propose that *E. multilocularis* utilizes EmTIP for early larval development, but in the process, the factor is released by the parasite larva and influences host T-cells by directing a parasitocidal Th1 immune response. Therefore, the authors recommend EmTIP as a promising lead for future studies on the development of anti-*Echinococcus* intervention strategies.

[14,15] hosts to *E. multilocularis* metacystodes is associated with a Th1-dominated immune response whereas a Th2-dominated immune response occurs as *E. multilocularis* metacystodes thrive in these murine [16,17] or human [15,18,19] hosts. In agreement with a parasitocidal role for Th1 responses during AE, administration of Th1-inducing immune-stimulants like Bacillus Calmette–Guérin (BCG) [20–23], IL-12 [13], IFN- α -2a [11,24] and IFN- γ [25,26] have all been shown to restrain parasite establishment, proliferation or dissemination in rodents experimentally infected with *E. multilocularis* larvae. Conversely, Th2-dominated immune responses have been tightly associated with progressive forms of AE both in humans [15,19] and mice [16,17] with compelling evidence pointing at an underlying expansion of regulatory T-cells [27,28]. As a result of these investigations, the current picture of a parasitocidal Th1 response and a permissive Th2 host response during AE has gradually emerged [4,9,29,30]. This picture has proven to be largely representative of the immunity to AE when considering studies which report the reversal of the dominating Th2 response to a Th1 response in patients with spontaneously died out lesions [14,15,31]. Understanding the basis of the anti-parasitic Th1 immune response would provide a wealth of information to interfere with AE. Although, like *E. multilocularis*, several tissue-dwelling parasitic helminths have been reported to show a similar Th1/Th2 immune polarity [32–34], the molecular basis for the early onset of the host protective Th1 response remains unknown.

We have developed an *in vitro* system for the cultivation of *E. multilocularis* primary cells that closely mimics the oncosphere-metacystode transition as it occurs early during an infection [35,36]. In the primary cell cultivation system, metacystode vesicles are formed from parasite stem cells within 1–2 weeks [36]. Since stem cells are the only cells in flatworms that are capable of proliferation [37], the primary cell system reflects the proliferative capacity of the parasite's stem cells. Using this system, we have already investigated the influence of excretory/secretory (E/S) products of the early developing parasite larvae on host immune cells [28]. In this previous study, a central role of larval E/S products in host immunomodulation by *E. multilocularis* was demonstrated. However, nothing is known so far on the role of

parasite E/S products in the onset of the Th1 immune response that governs the early phase of the infection by *E. multilocularis*.

In the current study, a new *E. multilocularis* E/S product, EmTIP, with homology to human and murine T-cell Immunomodulatory Proteins [38] was identified. EmTIP was expressed by primary cells and metacystode vesicles, but only found to be present in primary cell E/S products. TIP homologues are widely expressed by eukaryotes, suggesting an important function in fundamental biological processes [39]. However, except for a single study on T-cell immunomodulatory activities [38], their function is still unknown. As yet, human and murine TIPs have primarily been shown to elicit a strong IFN- γ release by murine and human T-cells, accompanied by elevated levels of IL-10 and TNF- α , translating into a protective effect in a murine model of Graft-versus-host disease [38]. Herein, we demonstrate that the TIP homologue of *E. multilocularis* is important for the proliferation and transition of parasite primary cells into metacystode vesicles and promotes IFN- γ release by murine CD4+T-cells *in vitro*. The characterization of EmTIP, a candidate target for anti-AE therapy, is reported.

Materials and Methods

Animals and ethics statement

C57BL/6 mice were purchased from Charles River and housed at the local animal facility of the Institute for Hygiene and Microbiology at least 1–2 weeks before experimentation as were Mongolian jirds (*Meriones uinguilatus*). Animal handling, care and subsequent experimentation were performed in compliance with German (*Deutsches Tierschutzgesetz, TierSchG*, version from Dec-9-2010) and European (European directive 2010/63/EU) regulations on the protection of animals. Ethical approval of the study was obtained from the local ethics committee of the government of Lower Franconia (*Regierung von Unterfranken* 55.2-2531.01-31/10).

Parasite material

E. multilocularis primary cells and metacystode vesicles were isolated, separated from host contaminants and maintained in axenic cultures as previously described [40]. For the collection of E/S products, axenically maintained parasite larvae were washed thrice in 1× PBS and resuspended in collection medium DMEM₁₀redox; i.e. dulbecco's modified eagle's medium, 4.5 g glucose/L (Gibco) supplemented with 10% fetal bovine serum Superior (Biochrom AG), 100 μ g/ml penicillin;streptomycin (Biochrom AG), 20 μ g/ml levofloxacin (Sanofi-Aventis), 143 μ M β -mercapthoethanol (Sigma-Aldrich), 10 μ M bathocuproine disulfonic acid (Sigma) and 100 μ M L-cysteine (Sigma) under axenic conditions (i.e. sealed in Nitrogen filled Ziploc freezer bag and placed in a 5% CO₂ incubator at 37°C). After 48 hours of culture, the supernatants containing the larval E/S products were collected and filtered through a 0.2 μ m sieve (Filtropur S filter, SARSTEDT). The total amount of proteins per supernatant was determined using the bicinchoninic acid assay (ThermoScientific) and normalized by addition of ddH₂O prior to storage at –80°C until use.

Identification, cloning and analysis of the *Emtip* gene and cDNA

For identification, a previously established *E. multilocularis* cDNA library for trans-spliced transcripts [41] was randomly sequenced and found by tblastn analyses to contain a recombinant clone encoding a homolog of the human T-cell Immunomodulatory Protein [38].

For RNA isolation and cDNA synthesis, total RNA was isolated from *in vitro* cultivated parasite primary cells and metacystode vesicles by guanidinium thiocyanate-phenol-chloroform extraction using Tri reagent (Invitrogen) according to the manufacturer's instructions. Due to the fluid-containing structure of metacystode cysts, the protocol was slightly modified. Briefly, a maximum of 5 cysts were ruptured using a sterile needle (23G, Dispomed Witt oHG). The released cyst fluid was removed upon centrifugation at 400 g for 3 minutes at RT, then the pellet was resuspended in 1 ml Tri reagent (Invitrogen) and subsequently processed as per the manufacturer's guidelines. The isolated RNA (1 μ g) was reverse transcribed into cDNA using a reverse transcription Kit (Qiagen) in a total volume of 20 μ l according to the manufacturer's instructions.

Amplification and cloning: The 5' and 3' ends of the coding sequence were used to design primers for full length amplification, namely Emtip_Dw (5'-CCT TGC AGT TTT GTA TGA AAA TG-3') and Emtip_Up (5'-GAT CAT TCG ACC TTC TAC ATT GC-3'). cDNA from the parasite larvae was used as a template in a polymerase chain reaction with the designed primer pair Emtip_Dw/Emtip_Up and a high fidelity polymerase chain reaction (New England Biolabs). Resulting amplicons were sub-cloned into the pDrive cloning vector (Qiagen) and sequenced in both directions.

In silico analyses: Sequence similarities between the deduced amino acid sequence of EmTIP and other members of the TIP family were determined through multiple sequence alignments using the BioEdit BLOSUM62 similarity matrix (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). The domains of T-cell immunomodulatory proteins were predicted using SMART online tool (<http://smart.embl-heidelberg.de/>) with default settings.

RT-PCR

Prior to the analysis of Emtip expression by *E. multilocularis* larvae, the Emtip cDNA was aligned to its genomic locus and the exon-intron boundaries defined. For unambiguous RT-PCR, the intron-flanking and Emtip-specific primer pair 5'-GAA ACG TTG AAA CAG ATC G-3' (FG-GAP_Dw) and 5'-GCT TAT GTT TCC GAG CTT G-3' (FG-GAP_Up) was designed. 1 μ l of *E. multilocularis* primary cell and metacystode vesicle cDNA preparations, reverse-transcribed (Qiagen) from 1 μ g of RNA, was amplified using the Emtip-specific primer set FG-GAP_Dw/FG-GAP_Up and the following amplification conditions: 30 sec denaturation at 94°C, 30 sec annealing at 55°C, and 30 sec extension at 72°C for 30 cycles. PCR products were separated by agarose gel electrophoresis (1%) and visualized under UV light after staining with ethidium bromide.

Immune serum production and purification of anti-EmTIP antibody

Production of an anti-EmTIP serum: For the production of polyclonal antibodies, EmTIP was expressed in the bacterial pBAD/topo thio fusion expression kit (Invitrogen). An *in silico* predicted, immunodominant portion (http://tools.immuneepitope.org/tools/bcell/iedb_input) of Emtip (from position 111 to 1119 bp of the coding sequence) was sub-cloned in pBAD/topo thio fusion following amplification with the primer pair AbTIP_dw (5'-GCT GAT TTG GCC GCT TTT G-3') and AbTIP_Up (5'-CTG AGA TTC AAC TCC AGG CAA AAG-3'). The Thioredoxin- fusion protein (Thio-EmTIP) with histidine tag, expressed in *E. coli* Top10 by Arabinose-induction (2 g/L; 4 hours), was purified on nickel-nitrilotriacetic acid resin (Invitrogen) according to the manufacturer's protocol. The purified Thio-EmTIP was then processed on centrifugal filter units (Millipore) against sterile PBS (1X) before

quantification using the BCA assay (ThermoScientific). Inbred rabbits (1 year old) were intradermally immunized repetitively with the Thio-EmTIP antigen suspensions (ImmunoGlobe Antikörpertechnik GmbH; project IG1149) generating 50 ml of polyclonal antiserum. An aliquot of pre-immune serum was used as specificity reference for the polyclonal anti-EmTIP immune serum.

Affinity purification of the anti-EmTIP serum: 200–500 μ g of purified Thio-EmTIP antigen was run on SDS PAGE and then blotted onto a nitrocellulose membrane. The band was visualized by ponceau S staining (Sigma-Aldrich) and excised prior to blocking in 5% skimmed milk in TBST (i.e. 5 g of skimmed milk in 100 ml of 1 x Tris Buffer Saline supplemented with 0.1% Tween 20) for 1 hour at room temperature on a rocking plate. The nitrocellulose strip was then incubated overnight at 4°C with 500 μ l of anti-Thio-EmTIP antiserum on a rotating wheel. Next, the strip was sequentially washed in 0.15 M NaCl, 1 x PBS, then the bound antibodies were eluted with 0.2 M glycine solution supplemented with 1 mM EGTA at pH 2.5. The eluted anti-EmTIP antibody solution was neutralized to pH 7 with Tris solution (1 M), then diafiltered on centrifugal filter units (Millipore) against sterile PBS (1X) and filtered (0.2 μ m; Millipore) before quantification of the protein content using the BCA assay (ThermoScientific) according to the manufacturer's instructions and, finally, stored at -20°C until use. In parallel, a whole γ class Immunoglobulin (IgG) solution from naïve rabbit (Santa Cruz Biotechnology, inc.) was similarly resuspended in 0.2 M glycine solution supplemented with 1 mM EGTA at pH 2.5, neutralized, diafiltered, filtered (0.2 μ m; Millipore) and quantified for protein content (ThermoScientific) before storage at -20°C.

Recombinant expression of EmTIP in HEK-293T cells

Human embryonic kidney- (HEK-) 293T cells were transiently transfected with an eukaryotic EmTIP-expressing vector system. Briefly, the full-length coding sequence of Emtip with or without a c-terminal stop codon (adding a short myc-tag to the C-terminus) was sub-cloned into the expression vector pSecTag2 Hygro A (Invitrogen) according to the manufacturer's instructions, yielding respectively the pSecTag2-Emtip and pSecTag2-Emtip_c-Myc vector constructs. HEK-293T cells were transfected with the Emtip constructs, pSecTag2 Hygro A vector as a negative control or with ddH₂O as a mock control. Transfections were performed using linear polyethyleneimine (25 kDa, Sigma) as instructed by the manufacturer. All transfections were performed in petri dishes (92 x 16 mm [\varnothing x height], SARSTEDT) seeded with cells 16 hours prior to transfection (3×10^6 cells/dish). 24 hours post-transfection, the culture supernatant was replaced by fresh medium. After another 24 hours, the supernatant was collected and the presence of recombinant EmTIP (rEmTIP) was verified by immunoprecipitation followed by Western blot. The harvested supernatants of transfected HEK-293T cells were filtered over a bottle top filter (SARSTEDT), normalized for the total protein content (BCA protein assay kit, ThermoScientific) and stored as aliquots at -80°C until use.

Immunodetection

Protein lysates were obtained from primary cell cultures, metacystode vesicles, and HEK-293T cells transfected with pSecTag2, pSecTag2-Emtip and pSecTag2-Emtip_c-Myc via treatment with 2 x STOPP mix (2 ml 0.5 M Tris-HCl pH 6.8, 1.6 ml glycerol, 1.6 ml 20% SDS, 1.4 ml H₂O, 0.4 ml 0.05% (w/v) bromophenol blue, 7 μ l β -mercaptoethanol per 100 μ l) for 10 min at 100°C.

E/S products of *E. multilocularis* primary cell cultures and metacystode vesicles as well as supernatant of HEK-293T cells

transfected with pSecTag2, pSecTag2-*Emtip* and pSecTag2-*Emtip_c-Myc* were first probed by immunoprecipitation with anti-EmTIP antibody (for the parasite E/S products, pSecTag2 and pSecTag2-*Emtip*- transfected HEK-293T supernatants) or anti-c-Myc antibody (Santa Cruz, for pSecTag2 and pSecTag2-*Emtip_c-Myc* -transfected HEK-293T supernatants). More precisely, anti-EmTIP antibody (1:20) or anti-c-Myc (2 μ g) was captured on protein G agarose (Cell Signaling Solutions) according to the manufacturer's instructions. The antibody-coated protein G agarose beads were packed into a column and used to capture rEmTIP proteins (i.e. rEmTIP and rEmTIP_myc) from supernatants. The captured antigens were eluted in 2 \times STOPP, mixed and boiled for 10 min at 100°C.

Immunodetection: To assess EmTIP expression/secretion by *E. multilocularis* larvae and transfected HEK-293T cells, the above-obtained somatic protein extracts and immunoprecipitated antigen preparations were separated by SDS-PAGE, electroblotted (Western blot), and probed with anti-EmTIP antiserum (1:10,000) as well as pre-immune serum (1:10,000). Control (pSecTag2-) and pSecTag2-*Emtip_c-Myc*-transfected HEK-293T supernatants were probed with anti-c-Myc antibody (1:1000). Affinity purified HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch) and Affinity purified HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch) were used to detect bound rabbit and mouse antibodies respectively. The secondary antibodies were detected using ECL reagents (Thermoscientific) as per manufacturer's instructions.

Immunolocalization

E. multilocularis primary cell cultures and metacystode vesicles axenically maintained *in vitro* [40] as well as non-infected and *E. multilocularis*-infested liver tissue from jirds were fixed and cut by a microtome into sections of 4 μ m. Briefly, the samples were fixed in 2% paraformaldehyde (in 1 \times PBS) and washed at 4°C overnight in 1 \times PBS containing 6.8% sucrose. The tissues were then dehydrated in 100% acetone for 1 hour at 4°C prior to embedding using a glycol methacrylate embedding kit (Heraeus Kulzer Technik) according to the manufacturer's instructions. Next, using a rotary microtome, 4 μ m sections were prepared and stained sequentially with purified anti-EmTIP antibody then peroxidase-coupled anti-rabbit-IgG (Jackson ImmunoResearch) followed by haematoxylin counterstaining according to the manufacturer's instructions (Heraeus Kulzer Technik). The sections were examined by light microscopy and images captured with a digital camera.

In vitro neutralization assay

Rabbit polyclonal anti-EmTIP antibody, obtained as described above by enrichment of anti-EmTIP immune serum over nitrocellulose-bound recombinant thio-EmTIP, was used for *in vitro* binding of EmTIP in *E. multilocularis* primary cell cultures. Primary cells were isolated as previously described [28] and adjusted to a turbidity of OD₆₀₀ nm 14/ml in culture medium DMEM10redox. All experiments were performed in a flat-bottom 96 well plates (Nunc, Thermo Scientific) with 10 μ l of primary cells suspension per well in a final volume of 100 μ l supplemented or not with different concentrations of purified anti-EmTIP antibody. As control, total IgG from naïve rabbit (Santa Cruz) resuspended in the anti-EmTIP antibody carrier solution was used. *In vitro* culture of primary cells without treatment was used as positive control. For the cultivation of primary cells, the plates were sealed in ziploc freezer bags filled with nitrogen before incubation in a CO₂ incubator at 37°C for 36 hours.

Next, 10 μ M BrdU solution (BrdU Cell proliferation Elisa kit; Roche Applied Science) was added to the cultures and the cells were further incubated for 4 additional hours. The cells were subsequently fixed and the amount of incorporated BrdU was revealed after DNA denaturation, probing with a peroxidase conjugated anti-BrdU monoclonal antibody and finally colorimetric detection with tetra methyl-benzidine (peroxydase substrate) according to the manufacturer's instructions (BrdU Cell proliferation Elisa kit, Roche Applied Science). In parallel, to identify any effects of anti-EmTIP antibody on general cell proliferation, 0.1 \times 10⁴ Rat hepatoma cells were seeded in 100 μ l of culture medium with or without anti-EmTIP antibody and the proliferation rate was assessed by BrdU incorporation as outlined above.

For the *in vitro* transition of primary cells into metacystode vesicles, 100 μ l of primary cell suspension in DMEM10redox was seeded in a 48 well plate format supplemented or not with anti-EmTIP antibody in flat-bottom 48 well plates (Thermo Scientific). The plates were sealed in ziploc freezer bags filled with nitrogen before incubation in a CO₂ incubator at 37°C. The medium was replaced with fresh DMEM10redox (control), total rabbit IgG- or anti-EmTIP- supplemented DMEM10redox every 48 hours. At day 8, the DMEM10redox medium was completely aspirated and fully replaced with medium that had been pre-conditioned by rat hepatoma cells (A4 medium) to support the *de novo* formation of metacystode vesicles from primary cell cultures [40]. The cultures were then further kept with frequent A4 medium change (twice per week). At day 22, when cyst generation was apparent, clusters of primary cells were dissociated by gentle pipetting of the supernatant and flushing of cell clusters with a 1-mL pipette to release hidden cysts. The total number of cysts per well was then determined by microscope-aided counting.

T-cell stimulation assays

CD4+ T-cells were isolated from spleen and lymph nodes of healthy C57BL/6 mice (6–8 weeks old) using a T-cell negative selection kit (mouse CD4+ T-cell enrichment kit, Stem Cell Technologies) to >90% purity according to the manufacturer's instructions. The CD25- fraction was further enriched using column-based cell isolation (Miltenyi Biotec) equipped with a suitable magnet-based cell separator achieving >90% purity for CD4+CD25- T-cells. Next, 2 \times 10⁵ CD4+CD25- T-cells were seeded in a 24-well tissue culture plate (Flat bottom, SARSTEDT) that had been coated with anti-CD3 (0.1 μ g/ml, eBioscience) overnight at 4°C. The cell suspension was supplemented with 5 μ g/ml anti-CD28 antibody (eBioscience). rEmTIP-containing and control HEK-293T cell supernatants were then added to T-cell cultures. Secretions of the parasite larvae were either directly added to T-cell cultures or first supplemented and pre-incubated for 30 minutes at room temperature with 30 μ g/ml of rabbit IgG or purified anti-EmTIP antibody before T-cell stimulation. 72 hours later, the culture supernatants were collected for determination of IL-10 and IFN- γ release by T-cells using ELISA kits (BD Biosciences) with detection limits of 19 pg/ml and 0.762 pg/ml respectively according to the manufacturers' instructions. Alternatively, Brefeldin A (5 μ g/ml; Sigma) was added during the final 6 h of stimulation and the cells were harvested for intracellular FACs analysis. Briefly, the cells were stained for surface markers (CD4-PE, BD Biosciences) in ice-cold PBS supplemented with 0.1% BSA and 0.1% sodium azide, followed by fixation in 2% formaldehyde and permeabilization in perm buffer (0.5% saponin in PBS) and then stained in perm buffer for intracellular IFN- γ (IFN- γ -FITC, BD Biosciences). Samples were

measured at a flow cytometer (BD) and data were analyzed with a flow cytometry analysis software (TreeStar).

Statistical analyses

All results were expressed as mean \pm standard deviation (SD). Differences observed between groups were evaluated using the Wilcoxon/Mann-Whitney U test, a nonparametric test that does not assume normality of the measurements (it compares medians instead of means). Values of $p < 0.05$ were considered statistically significant. Statistical analyses were performed with a statistical software analyzing package (GraphPad Software).

Accession numbers

Sequence data of *E. multilocularis* T-cell Immunomodulatory Protein reported in this manuscript are available from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) under accession number HF912277. Other GenBank accession numbers of genes and sequences used in this study include: Human TIP (Q8TB96); mouse TIP (Q99KW9) and *E. granulosus* tip (EgrG_000440000) on GeneDB (<http://www.genedb.org>)

Results

Characterization of the Emtip cDNA and gene

In previous investigations on the mechanism of trans-splicing in *E. multilocularis* [41], we isolated a cDNA clone that apparently encoded a protein with significant homology to human TIP. Since human TIP has been shown to influence cytokine production in T-cells [38], and considering immunomodulatory activities of *Echinococcus* larvae [4,9,28,30,42], we further investigated the parasite factor and named it EmTIP (*Echinococcus multilocularis* T-cell Immunomodulatory Protein homologue) (Genbank accession number HF912277). The corresponding cDNA sequence harbors an *Echinococcus multilocularis* spliced leader sequence at its 5' end (Fig. S1), suggesting that the Emtip mRNA is processed via trans-splicing. The full-length Emtip cDNA comprises 1,974 bp (excluding the polyA-tail) and encodes a protein of 592 amino acids (Fig. S1) with a hydrophobic region at the N terminus, indicating the presence of a signal peptide. The predicted gene product, EmTIP, shows moderate but significant sequence homology to human and mouse TIP-orthologs (36% and 34% identity, respectively; Fig. 1A). It contains two N-glycosylation sites, two FG-GAP (phenylalanyl-glycyl and glycyl-alanyl-prolyl) repeats and a transmembrane domain (Fig. S1). Thus, overall, EmTIP shows a structural architecture common to human and mouse TIPs (Fig. 1B).

The genomes of *E. multilocularis* and its close relative *E. granulosus* (dog-tapeworm) have recently been sequenced [43]. When using the Emtip cDNA sequence as a query, we identified the respective genomic locus as a single copy gene on scaffold 7614 (gene designation: EmuJ_000440000). The Emtip gene comprises 10 exons, separated by 9 introns. Extensive BLAST searches revealed that no other genes with significant homology to Emtip were present on the *E. multilocularis* genome. On the *E. granulosus* genome we also identified an orthologous gene, Egtip (EgrG_000440000) that encodes a protein with 98.5% identity to EmTIP. Hence, orthologs to human TIP are apparently encoded by the genomes of both important human parasitic *Echinococcus* species.

EmTIP expression pattern

To determine the expression patterns of Emtip, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on cDNA from axenically maintained primary cell cultures

and metacystode vesicles, the two larval stages that are relevant for the infection of the intermediate host [28]. Emtip transcripts could be detected in both larval stages (Fig. 2A, B). Western blot analyses using a polyclonal antibody against recombinant thio-EmTIP were then used to determine the protein expression profile. The anti-EmTIP serum recognized a single 58-kDa protein in primary cell preparations, metacystode vesicles and liver extracts from infected jirds (Fig. 2C), which correlates well with the predicted molecular mass of EmTIP (64 kDa). Interestingly, when examined for EmTIP secretion by immunoprecipitation with an affinity-purified anti-EmTIP antibody (Fig. S2), a band of the expected size was detected in E/S products of *E. multilocularis* primary cells, but not in those of metacystode vesicles (Fig. 2D).

To investigate the localization of EmTIP within the parasite, we performed immunohistochemical detection on sections of each larva. The affinity-purified anti-EmTIP antibody (Fig. S2) detected EmTIP mainly in the inter-cellular space and the surface of cells in primary cell culture aggregates and metacystodes (Fig. 2E). Poor association of EmTIP was seen with intracellular structures in both larvae or in parasite tissue present in infected jirds (Fig. 2E). No signal was obtained for liver tissue of non-infected jirds (Fig. 2E).

EmTIP is necessary for *E. multilocularis* stem cell proliferation and the formation of metacystode vesicles

To assess the role of EmTIP in *E. multilocularis* larval development, an affinity-purified anti-EmTIP antibody (Fig. S2) was used. When cultivated in the presence of this antibody, *E. multilocularis* stem cell proliferation was significantly impaired in a dose-dependent manner as assessed by BrdU incorporation assays (Fig. 3A). To exclude the possibility of a non specific effect of the anti-EmTIP antibody, an amount of total rabbit IgG equivalent to the most inhibitory dose of anti-EmTIP antibody (60 μ g/ml, Fig. 3A) was used, but revealed no inhibition of *Echinococcus* stem cell proliferation (Fig. 3B). In addition, a similar amount of anti-EmTIP antibody did not inhibit the proliferation of rat hepatoma cells (Fig. 3C), indicating that the antibody preparation did not exhibit general cytotoxic effects.

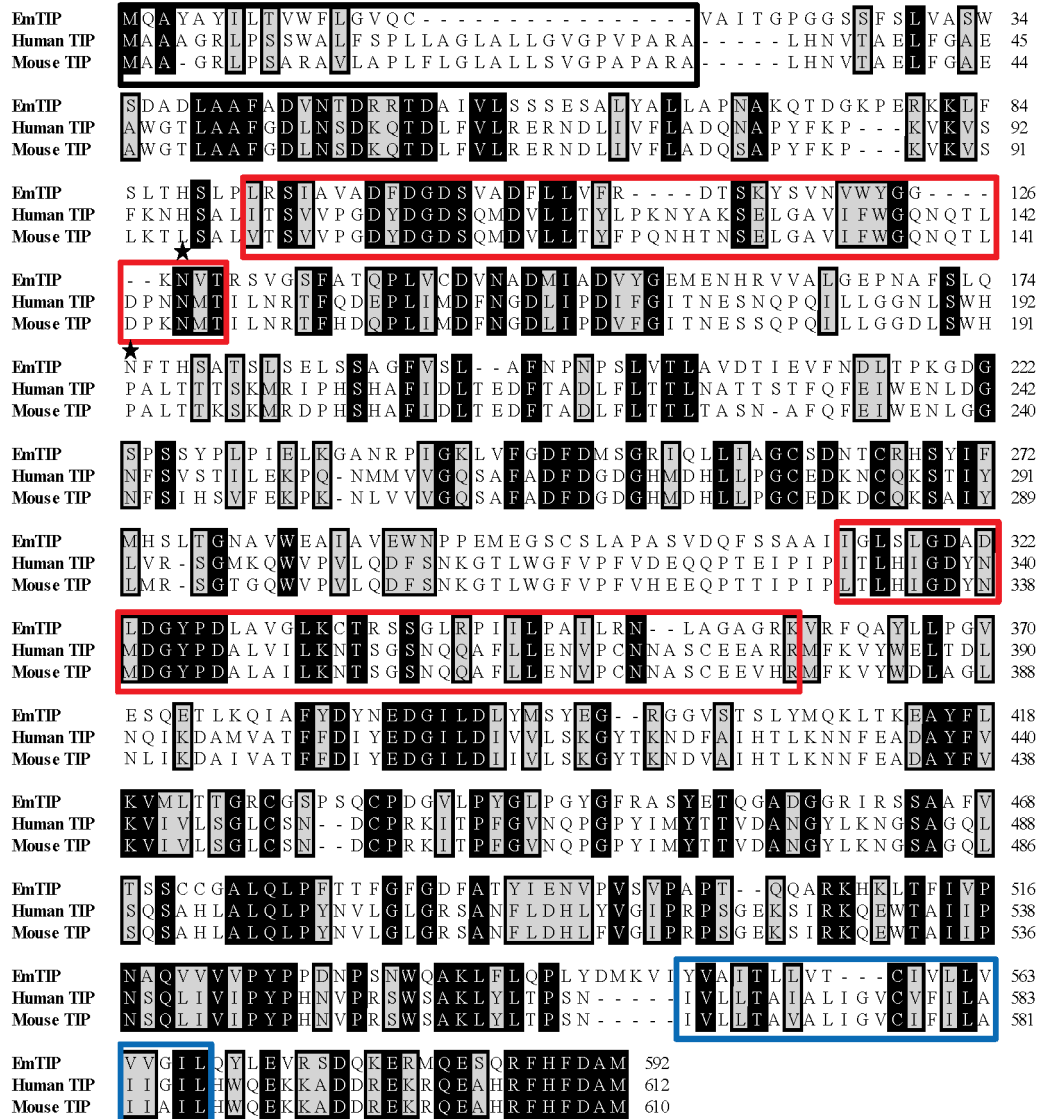
Using the primary cell culture system, we also investigated the effect of the anti-EmTIP antibody on the *de novo* formation of metacystode vesicles. As shown in Fig. 3D, we observed a significantly reduced rate of metacystode vesicle formation in anti-EmTIP supplemented primary cell cultures after 22 days of incubation ($p < 0.05$). Taken together, these data suggest that treatment of *E. multilocularis* primary cell cultures with anti-EmTIP antibody significantly affects parasite stem cell proliferation and larval development.

EmTIP promotes IFN- γ release by host CD4+ T-cells

Next, to assess the differential effects of primary cell vs. metacystode E/S products on T-cells, naive murine CD4+ T-cells were activated in the presence of secretions of each larva. When analyzing for the resulting cytokine profile, we observed a pronounced ability of primary cell E/S products to elicit IFN- γ but not IL-10 release when compared to those of metacystode vesicles (Fig. 4A).

Given that EmTIP could only be detected within the primary cell E/S products and not those of metacystode vesicles (Fig. 2D), we investigated the role of EmTIP in the IFN- γ release by CD4+ T-cells when exposed to primary cell E/S products. To this end, EmTIP was recombinantly expressed in HEK-293T cells, the transfected cells were probed for EmTIP expression, and their supernatant was analyzed for rEmTIP (rEmTIP) release (Fig. S3). rEmTIP proteins, as present in the supernatant of transfected HEK-293T cells (Fig. S3C, E), were added to CD4+ T-cell

A



B



Figure 1. EmTIP: a T-cell immunomodulatory protein homologue from *E. multilocularis*. (A) Alignment of EmTIP with human and mouse TIPs. Bioedit software, version 7.0.9, was used to align the amino acids sequences. Accession numbers for the sequences are as follows: EmTIP, HF912277; human TIP, Q8TB96; mouse TIP, Q99KW9. Identical residues are displayed in white on black background, biochemically similar residues in black on grey background. Gaps introduced to maximize the alignment are represented by dashes. Numbers at the end of each line correspond to the amino acid numbers in each respective sequence. EmTIP has 36% identity/48% similarity with Human TIP and 34% identity/42% similarity with Mouse TIP. The N-terminal signal sequences are shown in an open solid black box. Two FG-GAP repeats within EmTIP as predicted by SMART (<http://smart.embl-heidelberg.de/>) are shown within open solid red boxes. The C-terminal transmembrane domains are depicted in an open solid blue box. (B) Comparative structural architecture of *E. multilocularis*, human and mouse T-cell immunomodulatory proteins. Red boxes: signal sequences; Grey boxes: FG-GAP repeats; Blue boxes: Transmembrane domains. Sequence accession numbers are as described in (A), with structural architecture predicted with SMART (<http://smart.embl-heidelberg.de/>). doi:10.1371/journal.pntd.0002632.g001

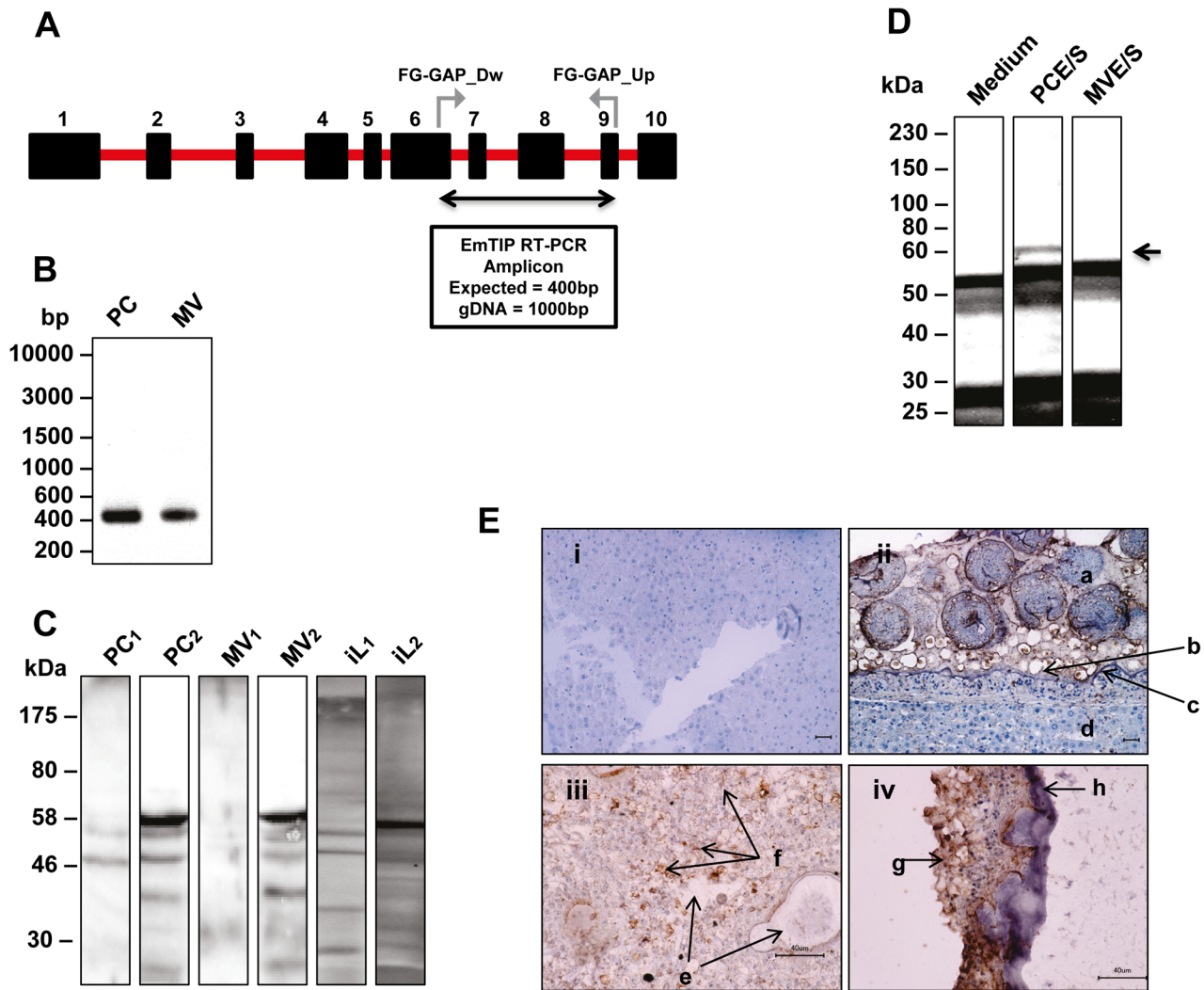


Figure 2. Expression pattern of EmTIP and localization within *E. multilocularis* larvae. (A) RT-PCR strategy for the amplification of the *emptip* transcript. Shown is an intron (red line)-exon (black boxes) arrangement of the EmTIP genomic locus. A 400 bp product specific for *emptip* was amplified using the primers FG-GAP_Dw and FG-GAP_Up spanning from exons 6–9. (B) *E. multilocularis* larvae (PC and MV) were separately used for qualitative assessment of *emptip* expression. 1 μ l of cDNA of each larva was used as template for PCR with a high fidelity DNA polymerase (High-Fidelity DNA Polymerase, New England Biolabs). 2 μ l of each PCR amplicon were resolved on a 1.5% agarose gel and stained with Ethidium bromide prior to visualization under a UV transilluminator. (C) Western blots of *E. multilocularis* larvae lysates probed with pre-immune rabbit serum (1) or anti-EmTIP immune-serum (2) followed by ECL detection and autoradiography. Whole larvae lysates (PC and MV) and *E. multilocularis*-infected jird liver (iL) were probed with pre-immune rabbit serum (1) or rabbit anti-EmTIP immune-serum followed by ECL detection and autoradiography. (D) Western blot of E/S products from *E. multilocularis* larvae after immunoprecipitation. Samples were probed with affinity-purified anti-EmTIP antibody followed by ECL detection and autoradiography. The positions of the molecular mass markers (in kilodaltons) are shown on the left. Arrow indicates the position of EmTIP around 58 kDa in primary cell E/S products. The blot is representative of two experiments with different biological isolates with similar results. (E) Staining with affinity-purified anti-EmTIP antibody followed by peroxidase-coupled anti-rabbit-IgG (Jackson ImmunoResearch) against haematoxylin counterstain of representative tissue sections from healthy jird liver (i), infected jird liver (ii), *in vitro* cultivated *E. multilocularis* primary cells (iii) and metacystode vesicles (iv). Note the EmTIP signal within the parasite intercellular spaces. Legend: a, *E. multilocularis* intra-cystic compartment with protoscolex; b, germinal layer; c, laminated layer; d, periparasitic jird liver tissue; e, central cavities surrounded by aggregates of *E. multilocularis* primary cells as previously defined [36]; f, EmTIP signal; g, germinal layer; h, laminated layer. Scale bars: 40 μ m. doi:10.1371/journal.pntd.0002632.g002

cultures during activation and the resulting cytokine profile was determined. We noted an elevated release of IFN- γ , but not IL-10, in rEmTIP-treated T-cell cultures when compared to control-treated T-cell cultures (Fig. 4B). We noted a similar ability of rEmTIP_myc to promote IFN- γ release by murine CD4+ T-cells (Fig. 4B).

To ascertain the role of EmTIP in the promotion of IFN- γ release by CD4+ T-cells, we pre-incubated the parasite E/S products with anti-EmTIP antibody before addition to T-cell cultures. We observed a significant reduction of IFN- γ release by

T-cells when primary cell secretions were supplemented with anti-EmTIP (Fig. 4C) confirming that EmTIP is important for the ability of *E. multilocularis* primary cell secretions to promote IFN- γ release by T-cells.

Finally, to confirm that CD4+ T-cells were the true source of IFN- γ release in our cultures, intracellular flow cytometric analysis was performed on treated T-cell cultures. It appeared that CD4+ T-cells were the principal source of the IFN- γ release promoted by primary cell E/S products or rEmTIP (Fig. 4D).

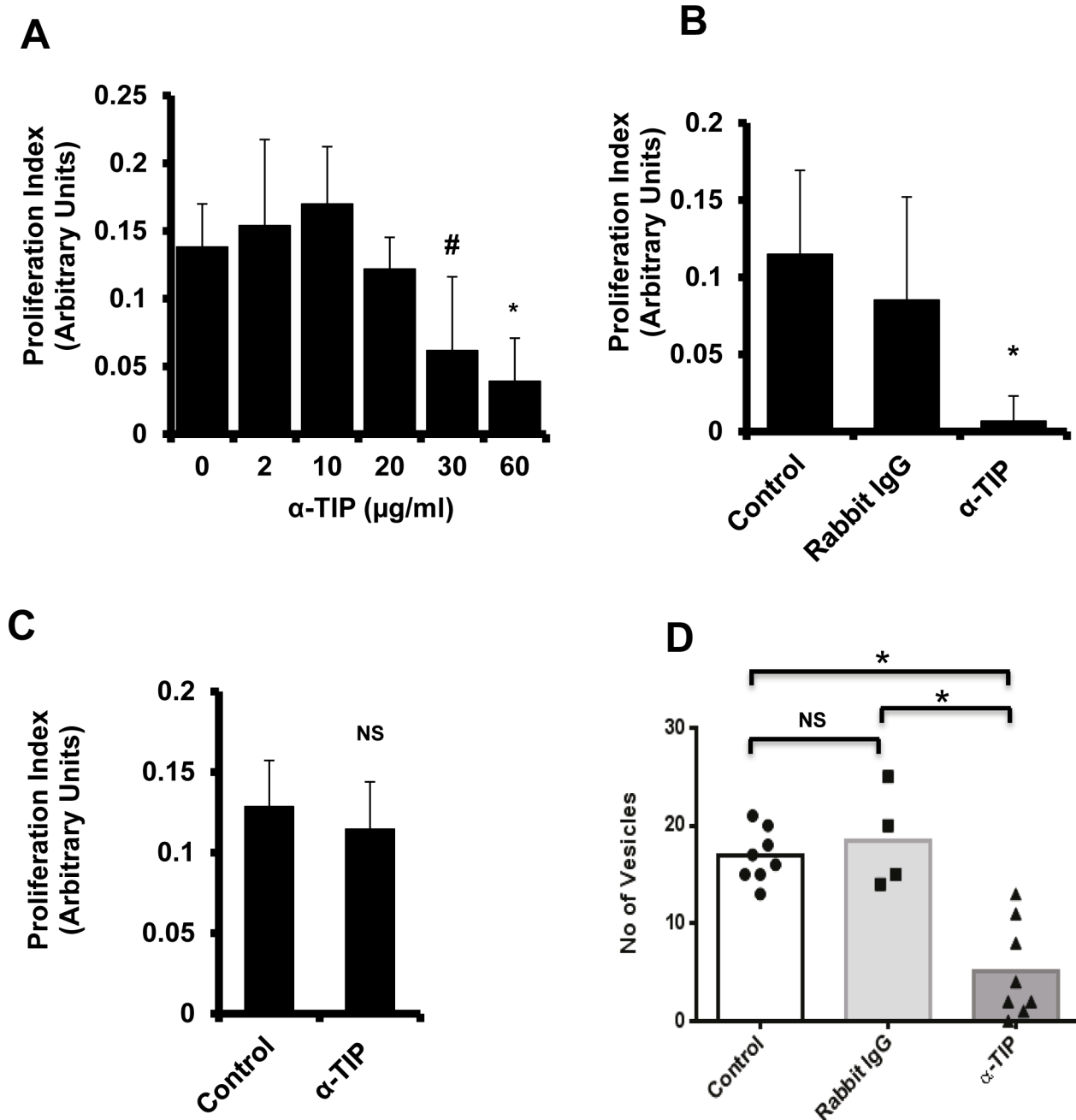


Figure 3. Anti-EmTIP antibody impairs *E. multilocularis* stem cell proliferation and metacystode vesicle formation *in vitro*. (A) Dose-response curve of the antiproliferative effect of EmTIP inhibition using affinity-purified anti-EmTIP antibody on *E. multilocularis* primary cell cultures. *E. multilocularis* stem cell proliferation was assessed by measuring the level of BrdU incorporation (see material and methods for details of the BrdU cell proliferation assay) here expressed as proliferation index. (B) 60 μ g/ml of anti-EmTIP antibody limits *E. multilocularis* stem cell proliferation whereas an equal amount of total rabbit IgG failed to do so. (C) Effect of anti-EmTIP antibody (60 μ g/ml) on rat hepatoma cell line proliferation. (D) Effect of anti-EmTIP antibody vs. rabbit IgG on the *de novo* formation of *E. multilocularis* metacystode vesicles from primary cell cultures. (A, B, C) Bars represent mean \pm SD for 4–6 replicates of two independent experiments. (D) Bars stand for mean levels. Data represent 4–8 biological primary cell isolates assayed individually. #, statistical trend $p < 0.1$; *, statistical significance $p < 0.05$; NS, not significant $p > 0.1$. doi:10.1371/journal.pntd.0002632.g003

Our data, thus collectively, suggest that EmTIP, secreted by primary cells of *E. multilocularis*, promoted IFN- γ release by murine CD4+ T-cells *in vitro*.

Discussion

IFN- γ and its associated effects dominate the very early post-oncospherical phases of an infection with *E. multilocularis* [3,29,30]

and are potentially deleterious to parasite larvae [12,13,15,25,44]. The resulting Th1 immune response is progressively suppressed as the metacystodes are formed and disseminate within the host [45]. The mechanisms mediating the early onset of host protective Th1 responses against *E. multilocularis* larvae are still not known. In the present study, we report on a parasite factor with significant homology to the human T-cell immunomodulatory protein (TIP).

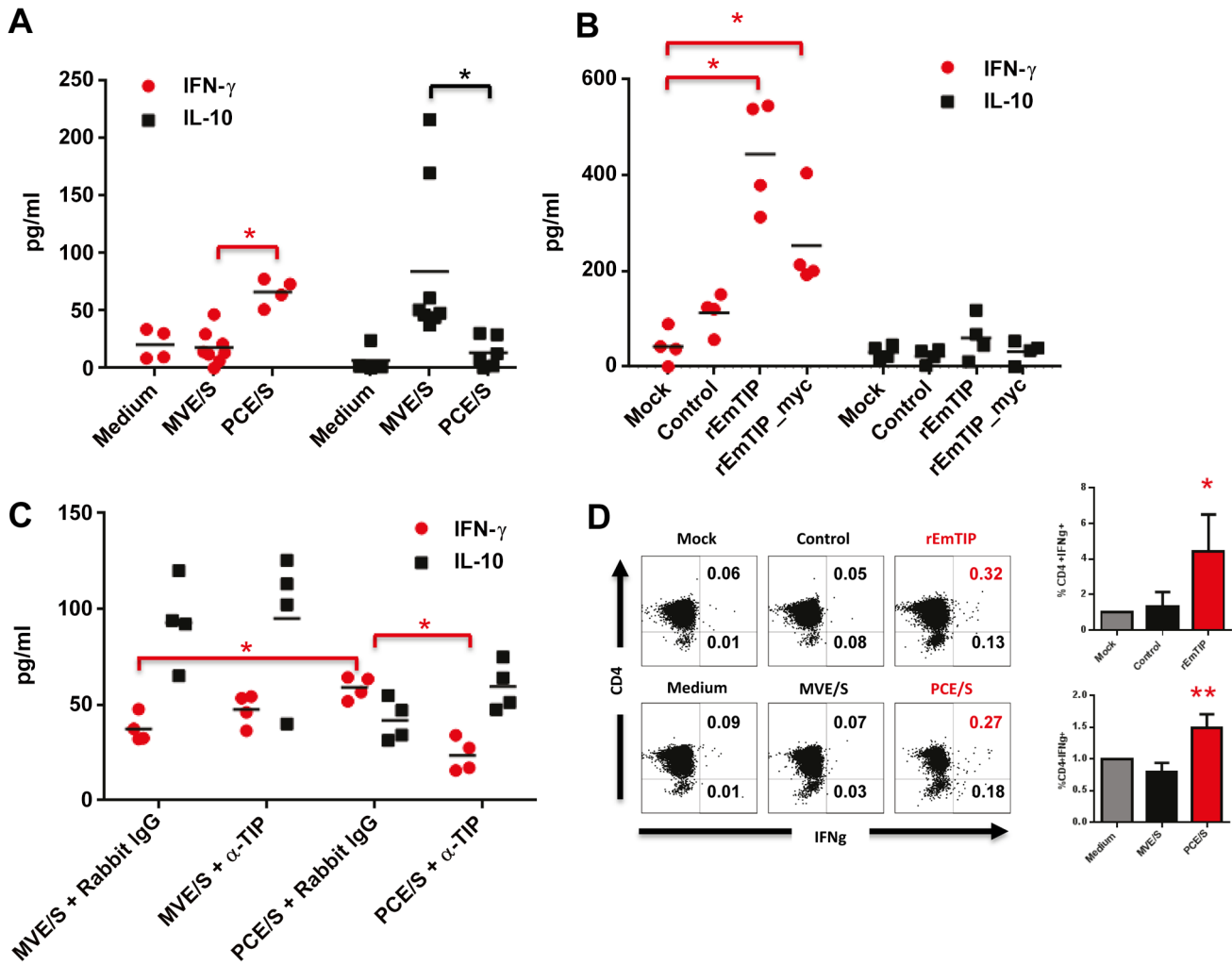


Figure 4. Elevated secretion of IFN- γ by CD4+ T-cells activated in the presence of EmTIP *in vitro*. (A) Freshly isolated CD4+CD25- T-cells (2×10^5 /ml) were stimulated with α -CD3 (0.1 μ g/ml) and α -CD28 (5 μ g/ml) in the presence of E/S products of *E. multilocularis* primary cells (PCE/S) or metacystode vesicles (MVE/S). Three days later, the T-cell supernatants were collected and probed for IFN- γ and IL-10 concentrations by Elisa. (B) Freshly isolated CD4+CD25- T-cells (2×10^5 /ml) were stimulated with α -CD3 (0.1 μ g/ml) and α -CD28 (5 μ g/ml) in medium supplemented with normalized supernatants of Mock-, control- (pSecTag2), *Emtip*- (pSecTag2-*Emtip*) or *Emtip_myc* (pSecTag2-*Emtip_myc*)- transfected HEK-293T cells. Three days later, the T-cell supernatants were collected and probed for IFN- γ and IL-10 concentrations by Elisa. (A, B) Individual results are displayed. Bars represent the mean from 4–8 independent biological replicates assessed within 3–4 independent experiments. (C) Freshly isolated CD4+CD25- T-cells (2×10^5 /ml) were stimulated with α -CD3 (0.1 μ g/ml) and α -CD28 (5 μ g/ml) in the presence of conditioned medium of primary cells (PCE/S) or metacystode vesicles (MVE/S) supplemented with total rabbit IgG or purified anti-EmTIP (anti-TIP) antibody at 30 μ g/ml. Three days later, the T-cell supernatants were collected and probed for IFN- γ and IL-10 concentrations by Elisa. Individual results are displayed. Bars represent the mean from experiments with 4 biological replicates. (D) Freshly isolated CD4+CD25- T-cells (2×10^5 /ml) were stimulated with α -CD3 (0.1 μ g/ml) and α -CD28 (5 μ g/ml) in medium supplemented with normalized supernatants of parasite larvae (PCE/S or MVE/S) or transfected HEK-293T cells (Mock, Control or rEmTIP). At day 3 of culture, 5 μ g/ml Brefeldin A was added for an additional 6 hours. Then the cells were harvested and the intracellular IFN- γ levels were determined by flow cytometry. Numbers in upper gates indicate the percentage of IFN- γ + CD4+ cells whereas the lower gate shows the percentage of IFN- γ + CD4- cells. One representative experiment of 3 conducted with similar results is shown (Left) and data from all three experiments are summarized as bar graphs (Right). * ($p < 0.05$), ** ($p < 0.01$) (D) Bars represent mean \pm standard deviations from three independent experiments with 3–7 biological replicates.
doi:10.1371/journal.pntd.0002632.g004

Since human TIP is known to promote the release of IFN- γ , TNF- α and IL-10 by T-cells [38], we characterized and functionally assessed the parasite TIP homologue, EmTIP, regarding its ability to promote the release of these cytokines by CD4+ T-cells. The corresponding gene, *Emtip*, codes for an atypical FG-GAP repeat containing protein, EmTIP. We showed that EmTIP is expressed by the larval stages that infect the intermediate host and that it is released in soluble form by *Echinococcus* primary cells, representing the early invading stage for the intermediate host. We also showed that EmTIP localized to the inter-cellular space of the parasite

larvae and that an antibody directed against EmTIP limited the proliferation of the parasite stem cells and restrained their ability to form metacystode vesicles *in vitro*. Finally, we found that EmTIP instructed murine CD4+ T-cells to release IFN- γ but not IL-10 *in vitro*. Taken together, these findings suggest that EmTIP is a parasite factor important for the early phase of *E. multilocularis* larval development and at the same time capable of promoting IFN- γ release by CD4+ T-cells.

Although an earlier study associated mammalian TIP with a protective effect in a graft-versus-host model, resulting in the

release of a complex cytokine profile (IFN- γ , IL-10 and TNF- α) by T-cells [38], the precise cellular and/or physiological function of the TIP protein family is elusive so far. TIP orthologs are widely conserved throughout the animal kingdom and are present in numerous invertebrates, suggesting that the modulation of adaptive immune responses is not their primary function. A structural feature of all TIPs is the presence of FG-GAP repeats, which are present in 2 copies in EmTIP. In mammals, FG-GAP repeat-containing proteins have principally been associated with cell-cell/ECM (extracellular matrix) interactions [46,47]. Alpha-integrins constitute such a group of proteins, harboring up to 7 copies of the FG-GAP repeat at their N-terminus through which they mediate ligand binding and cell-cell/ECM interactions [48–51]. Similar to alpha integrins, a signal sequence and a transmembrane domain are found associated with EmTIP and suggest a membrane-bound/extracellular localization. Furthermore, the localization of EmTIP to the inter-cellular space within parasite larvae indicates a possible role in the cell-cell/ECM interactome as described for FG-GAP repeat-containing proteins [46].

Although EmTIP is predicted to contain a transmembrane domain, we detected considerable amounts of the protein in the E/S fraction of *Echinococcus* primary cell cultures, suggesting that the transmembrane domain only confers transient membrane association or that the protein is post-translationally modified. Indeed, recombinant expression of EmTIP as a full-length protein, including transmembrane domain, did not impair its release from HEK-293T cells as a soluble form when compared to that of a truncated EmTIP variant that was depleted of the C-terminal transmembrane domain (data not shown). Likewise, human TIP also contains a predicted transmembrane domain but is nevertheless released by the cells in significant amounts [36]. Shedding of surface-associated components that mediate cell-cell/ECM interactions (e.g. integrins) is frequently observed during dynamic developmental processes that involve cell migration [52,53]. In these cases, the membrane-associated factors are usually cleaved off by intra-membrane proteases such as rhomboid proteases or the γ -secretase complex [54]. Interestingly, the dramatic morphological changes that occur early during the *Echinococcus* oncosphere-metacystode transition are highly suggestive of extensive cell migration events [55,56] and such processes can also be observed in the *in vitro* model of metacystode development from primary cells used in our study [36]. Furthermore, the *E. multilocularis* genome clearly encodes rhomboid/ γ -secretase proteases [43] and similar to murine and human TIP, EmTIP contains alpha-helix destabilizing glycine residues [57–59] known to facilitate the intra-membrane proteolytic cleavage and release of membrane-tethered proteins [60]. Hence, although further experimentation is clearly needed to shed more light on the function of TIP proteins, we suggest a role of EmTIP in cell-cell/ECM interactions during parasite development which involves cleavage of the protein from the cell surface during cell migratory events that are particularly prominent in the early phase of the oncosphere-metacystode transition.

An antibody directed against the FG-GAP repeat-containing portion of EmTIP, which is usually mediating cell-cell/ECM interactions [48,61,62], significantly inhibited *E. multilocularis* stem cell proliferation and, thus, the development of novel metacystode vesicles from stem cells. Since cell-cell/ECM interactions are necessary for stem cell proliferation in various organisms [49,63–65], this suggests that EmTIP antibody-mediated binding could affect *Echinococcus* stem cell interactions with the ECM and other cells. Although the precise mechanism through which the anti-EmTIP antibody inhibits *E. multilocularis* stem cell proliferation

remains unknown, it is conceivable that it sequesters soluble EmTIP or, more likely, covers membrane-associated EmTIP, and thus interferes with stem cell/ECM interactions. Irrespective of the mechanism, our data clearly indicate an important role of EmTIP in parasite development during the onset of AE and probably also during metastatic growth of the parasite in long-term infections, which all dependent on parasite stem cell proliferation and differentiation [36,37,66,67]. We therefore suggest EmTIP as a promising target for the development of anti-parasitic therapies that could involve the utilization of specific anti-EmTIP antibody, as reported in the present study, or vaccination strategies. An in-depth assessment of the influence of antibody-mediated EmTIP blockade *in vivo* during *E. multilocularis*-driven infections is currently underway in our laboratory and will shed more light on the potency of EmTIP as a host-protective antigen for AE.

A striking finding of this study is the observation that EmTIP promoted the release of IFN- γ by murine CD4+ T-cells *in vitro*, a cytokine known to promote anti-parasitic responses in *E. multilocularis*-infected hosts [12,15,25,44,68]. Despite the relatively limited overall sequence homology between EmTIP and human TIP (36% amino acid sequence identity), the parasite molecule obviously retained sufficient structural homology to elicit similar responses in human T-cells as mammalian TIP. This was clearly the case concerning the promotion of IFN- γ release, but not concerning IL-10 release, which could be due to structural differences. Although it still has to be determined which signaling pathways are influenced by either cytokine to result in the release of IFN- γ by T-cells, this effect of EmTIP is unprecedented and provides a first possible explanation for the early Th1 response reported during murine and human AE [3,4,9,29]. Importantly, despite the similarities of the murine and human systems, the promotion of IFN- γ release by EmTIP-stimulated T-cells of human origin needs to be confirmed. Nevertheless, if released *in vivo* as observed in our study *in vitro*, EmTIP could interact with host CD4+ T-cells that are present in the surrounding granuloma [69,70], potentially leading to IFN- γ release and T-cell polarization towards a potentially parasitocidal Th1 response. In this regard, we consider it unlikely that the parasite releases EmTIP primarily in order to modulate the host immune response. We rather suggest that the primary function of EmTIP lies in supporting early parasite development (e.g. by facilitating cell-cell/ECM interactions), that EmTIP is shed off/released by fulfilling this function and then, as an ortholog of human TIP, is inevitably inducing Th1 polarization when acting on host CD4+ T-cells. In later stages of development, when parasite cell migration events are rather limited and/or the release of EmTIP is prevented by the developing germinal and laminated layers of the metacystode, both the Th2 polarizing activities [15,17,71] and the immunosuppressive capabilities [28,72] of the metacystode products can then dominate the immunomodulatory activities of the parasite.

In conclusion, we herein present a novel *E. multilocularis* factor that could be exploited for the development of novel therapeutic measures against AE. In this regard, experiments to assess the potency of EmTIP blockade as a prophylactic measure for asymptomatic AE seropositive subjects and/or the efficiency of EmTIP as a protective antigen for vaccination against AE are needed and currently underway.

Supporting Information

Figure S1 Nucleotide and amino acid sequence of *Echinococcus multilocularis* T-cell Immunomodulatory Protein (EmTIP). Splice leader (Lower case) and Poly A tail are

boxed. The predicted N-terminal signal sequence is underlined. The FG-GAP repeats are shaded in grey. The potential glycosylation sites (NVT and NFT) are shown in solid boxes. The transmembrane region is delimited with dashed underlines. (TIF)

Figure S2 Assessment of affinity-purified anti-EmTIP antibody. Western blot of a similar amount of purified Thio-tagged EmTIP on (A) parasite-containing liver tissue from infected jirds (B), *in vitro* cultivated *E. multilocularis* primary cells (C), and *in vitro* cultivated *E. multilocularis* metacystode vesicles (D). Pre-immune serum (1), anti-EmTIP immune serum (2) or purified anti-EmTIP antibody (3) were used for detection followed by ECL detection and autoradiography. The positions of the molecular mass markers (in kilodaltons) are shown on the left. The arrow indicates the size of thio-tagged EmTIP (A) or natural EmTIP (B, C, D) in parasite lysates between 50–60 kDa. (TIF)

Figure S3 Recombinant expression and secretion of EmTIP in the HEK-293T cell line. (A) Schematic representation of the *Emtip* sequence cloned into pSecTag2 for recombinant expression. A modified version of *Emtip* with a c-terminal c-Myc tag (*rEmTIP_myc*) has been expressed in parallel

to investigate secretion of the factor by transfected HEK-293T cells. Western blots of control- (pSecTag2) *Emtip*- (pSecTag2-*Emtip*), and *Emtip_myc*- (pSecTag2-*Emtip_myc*) transfected HEK-293T cell lysates (B, D) and secretions (C, E). Following immunoprecipitation of E/S products with purified anti-EmTIP (C) or anti-c-Myc, 9E10 (E), the cell lysates and supernatant immunoprecipitates were probed with purified rabbit anti-EmTIP antibody (B, C) or anti-c-Myc antibody (D, E) followed by ECL detection and autoradiography. The arrows indicate the position of recombinant EmTIP on the blots. (TIF)

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Author Contributions

Conceived and designed the experiments: JKN MBL KB. Performed the experiments: JKN. Analyzed the data: JKN MBL KB. Contributed reagents/materials/analysis tools: JKN MBL KB. Wrote the paper: JKN MBL KB.

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