Immunomodulation through Excretory/Secretory Products of the parasitic Helminth *Echinococcus multilocularis*

Immunmodulation durch Exkretorisch/Sekretorischen Produkten der parasitären Helminthen *Echinococcus multilocularis*

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submitted by

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SUMMARY

Alveolar echinococcosis (AE) is a severe and life-threatening disease caused by the metacestode larva of the fox-tapeworm *Echinococcus multilocularis*. Parasite entry into the host evokes an early and potentially parasiticidal Th1 immune response that is gradually replaced by a permissive Th2 response. An immunoregulatory environment has also been reported in the host as the disease progresses. As a result of immunomodulation, *E. multilocularis* larvae persist in the host for decades without being expelled, and thus almost act like a perfect transplant. Very little is currently known on the molecular basis of the host immunomodulation by *E. multilocularis*. In this work, *in vitro* cultivation systems were used to assess the influence of metabolites released by the parasite larvae (E/S products) on host immune effector cells.

E/S products of cultivated larvae that respresent the early (primary cells) and chronic (metacestode vesicles) phase of AE induced apoptosis and tolerogenic properties (poor responsiveness to LPS stimulation) in host dendritic cells (DC) whereas those of control larvae (protoscoleces) failed to do so. These findings show that the early infective stage of *E. multilocularis* induces tolerogenicity in host DC, which is most probably important for generating an immunosuppressive environment at an infection phase in which the parasite is highly vulnerable to host attacks. Interestingly, metacestode E/S products promoted the conversion of naïve CD4+ T-cells into Foxp3+ regulatory T-cells *in vitro*, whereas primary cell and protoscolex E/S products failed to do it. Since Foxp3+ regulatory T-cells are generally known to mediate immunosuppression, the present finding indicates that Foxp3+ regulatory T-cells, expanded by E/S products of the metacestode larva, could play a role in the parasite-driven immunomodulation of the host observed during AE. Furthermore, a substantial increase in

number and frequency of suppressive Foxp3+ regulatory T-cells could be observed within peritoneal exudates of mice following intraperitoneal injection of *E. multilocularis* metacestodes, indicating that Foxp3+ regulatory T-cells could also play an important role in *E. multilocularis*-driven immunomodulation *in vivo*. Interestingly, a parasite activin ortholog, EmACT, secreted by metacestodes, was shown to expand host regulatory T-cells in a TGF-β-dependent manner, similarly to mammalian activin A. This observation indicated that *E. multilocularis* utilizes evolutionarily conserved TGF-β superfamily ligands, like EmACT, to expand host regulatory T-cells. Taken together, the present findings suggest EmACT, a parasite activin secreted by the metacestode and capable of expanding host regulatory T-cells, as an important player in the host immunomodulation by *E. multilocularis* larvae.

Another parasite factor EmTIP, homologous to mammalian T-cell immunomodulatory protein (TIP) was characterized in this work. EmTIP could be detected in the secretions of the parasite primary cells and localized to the intercellular space within the parasite larvae. EmTIP blockade inhibited the proliferation of *E. multilocularis* primary cells and the formation of metacestode vesicles indicating a major role for parasite development. Furthermore, EmTIP evoked a strong release of IFN-γ by CD4+ T-cells hence suggesting that the secretion of this factor as a result of its role in parasite development could “secondarily” induce a potentially protective Th1 response.

In conclusion, this work identified two molecules, EmACT and EmTIP, with high immunomodulatory potential that are released by *E. multilocularis* larvae. The data presented do provide insights into the mechanisms of parasite-driven host immunomodulation during AE that are highly relevant for the development of anti-parasitic immune therapies.

ZUSAMMENFASSUNG

Die Alveoläre Echinokokkose (AE) ist eine lebensbedrohliche Zoonose, die durch das Metazestoden-Larvenstadium des Fuchsbandwurms *Echinococcus multilocularis* ausgelöst wird. Nach Eintritt des Parasiten in den Zwischenwirt wird zunächst eine potentiell anti-parasitische, Th1-dominierte Immunantwort ausgelöst, welche anschließend in der chronischen Phase graduell durch eine permissive, Th2-dominierte Antwort ersetzt wird. Als Ergebnis einer zugrunde liegenden Immunmodulation durch den Parasiten können *Echinococcus*-Larven für Jahre bis Jahrzehnte im Wirt persistieren und verhalten sich ähnlich einem perfekt transplantierten Organ. Über die molekulare Basis der Immunmodulation durch den Parasiten ist derzeit wenig bekannt. In dieser Arbeit wurden geeignete Kultursysteme für verschiedene *E. multilocularis* Larvenstadien verwendet, um den Einfluss exkretorisch/sekretorischer Metaboliten (E/S-Produkte) auf Wirts-Immuneffektor-Zellen zu studieren.

E/S-Produkte kultivierter Larven, die die frühe (Primärzellen) und chronische (Metazestode) Phase der Infektion repräsentieren induzierten Apoptose und tolerogene Eigenschaften in Dendritischen Zellen (DC) des Wirts, während solche von Kontroll-Larven (Protoskolizes) keine derartigen Effekte zeigten. Dies zeigt, dass die frühen infektiösen Stadien von *E. multilocularis* in DC ein tolerierendes Milieu erzeugen, welches sehr wahrscheinlich die initiale Etablierung des Parasiten in einer Phase begünstigt, in der er höchst sensitiv gegenüber Wirtsangriffen ist. Interessanterweise förderten E/S-Produkte des Metazestoden in vitro die Konversion von CD4+ T-Zellen in Foxp3+, regulatorische T-Zellen (Treg) während E/S-Produkte von Primärzellen oder Protoskolizes dies nicht vermochten. Da Foxp3+ Tregs generell als immunosuppressorisch bekannt sind, deuten diese Daten an, dass der Metazestode aktiv eine Induktion von Tregs

herbeiführt, um eine permissive Immunsuppression während einer Infektion zu erreichen. Eine substantielle Zunahme von Anzahl und Frequenz Foxp3+ Tregs konnte zudem in Peritoneal-Exsudaten von Mäuuen nach intraperitonealer Injektion von Parasitengewebe gemessen werden, was anzeigt, dass eine Expansion von Foxp3+ Tregs auch während der *in vivo* Infektion von Bedeutung ist.

Interessanterweise konnte in dieser Arbeit ein Activin-Orthologes des Parasiten, EmACT, identifiziert werden, weleches vom Metazestoden sekretiert wird und ähnlich wie humanes Activin in der Lage ist, eine TGF-β-abhängige Expansion von Tregs *in vitro* zu induzieren. Dies zeigt an, dass *E. multilocularis* evolutionsgeschichtlich konservierte Zytokine nutzt, um aktiv die Wirts-Immunantwort zu beeinflussen. Zusammenfassend deuten die gewonnenen Daten auf eine wichtige Rolle Foxp3+ Tregs, welche u.a. durch EmACT induziert werden, im immunologischen geschehen der AE hin.

Ein weiterer Parasiten-Faktor, EmTIP, mit signifikanten Homologien zum T-cell Immunomodulatory Protein (TIP) des Menschen wurde in dieser Arbeit näher charakterisiert. EmTIP konnte in der E/S-Fraktion von Primärzellen nachgewiesen werden und induzierte die Freisetzung von IFN-γ in CD4+ T-Helferzellen. Durch Zugabe von anti-EmTIP-Antikörpern konnte zudem die Entwicklung des Parasiten zum Metazestoden in vitro gehemmt werden. EmTIP dürfte daher einerseits bei der frühen Parasiten-Entwicklung im Zwischenwirt eine Rolle spielen und könnte im Zuge dessen auch die Ausprägung der frühen, Th-1-dominierten Immunantwort während der AE begünstigen.

Zusammenfassend wurden in dieser Arbeit zwei *E. multilocularis* E/S-Faktoren identifiziert, EmACT und EmTIP, die ein hohes immunmodulatorisches Potential besitzen. Die hier

vorgestellten Daten liefern neue, fundamentale Einsichten in die molekularen Mechanismen der Parasiten-induzierten Immunmodulation bei der AE und sind hoch relevant für die Entwicklung anti-parasitischer Immuntherapien.

1-INTRODUCTION

1.1 Overview of helminth infections.

Helminthiases are the most prevalent parasitic diseases affecting close to a third of the people worldwide [1]. They prevail in the tropics and are mostly poverty-associated [1]. These parasitoses are caused by a diverse group of metazoan invertebrates namely helminths or parasitic worms [1] which have colonized humans for hundreds of thousands of years. One of the oldest evidence of human helminthiasis is provided in the Egyptian medical papyri, the Ebers papyrus, referring to intestinal worms [1]. These evidences are strongly supported by paleo-parasitological analyses which reveal calcified helminth eggs in mummies dating from 1200 BC [1]. The burden of parasitic helminths has raised a considerable interest throughout the ages resulting in a better understanding nowadays of the medically relevant classes of helminths. Parasitic helminths have been clustered into two major phyla characterized by elongated, flat or round bodies. In medically oriented schemes this subdivision comprises (i) The nematodes (also known as roundworms) which include the major intestinal worms (also known as soil-transmitted helminths) and the filarial worms that cause lymphatic filariasis and onchocerciasis, and (ii) the platyhelminths (also known as flatworms) which include the flukes (also known as trematodes), such as the schistosomes, and the tapeworms (also known as the cestodes), such as the pork tapeworm that causes cysticercosis or the fox-tapeworm, the subject of the present study, that causes Alveolar echinococcosis (AE).

1.2 The fox-tapeworm *Echinococcus multilocularis*.

1.2.1 Phylogeny.

Parasitic cestodes including the fox-tapeworm *Echinococcus multilocularis* are metazoans and tissue-forming organisms that can establish an infection in mammalian hosts. Figure 1 provides a consensus view of the phylogenetic position of *Echinococcus multilocularis*, its interrelationships with other helminths and the shared ancestry with its mammalian hosts based on molecular and morphological estimates.

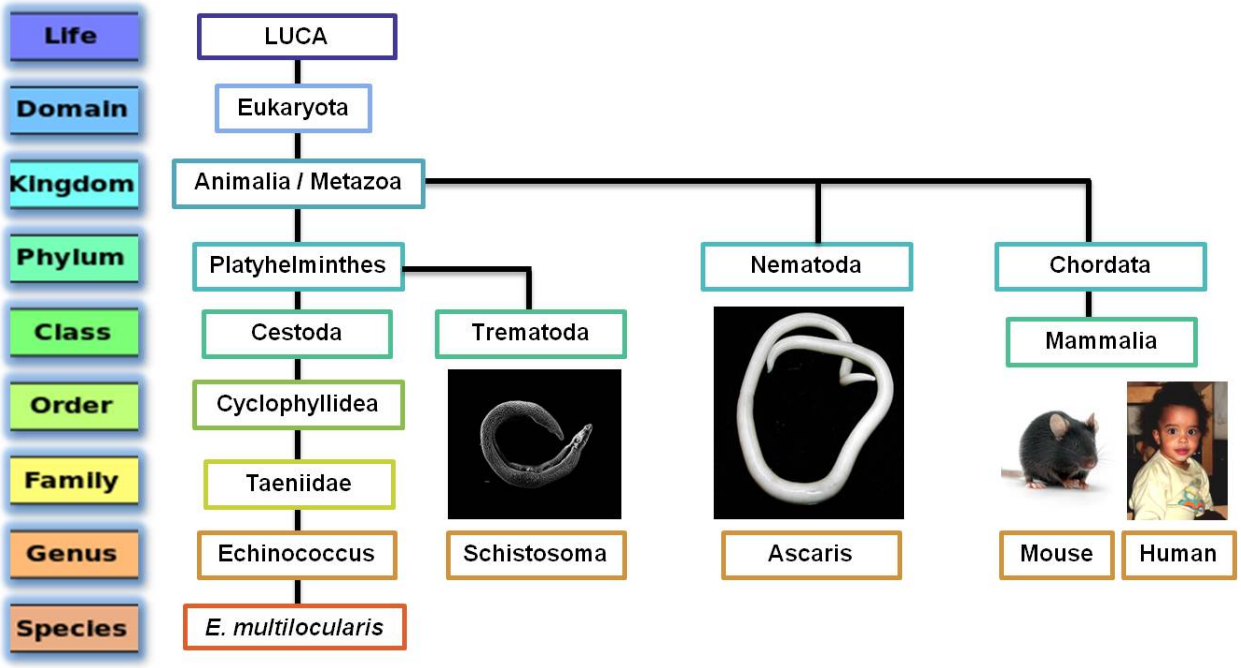


Figure 1: Position of *Echinococcus multilocularis* in the tree of life (adapted from [2]). The phylogenetic position of the fox tapeworm *Echinococcus multilocularis*, the Schistosomes of the class of trematoda, the nematodes of the genus ascaris and the murine and human hosts of such parasitic helminths are shown. LUCA: Last Universal Common Ancestor.

The fox-tapeworm *Echinococcus multilocularis* belongs to the Taenidae family of the class of parasitic cestodes within the phylum Platyhelminthes (Fig. 1). It is closely related, molecularly and morphologically, to the dog tapeworm *Echinococcus granulosus* and to a lower extent to the pork tapeworm *Taenia solium* which are altogether encompassed within the class of parasitic cestodes [2]. Another medically relevant class of platyhelminths is that of the trematodes, represented in Fig. 1 by the genus *Schistosoma*. Trematodes like schistosomes show several morphological and molecular similarities with parasitic cestodes as they both belong to the flatworms/platyhelminths phylum as opposed to the distantly related phylum of roundworms or nematodes (Fig. 1).

1.2.2 Epidemiology.

Alveolar echinococcosis is rare in humans [3] and the progression of the disease rather appears as an exception in exposed hosts [3]. The disease is confined to the northern latitudes (Fig. 2).

Figure 2: Global Distribution of Alveolar echinococcosis [4,4,4]. Alveolar echinococcosis is shown primarily confined to northern latitudes. A complete picture for all the geographical foci might not be represented here because of incomplete or lacking data for some areas.

Its causative agent, the fox-tapeworm *Echinococcus multilocularis*, has a scattered geographic distribution in the northern hemisphere, with endemic regions in central Europe, most of northern and central Eurasia and parts of North America. Eurasia, parts of Turkey, Iran and possibly northern India (report of 1 human case) seem to represent the southern limits of the range of *E. multilocularis*, but little information is available in this respect [4]. In North America, the range of the cestode reaches from Alaska southward to the States of Nebraska, Iowa, Illinois, Indiana and Ohio (4). Two autochthonous human cases of AE were reported from a mountainous region of northern Tunisia, which might be an indication of the occurrence of *E. multilocularis* in northern Africa, but further information is not available [5].

A critical aspect of *E. multilocularis* distribution is the geographical spreading of the parasite [4]. In central Europe, endemic areas were known to exist in only four countries by the end of the 1980s, but recent studies revealed a much wider geographic range, including at least twelve countries (Austria, Belgium, the Czech Republic, Denmark, France, Germany, Liechtenstein, Luxembourg, the Netherlands, Poland, the Slovak Republic and Switzerland) [4]. A new focus was detected in 1999 on the Norwegian Islands of Svalbard [4] and in 2012 in the Netherlands [4]. Although it is not known whether these new foci are a result of increased surveillance, there are nevertheless indications for emerging risk factors, such as increasing parasite prevalence in foxes [6], growing human and fox populations [7]. As a direct consequence of the latter, human and fox habitats initially separated started to overlap as evidenced by an increasing number of foxes reported in/near cities [7]. The current estimates suggest an annual incidence of 20,000 new human cases worldwide with 91% of them occurring in the people´s republic of China [8] and 0.3% (~60) in Germany [9]. The prevalence of AE in central Europe approximates 2-40 cases per 100,000 inhabitants [10].

1.2.3 Life cycle.

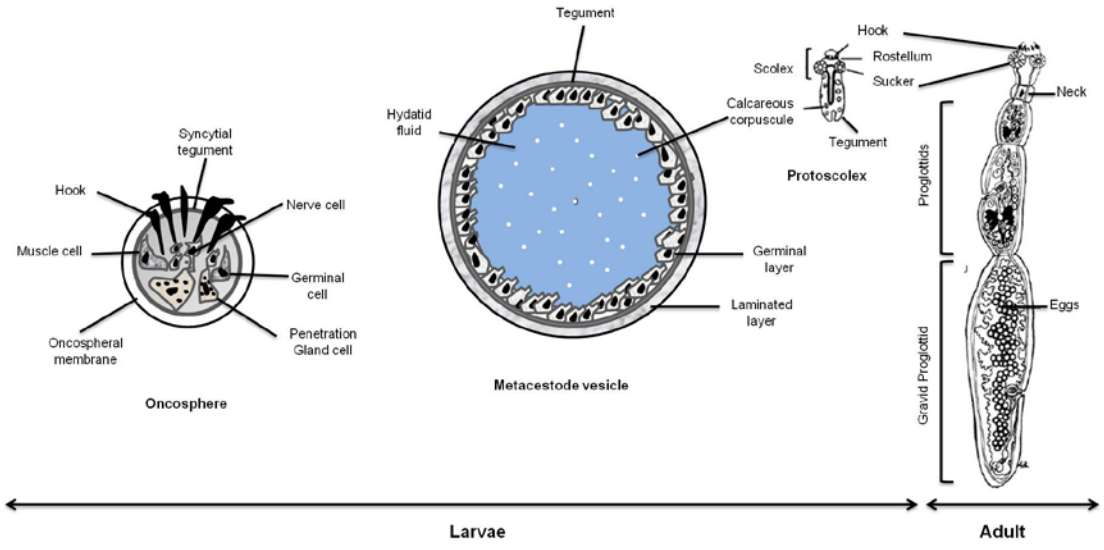


Figure 3: Developmental stages of *Echinococcus multilocularis* (adapted from [11]).

E. multilocularis transitions from three larval stages to an adult form (Fig. 3). The obligate two-host parasitic cycle of the parasite is predominantly sylvatic (Fig. 4).

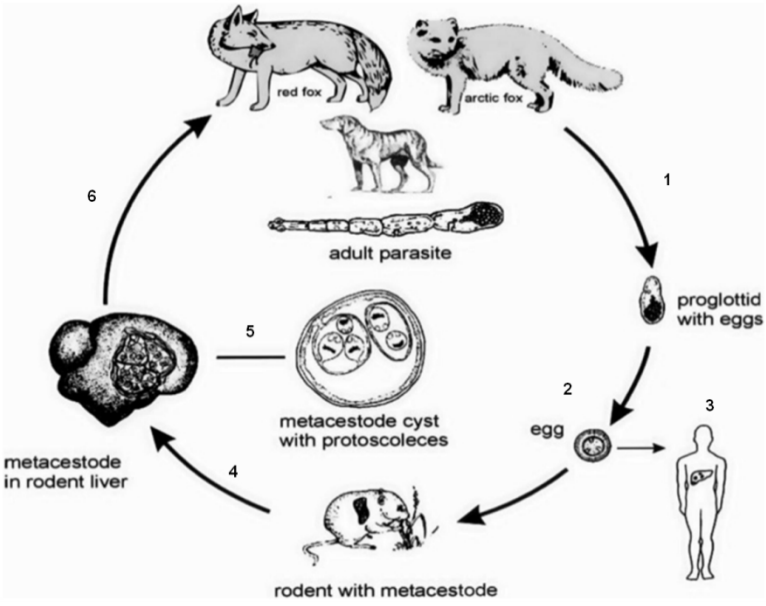


Figure 4: Life cycle of *Echinococcus multilocularis* [11,12]. (**1**) The adult worm resides within the gut of the definitive hosts and sheds gravid proglottids containing eggs along with faeces. (**2**) Eggs are released in the environment after the proglottids have dried out contaminating food, water or attaching to pets fur. (**2**) Eggs are accidentally taken up by rodents or humans. Upon ingestion, eggs hatch and release the oncosphere that gets activated and penetrates the intermediate human (**3**) or rodent (**4**) host intestinal epithelium to lodge by passive drainage to the liver forming metacestodes. (**5**) In the rodent but rarely the human host, protoscoleces form from the germinal layer of the metacestodes. (**6**) The enclosed protoscoleces are taken up by definitive hosts as they predate voles. Once in the definitive carnivorous host, the protoscoleces excist, get activated and anchored to the small intestine so as to mature into adult worms.

In the carnivorous definitive hosts, the adult worms of 2-4 mm in size parasitize the posterior small intestine for up to twelve months. In Europe, the red fox represents the main definitive host but could, albeit to a lesser extent, be dogs or cats [12]. After a prepatent period of nearly 4

weeks, the adult distal segments or gravid proglottids of the adult worm containing eggs are passed in the faeces of the definitive host to reach the environment. Different species of rodents are involved in the parasitic cycle as next recipients. They get infected by oral uptake of eggs which contain the oncosphere larvae. Equally at risk, suburban human populations and especially farm workers [13] can acquire the infection by the ingestion of eggs. Following the action of enzymes in the stomach and small intestine, the oncospheres are released from the eggs and are activated by intestinal enzymes and bile salts [11]. Equipped with hooks and penetration gland secretions, the activated oncospheres penetrate the wall of the small intestine, gain access to a venule or lacteal, to be passively transported to the liver, where some are retained [11]. Others reach the lungs, and a few may be transported further to the kidneys, spleen, muscles, brain or other organs [11]. Once directed, presumably by organ-specific signals few weeks post ingestion [11], the oncosphere undifferentiated germinal cells initiate a transformation process into a fluid filled bladder-like structure enclosed within an outer carbohydrate and high molecular glycan-rich laminated layer and an inner cellular germinal layer: the metacestode larvae or cyst [11,14]. All mammals (including man) in which metacestodes develop after infection with eggs are referred to as 'intermediate hosts'. The metacestode vesicles via the cells of its germinal layer then infiltratively grow within the intermediate host tissues forming daughter cysts, expanding like a tumor leading to the condition termed alveolar echinococcosis (AE) [11]. From the epidemiological point of view, it is useful to differentiate between 'intermediate hosts', which play a role in the perpetuation of the cycle, and 'aberrant or accidental hosts' which represent a 'blind alley' for the parasite as the latter are not involved in disease transmission [11]. This may be because metacestodes fail to become fertile in these hosts [11] thus leading to an interruption of the transmission cycle. With a few rare exceptions, humans belong to the group of 'aberrant hosts' [11] whereas in several species of rodents, buds are then formed in the germinal layer of the metacestode vesicles and grow toward the fluid filled cavity of the hydatid cyst [11]. The buds later become stalked and vacuolated [11]. A new process of budding is initiated from the inner layer of the cells of these cavities, which leads to the formation of hypobiotic protoscoleces within the cyst now fertile [11]. As a result of natural predator-prey relationships, the intermediate hosts (rodents) are devoured by carnivorous definitive hosts permitting the uptake of the protoscoleces in the definitive host's gastro-intestinal tract. The protoscoleces will then excyst, evaginate and become activated as a result of the low pH of the stomach and bile salts of

the intestine [11]. The activated protoscoleces will then attach to the intestinal mucosa initiating the formation of proglottids and ensuing egg production [11].

1.2.4 Clinical manifestations of AE.

Caused in the intermediate hosts (humans, rodents) by metacestodes of *E. multilocularis* and characterized by a tumor-like, infiltrative and destructive growth with the potential to induce serious disease, the condition known as AE was elucidated by the German pathologist Rudolf Virchow in 1855 [15]. Since then, a large body of studies has drawn a rather comprehensive picture for the clinical course of the disease [16,17]. An initial asymptomatic incubation period of 5 to 15 years duration is followed by a subsequent chronic phase primarily featured by cholestatic jaundice (about a third of the cases) and/or epigastric pain (about a third of the cases) and incidentally detected in the other third of patients during medical examination for symptoms such as fatigue, weight loss or hepatomegaly [16,17]. Consistent with the symptoms, the primary site of metacestode development is almost exclusively found to be the liver [15–17] as depicted in Fig. 5.

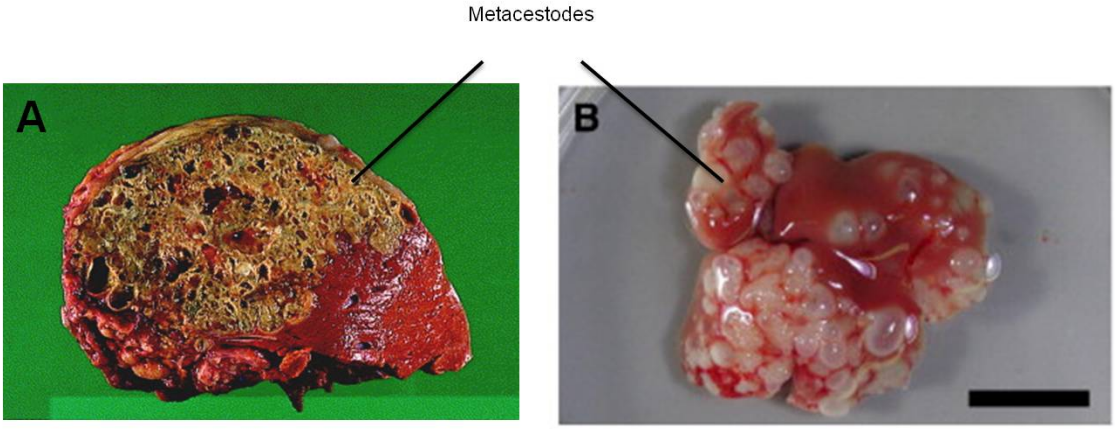


Figure 5: Livers of hosts (intermediate) infested with *E. multilocularis* larvae. Human (**A, [12]**) and mouse (**B, [18]**) livers extensively colonized by metacestode larvae.

The right lobe is predominantly infected, but the liver hilus together with one or two lobes may also be involved. Extrahepatic primarily locations are rare. During the infection, secondary echinococcosis (= metastasis formation) may occur in variety of adjacent or distant organs where the parasite cysts will establish new niches and grow, aggravating the medical condition [2,12,16–18].

1.2.5 Prevention and control of alveolar echinococcosis.

When no intervention measures are taken, the fatality rate is high as illustrated by a follow-up of a series of 66 individuals with AE from Germany (period 1960-1972), 70% died within 5 years and 94% within 10 years after diagnosis of the disease [16] . However, data obtained from more recent series (diagnosis after 1983) show an improvement of the survival rate due to improved control measures [16,17]. This is further supported by an increased life expectancy at diagnosis in Europe from averagely 3 years in the 1970s to 20 years in 2005 [8]. Early diagnosis and improved treatment have been instrumental in this progress [8] and continue nowadays to gather considerable attention in anti-AE research agendas.

1.2.5.1 Diagnosis.

The diagnosis of AE in individual patients is based on a consensus approach directed by the guidelines of the WHO-Informal Working Group on echinococcosis [16,19] i.e. from clinical examination to epidemiological data, imaging techniques, histopathology and/or nucleic acid detection, and serology as summarized in table 1.

1.2.5.2 Treatment.

Once confirmed, AE treatment is planned in a multidisciplinary discussion, taking all elements of available pre-treatment imaging into account [16,19]. The probability of effective cure strongly depends on the time point of diagnosis and ensuing therapeutical measures [16,19]. Surgery is the basic form of treatment for early AE [16,19], but radical resection of parasite material is almost impossible and recurrences therefore cannot be excluded [16,19,20]. Therefore

supportive chemotherapy [21] has been introduced to improve the first line surgical therapy. In advanced stages of AE, radical resection is impossible and long-term chemotherapy with benzimidazoles (BZs) and palliative surgical measurements, e.g., hepaticojejunostomy, drainage of necrotic cavities, or portocaval shunts, may prevent complications and improve the survival rate [22]. Two BZ derivatives, mebendazole (MBZ) and albendazole (ABZ) have been introduced for chemotherapy against AE in humans [22–25]. Benzimidazoles are known to cause degenerative alterations in the tegument and intestinal cells of the parasite by binding to the

Table 1: Approaches for immunodiagnosis of AE in humans (adapted from [16,19]).

<table>
<tr><td colspan="4">Primary Antibody test (ELISA)
of patients serum using E. multilocularis antigens (Em2, Em18, EmAP, C-Ag, Em10) for detection of IgG – High sensitivity</td></tr>
<tr><td colspan="3">Seronegative</td><td>Seropositive</td></tr>
<tr><td>Asymptomatic and no Imaging evidences
(Ultrasonography, Computed tomography)</td><td colspan="2">Imaging evidences of parasite cysts</td><td>/</td></tr>
<tr><td>Repeated Serological examinations and Imaging after 3 and 6 months to exclude risk</td><td>Asymptomatic
In depth imaging
Needle biopsy and PCR
Immunohistology
If lesions calcified >Repeated Serological examinations and Imaging after 6 months to confirm parasite death</td><td>Symptomatic
Consider
Treatment</td><td>Secondary IgG4 antibody test (ELISA) for verification and exclusion of false positive using Em2plus ELISA kit – High specificity
Western Blot patterns to discriminate with E. granulosus infection
Treatment</td></tr>
</table>

colchicine-sensitive site of beta-tubulin, thus inhibiting its polymerization or assembly into microtubules [26]. The loss of the cytoplasmic microtubules leads to impaired uptake of glucose

by the larvae and depletes the glycogen stores [26]. Degenerative changes in the endoplasmic reticulum, the mitochondria of the germinal layer, and the subsequent release of lysosomes result in decreased production of adenosine triphosphate (ATP), which is the energy source required for the survival of the helminth [26]. Due to diminished energy production, the parasite is immobilized and slowly dies [26]. Indirectly, the metabolic blockade and the ensuing disintegration of the tegument renders the larvae more antigenic thus better targeted, attacked and destroyed by the host immune system, since not efficient anymore at eluding/evading the host immune recognition [27]. Although long-term application of MBZ caused complete death of the parasite in one Alaskan study [28], complete eradication of the parasitic mass by BZs cannot be achieved in the majority of patients [29]. In summary, there is circumstantial evidence from the clinical outcomes of treated patients that BZs should rather be considered parasitostatic *in vivo*. Moreover, BZ derivatives, principally ABZ have been reported to be teratogenic [30] and to cause neurological deficit, gastrointestinal disorders, bone marrow suppression, agranulocytosis or aplastic anemia which may be permanent [22,31,32]. The risk of developing some of these side effects seems to be increased in patients with liver disease, including echinococcal cysts [33]. Because of the double-edged sword of chemotherapy with BZs and the severity of the disease, it is important to regularly monitor AE-patients [22]. Comparison of the survival of patients on chemotherapy with that of untreated historical controls shows that the 10-year survival rate of the treated group has increased from <10% to 85-90% [22]. Increased survival rates might not only be associated with chemotherapy but also with early diagnosis, improved surgery, and medical care of the patients [22]. As for chemotherapy, Praziquantel has also been used but experimental data obtained from animal models indicate that it failed to show efficacy against the metacestode stage of *E. multilocularis* in contrast to BZs [34,35]. Praziquantel is however highly efficient against *E. multilocularis* protoscoleces and adult worms [36–38] and is thereby commonly used for deworming carnivorous host in endemic areas as an anti-AE transmission-blocking measure [37,39,40].

1.2.5.3 Vaccination.

Concerns raised by the parasitostatic rather than parasiticidal effect of benzimidazole-derivatives in patients with AE and the limitations of these drugs have driven a growing interest

of the scientific community in the development of a vaccine for populations at risk. Compelling evidences suggesting the feasibility of vaccines against *Echinococcus* sp. in particular and cestodes in general are summarized into 4 major points:

(i) after infection with oncospheres the host develops strong immunity to re-infection [41].
(ii) the oncosphere and early developing larvae are highly susceptible to immune attack whereas mature metacestodes are relatively resistant [42,43].
(iii) immunity to infection can be transferred to naive recipients following injection of infected host serum [42].
(iv) high levels of immunity to egg challenge can be stimulated by immunization of naive hosts with non-living extracts of the parasites [44,45].

An important role for oncosphere diffusible antigens as host protective antigens was gradually unveiled for cestodiasis as per observations that the use of oncosphere diffusible antigens or E/S products could confer a complete protection against infection by cestode eggs in animals [44,46]. Lightlowers and collaborators [47] then initiated the design of a vaccine against echinococcosis. Experimentating on the closely related tapeworm *E. granulosus*, they identified an oncosphere antigen Eg95, with a fibronectin type III domain, able to confer 96-100% protection against infection to sheeps over a year period [47]. A closely related antigen named Em95 from *E. multilocularis* could similarly show up to 83% protection against infection with eggs in mice [48]. The Em95 oncospheral antigen constitutes hitherto the most promising candidate for the development of a vaccine against human AE.

Despite the efforts and achievements in the development of better control measures for AE, the lack of early prophylactic tools for patients at risk (asymptomatic but seropositive), the ineffectiviteness of area-specific vaccination, the parasitostatic rather than parasiticidal character of the available chemotherapy are altogether warranting more attention from the scientific community. On the one hand, such undertakings, if successful, would help alleviate the current burden of the disease. On the other hand, since AE manifests in patients like a slow-growing cancer where the parasite larvae establish and persist for decades within the host tissues in the face of the immune system like perfect transplants, the informations that could be gained from a better understanding of the interactions between *E. multilocularis* larvae and the host immune system are invaluable. This is especially true when considering the high incidence of non-

communicable immunopathologies (allergic and autoimmune diseases) in developed countries [49] that could certainly benefit from such knowledge on how to keep detrimental immune responses in check. To set the stage for such a quest on mechanisms used by *E. multilocularis* to survive within its host, it is paramount to first know the current picture of the host immune response to *E. multilocularis*.

1.3 Host immune response to *Echinococcus multilocularis.*

1.3.1 The host immune system.

The physiologic function of the immune system is defense against infectious microbes resumed into 4 major tasks (Fig. 6): recognition of the pathogen, effector functions, immune regulation (contraction) and immunological memory [50].

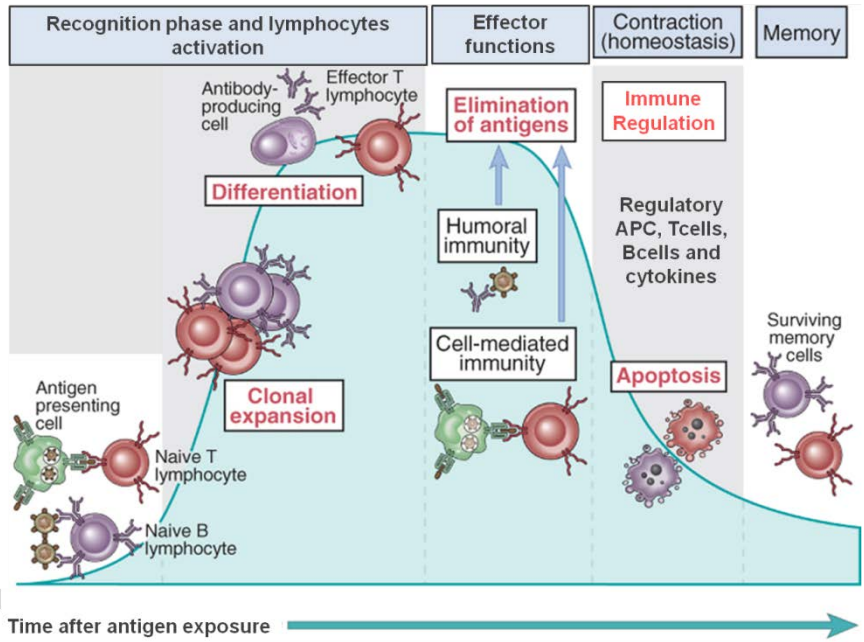


Figure 6: Phases of the immune response (adapted from [50])

1.3.1.1 Recognition of the pathogen.

Phagocytes of the myeloid lineage (neutrophils, macrophages and DC) gather to the site of infection and engulf the invaders or its derived antigens [51]. Unlike the neutrophils that are relatively short lived and are produced solely in response of an inflammatory signal, macrophages and DC reside within the host tissues and permanently circulate throughout the body for surveillance [51,52]. These phagocytes are poised to transform upon challenge by pathogen signatures or pathogen-associated-molecular-patterns (PAMPs) [51] like lipopolysaccharide (LPS) from the gram-negative cell wall, peptidoglycan and lipotechoic acids from the gram-positive cell wall, the sugar mannose (a terminal sugar common in microbial glycolipids and glycoproteins but rare in those of humans), bacterial and viral unmethylated CpG DNA, bacterial flagellin, the amino acid N-formylmethionine found in bacterial proteins, double-stranded and single-stranded RNA from viruses, glucans from fungal cell walls or DAMPs (damage-associated molecular patterns) from damaged tissue [53]. These phagocytes become activated as a result of the recognized threat [51,52]. Considerably important in this process, DC, which provide the link to the propagation of the defense mechanisms i.e. the activation of the adaptive immune response [50,52], will phenotypically and functionally alter their profile [52,54]. From their immature state, poised to engulf invader antigens with a minimal level of expression of activation markers (MHC, CD80, CD86, CCR7), these latter will then change to a minimal level of phagocytosis, a higher surface expression of migration markers (e.g. CCR7) and activation markers (CD80, CD86) exposing the molecular signature of the invader (antigen epitopes coupled to increasingly expressed surface MHC molecules) [52,54]. Driven by their chemokine receptor (CCR7) in response to the chemokine gradient (CCL19 and CCL21), antigen-loaded DC now mature, migrate to the T-cell area of the draining lymph node to principally present the antigen to naïve CD4+ T-cells [52,54]. The process of antigen presentation requires 3 major signals (Fig. 7) that will direct the T-cell polarization and influence the ensuing effector functions [52,55–57]:

i-The interaction between the MHC-coupled Ag and the T-cell receptor as signal 1 or recognition signal where the strength of the interaction defines the immunogenicity of the ensuing response [52,55–57].

ii- the interaction between the costimulatory molecules, upregulated at the surface of the mature DC (e.g. CD80, CD86) as signal 2 or verification signal and the T-cell costimulatory receptor

(e.g. CD28) [52,55–57]; Failure to provide this verification signal would lead to T-cell inertia or as commonly termed T-cell anergy [58–60].

iii- the release by DC of soluble mediators or cytokines as signal 3 that will together with the strength of the signal 1 and 2 polarize the naïve T-cells towards activated T-cells poised to install and propagate adequate immune effector mechanisms [55,57].

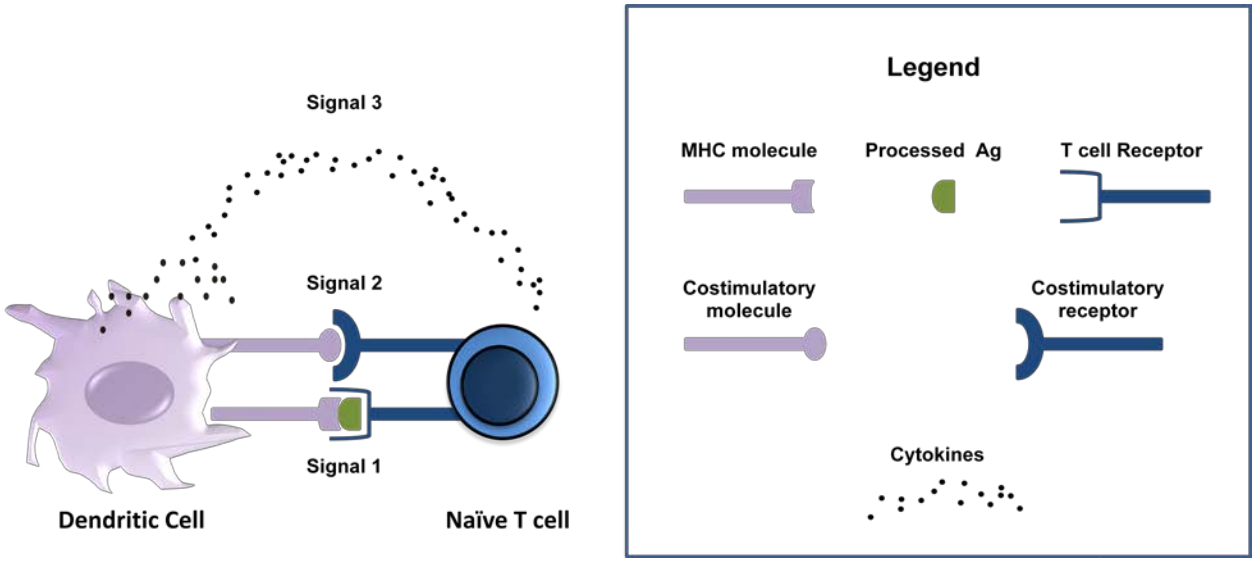


Figure 7: "Three signals theory" of T-cell polarization by DC (adapted from [55]). Shown is an ideal setting of T-cell priming with the distinct interactions between the mature DC and the T cell i.e. antigen presentation (signal 1), co- stimulatory molecule upregulation (signal 2) and cytokine secretion (signal 3).

1.3.1.2 Effector functions.

The level of antigen presentation, the rate of expression of costimulatory molecules and the cytokine profile of challenged DC are altogether crucial to direct the polarization of naïve T-cells towards a particular effector function [55,57] and therefore define the type of T-cell response (Fig. 8). The release of Interleukin 12p70 is important as a cytokine for the onset of CD4+ T helper 1 cells [54,55,61,62]. T helper 1 cell responses intervene in cases of bacterial or viral infections preferentially, releasing soluble mediators like IFN-γ and in a CD154-dependent

manner increase the cytotoxic potential of CD8+ cytotoxic T cells, neutrophils and macrophages [61]. Secondly, Th1 cells migrate into B-cell follicles to promote CD154-dependent B-cell clonal expansion and Antibody (Ab) production which altogether synergizes via a coordinated cellular mediated response to arrest the development of the intruder [61].

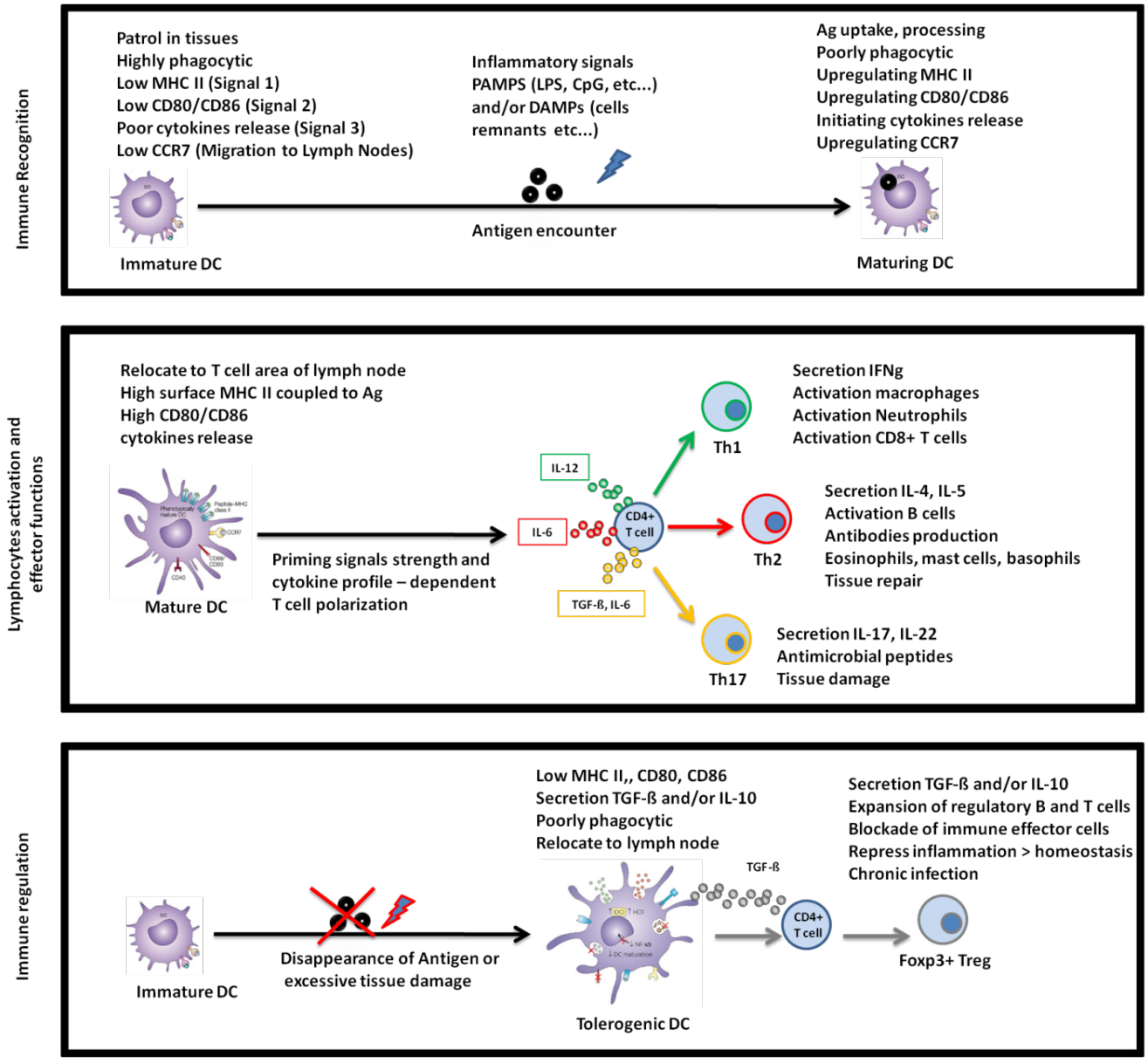


Figure 8: Model of DC-driven T-cell priming from the effector to the regulatory phases of the immune response (adapted from [50–55,57,58,61]).

In instances of encounters with macroparasites (helminths) or allergens, DC rather show a weak signal 1 and 2 and have been collectively described by the poor release of IL-12p70 and T helper 2 responses [63]. IL-6 a pleiotropic cytokine has been shown in several settings to participate in DC-driven polarization of naïve T cells into Th2 [64–68] and stimulate B-cell growth [62]. Th2 cells principally produce IL-4 and IL-5 [61]. Whereas IL-4 induces B-cell switching to IgE production, IL-5 activates Eosinophils [61,62]. IgE and Eosinophils are important to eliminate a wide range of helminth parasites [69]. As shown in Figure 8, Th2 cells activate B-cells, stimulate the antibody production and limit inflammatory responses overall driving macroparasite eviction and minimizing tissue damage primarily via mediators of the humoral immune response (antibodies, cytokines, major basic proteins etc...) [61,69,70].

When opportunistic commensals like *Candida* spp. or *Staphylococcus* spp. are encountered but also when the altered "self" appears threatening to the host himself during the initiation of autoimmune diseases i.e. in a highly inflammatory setting, DC are releasing IL-6 [71] and CD4+ T-cells are producing TGF-β [72] which jointly direct the onset of Th17 cells [61]. A recent study demonstrated a TGF-β-independent mechanism of Th17 cell onset with IL-6, IL-23 and IL-1β as sufficient actors [73]. Th17 cells secrete IL-17 which fosters the recruitment of neutrophils and macrophages to the infected tissues and IL-22 which prompt the release of anti-microbial peptide by IL-22R+ cells potentiating the response at mucosal sites like lungs and gut [74–76]. The high early influx of inflammatory cells driven by IL-17 to host tissues [76], the driving cause of auto-immunity is also the attribute of Th17 cells (Fig. 8).

Following pathogen eradication (i.e. loss of antigen) or tissue damage from persisting and detrimental immune response, the host coordinates a dynamic removal of immune effector cells in order to temper the ongoing inflammatory processes and restore homeostasis [58].

1.3.1.3 Immune regulation.

Unchecked Th1, Th2 or Th17 immune effector functions can lead to severe immunopathology thus prove disastrous for the host [77]. The observation that immune effector cells are rapidly dying by negligence following antigen removal and that this phenomenon can be antagonized in a timely manner by re-introduction of antigen supports a causal link between antigen clearance

and T-cell contraction [58,78]. Intriguingly, in instances of chronic infection where the invader antigens are still present, the contraction of the immune effector functions may also be initiated by the host as an attempt to restore homeostasis and avoid exacerbated and tissue damaging responses [79]. The emerging consensus is that the termination of immune responses begins ideally with invader clearance but additionally can be set on by untoward immune reactivity appearing as a threat to the host integrity [58,79,80]. In either case, the expanded T-cells need to be removed, bringing the immune system to a new homeostatic status. Early studies on T-cell apoptosis have proposed that programmed death of activated T-cells serves as a suicidal retrenchment mechanism which counterposes the proliferation of T-cells in a clonally specific manner [78,81–85]. Indeed, a significant increase of apoptotic T-cells coincides with the contraction phase of the immune response substantiating this assumption [78,82–85]. The decline of activated helper T-cells translates into a shrinking of the inflammatory events as per the disappearing antigen or the excessive tissue damage [78,81]. Additionally, DC which are short-lived become more prone to apoptosis as a result of lack of supportive immune effector cytokines or repeated antigen exposure in case of antigen persistence therefore considerably antagonizing the ongoing immune response [58,86,87]. As suggested by several studies, immune sentinels like DC might additionally take up apoptotic bodies from degenerating cells and as a result appear less maturation-sensitive (lower MHC, CD80, CD86) releasing immunoregulatory mediators and priming naïve T-cells in tolerogenic ways [88–90]. As a consequence, the strength of the T-cell priming by DC is diminished and primed T helper cells become more prone to anti-inflammatory responses and eventually predisposed to convert into immune suppressor T-cells or regulatory T-cells [87–90]. Finally, apoptotic bodies-loaded DC at a tolerogenic state potent source of regulatory cytokines (IL-10, TGF-β etc...) [87–90] and T-cell antagonizing enzymes like IDO (Indoleamine 2,3-dioxygenase) [91,92] would continue to support T-cell contraction and expansion of regulatory T-cells [88–92] so as to restore immune homeostasis (Fig. 8). Regulatory T-cells are instrumental in the restoration of immune homeostasis. From the production of inhibitory cytokines like IL-10, IL-35 and TGF-β, the granzyme/perforin-dependent cytolysis of effector cells, the metabolic disruption by IL-2 deprivation, cAMP-mediated inhibition, CD39/CD73-driven generation of immunosuppressive adenosine to the negative regulation of DC, these cells veto ongoing immune effector mechanisms [93].

It is now firmly established that there are CD4+CD25+ natural (or constitutive) and inducible (or adaptive) populations of regulatory T-cells, which probably have complementary and overlapping functions in immune homeostasis [94,95]. Natural regulatory T-cells congenitally generated from the thymus, expand concomitantly to the developing immune response and enter peripheral tissues where they repress self-reactive effector T-cells [94,95]. Foxp3 is the transcription factor that specifies the regulatory T-cell lineage [96]. Regulatory T-cells can also be generated in the periphery. Termed inducible regulatory T cells, they are separated into T regulatory 1 cells, T helper 3 cells and inducible Foxp3+ regulatory T-cells [95,97]. Indeed IL-10- or TGF-β-secreting regulatory T-cells, which are known as T regulatory 1 (Tr1) or T helper 3 (Th3) cells respectively, are generated from naive T-cells in the periphery after encounter with antigen presented by tolerogenic DC [95,97].

Human and murine Tr1 cells can be induced *in vitro* by chronic activation of CD4+ T-cells in the presence of IL-10, co-culture with tolerogenic DC or with immunosuppressive drugs, vitamin D3 and Dexamethasone [97,98]. These Tr1 cells are poorly proliferative with a low level of IL-2 production but have a higher propensity to release IL-10 and TGF-β amid an absence of IL-4 production [94,98,99]. Adoptively-transferred Tr1 cells show strong potency in resolving inflammatory disorders *in vivo* [99].

Thelper 3 cells are still poorly understood. They can be readily generated *in vitro* by high TGF-β levels and are also poised to release TGF-β [94,95,100,101]. Mainly found at mucosal sites where it is assumed that TGF-β is released by apoptotic cells, Th3 cells can rescue IL-2 deficient animals from autoimmunity by driving the onset of Foxp3+ Tregs [100,101]. Their strict separation from Foxp3+ regulatory T-cells is still ambiguous.

Foxp3+ regulatory T-cells induced in the periphery by the onset of Foxp3 expression in actively primed naïve T-cells represent the most studied class of inducible regulatory T-cells [94,95,97,102–105]. Generated by sub-immunogenic priming of naïve T-cells [106–108] via activation of the TGF-β pathway [103,109] and potentiated by IL-2 [110,111], Foxp3+ regulatory T-cells have been widely associated to the limitation of immune effector functions [110–112]. Upon binding of the TGF-β type II receptor and signal transduction to the cytoplasmic Smads which translocate to the nucleus and lead to the up-regulation of the transcription factor Foxp3, T-cells become potent immune suppressor cells [113].

Although poorly known, some other types of regulatory cells might be instrumental in the termination of immune responses namely, CD8+ regulatory T-cells [95,114–116] and IL-10-producing regulatory B-cells [117,118]. All these regulatory cells may act altogether [58,119–121] and in conjunction with several other mechanisms like upregulation of inhibitory receptors on immune cells and B-cell decay in the absence of helper T-cells [58] to provoke the loss of most of the immune effector cells and thereby restore immune homeostasis during the contraction phase. Other regulatory cells might contribute to immunological tolerance and are discussed in more details elsewhere [118–120,122–125]. The above description is tailored to give enough background for the present work and might not entails all mechanisms of immunological tolerance which could be at play for the restoration of immune homeostasis following an immune response. Importantly, some effector cells are retained after the contraction phase providing the raw material for immunological memory [126].

1.3.1.4 Immunological memory.

Not only does the immune system, its adaptive arm in particular, foster the removal/containment of infectious agents but ultimately prevent re-infection with the same pathogen [50,126]. It has now become clear that as the most important consequence of adaptive immunity, immunological memory enables the host to respond more rapidly and effectively to pathogens that have been encountered previously [50,126]. These subsequent responses to a given pathogen qualitatively differ considerably from the first or primary response [50,126]. This is noticeable in humoral immunity where plasma B-cells alter the characteristics of the antibodies produced at pathogen secondary or tertiary encounters as judged by a faster pool of highly specific antibodies [126]. This feature of memory i.e. preformed antibody-secreting B-cells poised to respond rapidly and fiercely is also present for cellular immune effectors T-cells [126]. These cells which similarly to memory B-cells are also long-lived are set to respond promptly to the antigens previously encountered [126]. CD4+ T cells play a central role in the design of this cellular memory response as they both direct the formation of CD4+ and CD8 + memory T-cells [126]. Of note, the artificial induction of protective immunity by vaccination, which stems from the principle of immunological memory, is arguably the most outstanding accomplishment of immunology [127].

1.3.2 Immunity and susceptibility to *Echinococcus multilocularis.*

An exciting but yet-complex issue is that of the immunological events governing the interactions between *E. multilocularis* larvae and their hosts. It has become clear over the past decade that upon entry of eggs, hatching and activation of oncospheres within the intermediate human or rodent host gut, a mucosal humoral response occurs as illustrated by a rise in IgG and principally IgA specific antibodies [128]. IgA responses in the mucosa have been hypothesized to target the parasite oncosphere by attenuating parasite-host interactions thus interfering with parasite feeding and survival on the one hand and on the other hand possibly playing a part in the tolerance necessary for the early oncosphere survival within the intestine [128]. The observation that following penetration of the intestinal epithelium by the activated oncospheres, the mucosal permeability decreases and the level of intestinal antibodies declines [128] argued for the first possibility, supporting a role for local antibodies production as a host attempt to control *Echinococcus multilocularis* oncospheres within the gut.

Next, passively drained via the portal vein or lymphatics [11], activated oncospheres elicit an IFN-γ-dominated response [129,130], illustrative of a Th1 immune response [131] favoring cell-mediated immunity (Fig. 9). The consensus now emerging is that the early Th1 immune effector functions are detrimental to the oncosphere establishment within the host and efficiently counter the development of the metacestode larvae [132–137]. Exciting but still not clinically effective approaches have stemmed from this observation, using pro-Th1 immune stimulants like BCG [138–140]and more specifically IL-12 [133] or interferons [132,137,141] to control the disease onset and progression.

In some limited instances following exposure to eggs, the disseminated oncospheres, principally lodged in the liver, initiate a metamorphosis into metacestodes [11] gradually shifting the host response towards a IL-4^{high}, IL-5^{high}, and IL-10^{high} dominated Th2 profile [142–145]. This humoral response of the host characterized majorly by IL-5 production [142] and increased IgE levels [146–148] amid a very infrequent hypereosinophilia [142,146,149,150] has now been epidemiologically and clinically associated to the condition known as Alveolar echinococcosis [142–146,149,151]. In fact, from observations on humans and murine studies, it is now comprehensively assumed that cell-mediated Th1 immune response is protective whereas humoral Th2 immune response is rather permissive during AE [152–154].

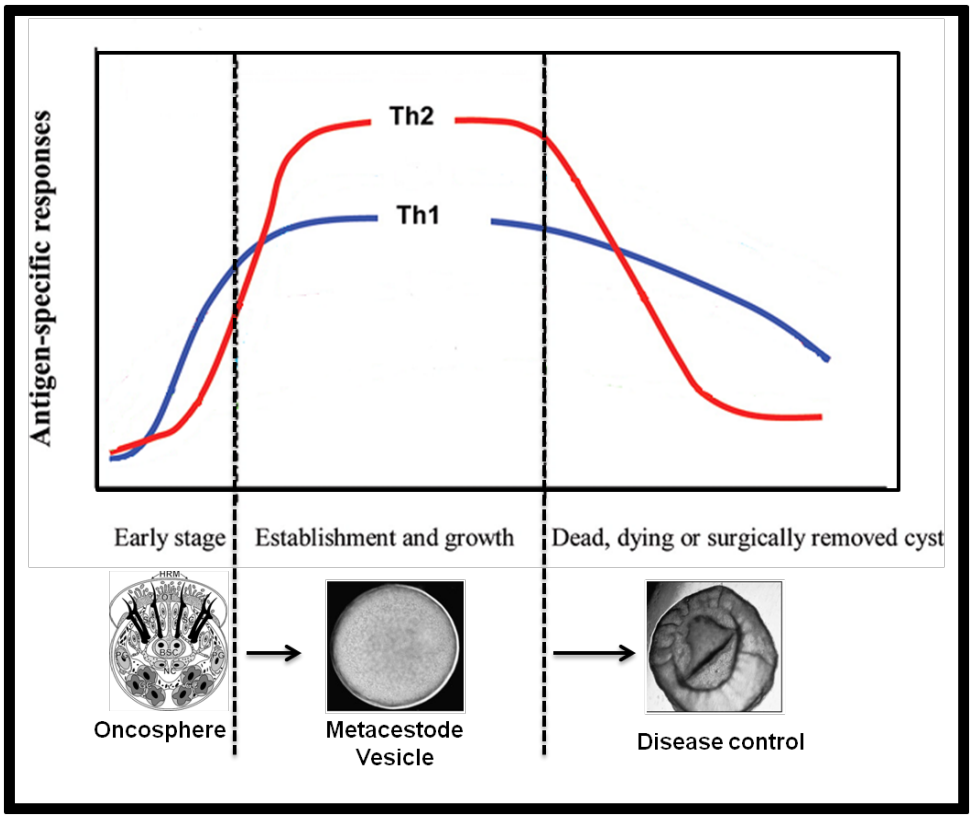


Figure 9: Kinetics of the intermediate host immune response to *Echinococcus multilocularis* larvae (adapted from [155])**.** Activated oncosphere triggers an early parasiticidal Th1 immune response that progressively switches to a permissive Th2 response coinciding with the metacestode vesicles formation and proliferation. The effective control of the disease and parasite removal/death associates with a parasiticidal Th1 immune effector functions.

In this respect, it was initially observed that the control of AE was dependent on cell-mediated immune responses as evidenced on the one side by the intense granulomatous infiltration observed in the periparasitic area of lesions in experimentally infected mice [156] and on the other side by the fact that Immunodeficient athymic nude [157] and SCID mice [158] as well as HIV-co-infected patients [159,160] exhibited high susceptibility to infection and disease. Then, by using transgenic mice a central role for CD4+ T-cells was unveiled in the control of murine AE [161]. Accordingly, a comparison of lesions from susceptible vs. resistant human patients unambiguously showed an association of CD4+ T-cells with abortive lesions and CD8 + T-cells

with progressive masses [162]. Finally, attempts to artificially boost Th1 immune effector functions ameliorated AE prognosis in humans and mice altogether pointing at type 1 immune effector functions in particular as instrumental to control AE [132–134,136–140] contrarily to permissive Th2-related humoral effector functions [142,143,145,151,161,163,164].

In some rodents, the metacestodes become fertile and produce protoscoleces that are released within the definitive carnivorous host when the latter devours the infected rodent(s) [11]. Protoscoleces instruct a strong mucosal IgA [165] which strongly depends on IL-4 and innate cytokines like IL-10 and IL-6 [166] characteristic of the Th2 immune response within the gut of the definitive hosts [165]. This mucosal response, as shown for intestinal helminths, fosters the worm containment and expulsion [167].

As AE progresses, a superimposition of a regulatory immune response has been demonstrated in the intermediate human and murine hosts (Fig. 8) [144,145,163,164,168]. This regulatory response probably blunts the efficiency of the immune response and thereof fosters the parasite growth and development towards a chronic stage of infection [152,153]. Regulatory cytokines like TGF-β and IL-10 abound in the periparasitic environment and circulate at higher rates in the bloodstream of infected patients supporting the assumption of such a regulatory network during AE [151,163,164,168]. This is further consolidated by the observation that AE-infected hosts exhibit a reduced immune reactivity when compared to healthy controls [162,163,169–172]. Although larvae somatic products would clearly account in part for the host immunomodulation observed during AE [173–176], current evidences point at a central role for *E. multilocularis* larvae excretory/secretory (E/S) products in this process. First, the observation that following chemotherapy or spontaneous calcification, dying/dead *E. multilocularis* larvae failed to maintain the alterations of the host immune response associated with progressive AE [170,177,178] resulting in a reversion of the permissive Th2 into a parasiticidal Th1 [141,179] and ultimately, the ability of collected E/S products from the parasite larvae to alter the host immune effector cells similarly to the full larvae [169,180] altogether compels to acknowledge the parasite larvae E/S products as the major source of *E. multilocularis* immunomodulators.

However, the influence of *E. multilocularis* E/S products on the host immune response has yet to be investigated. Since the immune axis DC-CD4+ T-cell is central to the onset of host immune responses [54,181], there is consequently an urgent need to study the effects of *E. multilocularis*

larvae E/S products on this axis. If not for a recent study using crude extracts of metacestode vesicles to stimulate human DC *in vitro* [182] where the latter failed to mature in response to the parasite products, very little is currently known in this regard. In this study [182], and a couple of other studies using the late protoscoleces [183–185] for studying parasite-driven host alterations which govern the infection of the intermediate hosts by *E. multilocularis* larvae, one could not exclude the presence of contaminating host remnants within parasite material. Protoscoleces are arguably the most accessible *Echinococcus multilocularis* larvae [11,22]. This accessibility would therefore explain their frequent use in host stimulation assays ignoring their natural occurrence at a dormant state within fertile cysts of some infected rodents unable to directly interact with the host interior milieu [11,22]. Clearly, the initial point of contact between protoscoleces and the carnivorous host is the lumen of the gut [11,22]. A breakthrough came with the work of Spiliotis and collaborators [186] who coined the establishment of sophisticated cultivation systems enabling the isolation and axenic maintenance of each of the parasite larvae.

1.4 Maintenance of *E. multilocularis* larvae in the laboratory.

E. multilocularis offers a unique model for *in vitro* maintenance of parasitic cestodes in the sense that its larvae exhibit a rapid growth, and naturally colonize rodents in contrast to other cestodes like *E. granulosus* (dog tapeworm) or *T. solium* (pork tapeworm) [186]. The development of *in vitro* cultivation systems for *E. multilocularis* larvae were undertaken in the 1990s by Hemphill and Gottstein [187] and Jura *et al* [188] using co-cultivation of host feeder cells and metacestode vesicles. These systems failed to provide a suitable platform for the assessment of the parasite larvae E/S products. Spiliotis and collaborators [189] then achieved a major breakthrough by defining a liquid medium system for long term maintenance of *E. multilocularis* metacestodes. Using culture medium conditioned with host feeder cell metabolites, an axenic maintenance of the metacestode vesicles could be achieved for the first time [189]. Interestingly, by digesting the cellular matrices and releasing cells from the germinal layer of the metacestodes, this system permitted the isolation of parasite primary cells. The parasite cells proved to be a suitable model for the post-oncospheral development towards the metacestode vesicles, as judged by their ability to completely regenerate into infective metacestodes *in vitro* [190] and *in vivo* (my own observation). Furthermore, transcriptome analyses demonstrated that regenerating primary cells

express factors, originally described as being oncosphere-specific [191]. The axenization procedure developed in this system also proved to be useful for the decontamination of *E. multilocularis* protoscoleces following isolation [186]. Thanks to these cultivation systems, the *E. multilocularis* genome is now completed [192] and the larval stage-specific transcriptome is currently underway. Equipped with both these cultivation systems for isolation of host-free parasite material [186] and the wealth of knowledge provided by the *E. multilocularis* genome sequencing project [191], I initiated the present work to try understand the basis of the host immunomodulation by *E. multilocularis*, the role of the parasite E/S products in this process and the molecular means underlying such a role.

1.5 Objectives.

Alveolar echinococcosis is a life-threatening disease in humans. A striking feature of the disease is the asymptomatic persistence of parasitic larvae in the host for decades, almost like a perfect transplant. A strong regulation of the host immune system has therefore been suggested. This is supported by the observation that immune cells of AE patients have an impaired reactivity. Understanding how *E. multilocularis* larvae modulate the host immune response could prove valuable not only for developing novel anti-AE intervention measures but could ultimately provide novel means to control detrimental immune responses and associated pathologies (allergies, autoimmunities, transplant rejections). Given the scarcity of information on the molecular bases of the host immunomodulation by *E. multilocularis*, the present work sought to:

- characterize the effects of E/S products of *Echinococcus multilocularis* larvae on DC

- characterize the effects of E/S products of *Echinococcus multilocularis* larvae on CD4+ T-cell polarization with regards to regulatory T-cell expansion

- identify and characterize candidate proteins released by the parasite larvae that could influence the host immune system.

In combination, these objectives are meant to help understand how *E. multilocularis* through its larvae E/S products affect the host immune response.

2-MATERIALS AND METHODS

2.1 Material.

2.1.1 Equipment.

Developer for radiographic films (Agfa Graphics Germany, Duesseldorf)

ELISA reader Multiscan Ex Primary EIA V.2.1-0 (Thermo)

FACSCalibur ™ (Beckton Dickinson)

Gel documentation system: MidiDoc (Herolab, Wiesloch)

Gel electrophoresis camber (Bio-Rad, Muenchen)

Heating block: DB-3 (Techne, Cambridge, UK), Heizblock (Liebisch, Bielefeld)

Heating stirrer: Typ RCT (Jahnke & Kunkel, Staufen i. Br.)

HERA safe (Heraeus, Thermo Electron, Langenselbold)

Incubator: Heraeus (Thermo Electron, Langenselbold)

Leica IRB (Leica Microsystems, Wetzlar)

Macs columns adapter (Miltenyi Biotec)

Mikro 200 table centrifuge (Hettich, Tuttlingen)

Micro scales: R160P (Sartorius, Goettingen)

Microtom 2065 (Leica Microsystems, Wetzlar)

Mini-PROTEAN II System (BioRad, Muenchen)

MoFlo® high-speed sorter (Cytomation)

MSC advantage laminar flow (Thermo Scientific, Braunschweig)

NanoDrop 1000 (PeqLab Biotechnologie, Erlangen)

Neubauer chamber (0.1 mm, 0.0025 mm2; (Hartenstein, Wuerzburg)

Nuaire laminar flow (Nuaire)

Power Pack P24 and P25 (Biometra, Goettingen)

Refrigerated Centrifuge 3K30 (Sigma-Aldrich, Muenchen)

Research Plus pipette set (Eppendorf, Hamburg)

Scale: 10-1000 g (Sartorius, Goettingen)

Selectomat S2000 autoclave (Muenchner Medizin Mechanik, Muenchen)

G24 shaking incubator (New Brunswick Scientic, Edison, N.J., USA)

Spectrophotometer U-2000 (Hitachi, NY, USA)

Tecan ELISA reader (Tecan Group, Crailsheim)

T-Gradient Thermocycler (Biometra, Goettingen)

TH30 shaking incubator (Hartensein, Wuerzburg)

Trio-ThermoblockTM heated lid (Biometra, Goettingen)

Trio-ThermoblockTM oil (Biometra, Goettingen)

UV transilluminator (UVT-28MP, HeroLab)

Vacuum pump (ILMVAC, Ilmenau)

Vortex mixer: L46 (Gesellschaft fuer Laborbedarf, Wuerzburg)

2.1.2 Consumables.

0.2 µm Syringe Filters (SARSTEDT)

96-well plates (Sarstedt, Nuembrecht)

12-well plates (Nunc, Roskilde, Denmark)

24-well plates (Nunc, Roskilde, Denmark)

96-well plates (Sarstedt, Nuembrecht)

Cell Strainer, 70µm (BD Biosciences)

Filtropur Bottle Top Filters (BT50, SARSTEDT)

Macs columns, MS (Miltenyi Biotec)

Microtom sample adapter (Rudens Plastinindusti, Hestra, Sweden)

Nitrocellulose membran (GE Healthcare, Muenchen)

PD-10 column (GE Healthcare, Muenchen)

Radiographic film (Fujifilm Europe, Duesseldorf)

Safe-Lock Tubes 0,5, 1,5 and 2 ml (Eppendorf, Hamburg)

Semi-micro cuvettes (Sarstedt, Nuembrecht)

Sterilfilter (Nalgene, New York, USA)

Sterile tubes, 15 and 50 ml (Greiner, Nuertingen)

Superfrost Plus slides (Thermo Scientific, Braunschweig)

Syringes and canula, sterile (Braun Melsungen AG, Melsungen)

Tissue culture flasks 25 cm2 (Sarstedt, Nuembrecht)

Tissue culture flasks 75 cm2 (Sarstedt, Nuembrecht)

Tissue culture flasks 175 cm2 (Sarstedt, Nuembrecht)

Tissue culture inserts, 1µm (Greiner Bio-One)

Vacutainer ® Blood Collection Tubes (BD Biosciences)

Whatman blotting paper (GE Healthcare, Muenchen)

X-Ray film HyperfimTM -MP (Amersham, Braunschweig)

2.1.3 Chemicals, media, commercially available kits and enzymes.

7-Amino-actinomycin D (7-AAD, BD Pharmingen^{TM})

Agarose (ROTH, Karlsruhe)

Albumin fraction V (pH 7) Blotting grade (BSA) (AppliChem, Darmstadt)

Ammonium peroxodisulfate (APS), (Carl Roth, Karlsruhe)

Aqua demin. (VE-water)

Ampuwa/double distilled H2O (Fresenius, Bad Homburg)

Ampicillin (Sigma-Aldrich, Muenchen)

Annexin V staining solution (BD Biosciences)

Antarctic Phosphatase (New England Biolabs Inc., Schwalbach)

AS601245 (Calbiochem, Merck, Darmstadt)

Bacto agar (Difco Laboratories, Augsburg)

Bathocuproine disulfonic acid (Sigma-Aldrich, Muenchen)

ß-Mercaptoethanol (Sigma-Aldrich, Muenchen)

Chloroform (Merck, Darmstadt)

Carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Invitrogen)

CD25 MicroBead Kit (Miltenyi Biotec)

Color Plus Prestained Protein Marker (10- 230 kDa) (NEB, Schwalbach)

Colorimetric Cell Proliferation ELISA BrdU (Roche, Grenzach)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Muenchen)

DNeasy Isolation Kit (Qiagen)

dNTP lyophilised (ROTH, Heidelberg)

DMEM- GlutaMAXTM (DMEM) (Invitrogen, Darmstadt)

EasySepTM Mouse CD4+ T cell Enrichment Kit (STEMCELL™ Technologies)

Fetal Bovine Serum (FBS, Biochrom)

Fetal Calf Serum (PAA Laboratories)

LPS (Sigma)

Luria-Broth (LB; Invitrogen, Darmstadt)

L-Cystein (Sigma Aldrich, Muenchen)

L-Glutamine (Sigma)

Mouse IFN-γ ELISA Set (BD OptEIA™ , BD Biosciences)

Mouse IL-6 ELISA Set (BD OptEIA™ , BD Biosciences)

Mouse IL-10 ELISA Set (BD OptEIA™ , BD Biosciences)

Mouse IL-12 (p70) ELISA Set (BD OptEIA™ , BD Biosciences)

NucleoSpin® Extract II (Macherey-Nagel, Dueren)

NucleoSpin® Plasmid Kit (Macherey-Nagel, Dueren)

Oligonucleotides (Sigma, Muenchen)

Omniscript® RT Kit (QIAGEN, Hilden)

OVA peptide (323-339, Sigma)

pBAD/TOPO®ThioFusionTM Expression Kit (Invitrogen, Darmstadt)

Penicillin (Sigma)

Penicillin/Streptomycin (Invitrogen, Darmstadt)

Phusion® DNA Polymerase (New England Biolabs)

Pierce® BCA Protein Assay Kit (Thermo Scientific, Braunschweig)

Pierce® ECL westen blot substrate (Thermo Scientific, Braunschweig)

Ponceau S (Sigma)

ProBond™ Nickel-Chelating Resin (Invitrogen, Darmstadt)

Proteinase K (AppliChem, Darmstadt)

pSecTag2/Hygro A mammalian expression vector (Invitrogen)

Restriction enzymes (New England Biolabs Inc., Schwalbach)

Rotiphorese® Gel 30 (37,5:1) (Carl Roth, Karlsruhe)

Rotiphorese® Gel 40 (Carl Roth, Karlsruhe)

RPMI-1640 (GIBCO BRL)

SB431245 (Sigma)

SOC (Invitrogen, Darmstadt)

Streptomycin (Sigma)

T4 DNA Ligase (New England Biolabs Inc., Schwalbach)

Taq-Polymerase (New England Biolabs Inc., Schwalbach)

Tavanic® (Tava, active component levofloxacin, 5mg/ml) (Aventis)

Technovit® 8100 (Heraeus Kulzer Technik, Wehrheim)

TEMED (N,N,N',N'-Tetramethylethylendiamin) (Merck, Darmstadt)

TGF-β1 (Human, R&D Systems)

TGF-β1 (Porcine, R&D Systems)

Treg Detection Kit, mouse (Miltenyi Biotec)

Triton® X-100 (Sigma-Aldrich, Muenchen)

Trizol® Reagent (Invitrogen, Darmstadt)

Trypan Blue (Biochrom, Berlin)

Trypsin/EDTA (0,05%/0,02% (w/v)/ PBS w/o Ca2+, Mg2+) (Biochrom, Berlin)

Tween® 20 (Merck, Darmstadt)

2.1.4 Antibodies.

2.1.4.1 Primary antibodies.

Anti-CD3 (eBioscience)

Anti-CD11c (Caltag Laboratories)

Anti-CD19 (BD Pharmingen^{TM})

Anti-CD86 (eBiosciences)

Anti-MHC II (eBioscience)

Anti-TGF-β (1D11, R&D Systems)

Normal Rabbit IgG (Santa Cruz Biotechnolocies)

2.1.4.2 Secodary antibodies (peroxydase-conjugated).

Anti-mouse-IgG (Jackson ImmunoResearch)

Anti-rabbit-IgG (Jackson ImmunoResearch)

2.1.5 Softwares, online bioinformatics tools and databases.

Bepipred Linear Epitope Prediction based Tool (tools.Immunoepitope.org)

BD CellQuest™ (BD Biosciences)

BioEdit 7.1.3

Excel 2007

Flowjo (Treestar, USA)

GraphPad Prism® 6.0.0

GeneDB (http://www.genedb.org/)

ImageJ (http://rsb.info.nih.gov/ij/)

MEGA 4 (Molecular Evolutionary Genetics Analysis)

SMART (http://smart.embl-heidelberg.de/)

Uniprot (http://www.uniprot.org/)

2.1.6 Animals.

Animals were bred in the animal facilities of the Institute for Hygiene and Microbiology (IHM) and of the Institute for Virology and Immunobiology (VIM), University of Würzburg, Würzburg, Germany, or at Charles River.

Meriones unguiculatus

Commonly termed Jirds or gerbils, they represent highly susceptible species of rodent to experimental infection with *E. multilocularis* larvae and were used throughout these studies for *in vivo* maintenance of the parasite.

C57BL/6 mice

An inbreed strain of *Mus musculus*: C57BL subline number 6 or commonly termed Black 6.

OT-II C57BL/6 mice

These mice are transgenicly altered so that the T-cell receptor of CD4 T-cells is specific for the recognition of OVA peptide (amino acids 323-339). They were kindly provided by Francis Carbone, Melbourne, Australia [193].

OT-II×RAG-1(-/-) C57BL/6 mice

These are transgenic mice resulting from the crossing between OT-II mice and RAG-1(-/-) mice (devoid of mature B and T cells), giving rise to mice having for only mature CD4+ T cells those whose TCR is altered to recognize OVA peptide. These mice were a generous gift from Thomas Winkler, Erlangen, Germany [194].

2.2 Effects of E/S products of *Echinococcus multilocularis* larvae on murine DC.

2.2.1 Ethics statement.

All experiments were in compliance with European and German regulations on the protection of animals (*Tierschutzgesetz*). 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).

2.2.2 *In vivo* maintenance of *Echinococcus multilocularis* larval tissue.

All starting with *E. multilocularis* metacestode tissue, isolated from naturally infected hosts (small rodents or human patients), the larvae can be maintained for years in laboratory rodents (*Meriones unguiculatus*) through serial intraperitoneal passages as described previously [195].

2.2.3 *In vitro* cultivation of *E. multilocularis* larval tissue.

2.2.3.1 Isolation of parasite homogenate.

Parasite tissue was extracted with a fresh pair of tweezers and scalpel from the viscera of intraperitoneally infected Jirds (*Meriones unguiculatus*) or infected human tissues. In order to render the material suitable for *in vitro* maintenance, the material was homogenized and sieved as previously described [186]. The parasitic tissue homogenate obtained was subsequently used for the *in vitro* purification/expansion of the various parasite larvae or alternatively injected into jirds´peritoneum for *in vivo* propagation (section 2.2.2).

2.2.3.2 Cultivation media.

2.2.3.2.a Co-cultivation (DMEM10).

Dulbecco´s Modified Eagle´s Medium, 4.5g glucose/L (DMEM + Glutamax^{TM}, GIBCO) supplemented with 10% Fetal Bovine Serum Superior (Biochrom AG), 100ug/ml Penicillin/streptomycin (PenStrep solution, Biochrom AG) and 20ug/ml Levofloxacin (Tavanic, Sanofi-Aventis, Deutschland GmbH).

2.2.3.2.b Axenic maintenance for E/S collection (DMEM10$_{redox}$).

Dulbecco´s Modified Eagle´s Medium, 4.5 g glucose/L (DMEM + Glutamax^{TM}, GIBCO) supplemented with 10% Fetal Bovine Serum Superior (Biochrom AG), 100ug/ml Penicillin/streptomycin (PenStrep solution, Biochrom AG), 20ug/ml Levofloxacin (Tavanic, Sanofi-Aventis, Deutschland GmbH), 143 µM ß-mercapthoethanol (Sigma-Aldrich, cat. M6250), 10 µM Bathocuproine disulfonic acid (Sigma, cat. B-35) and 100 µM L-Cysteine (Sigma, cat. C-1276).

2.2.3.2.c Axenic cultivation (A4).

To generate A4 medium, DMEM10redox (Section 2.2.3.2b) was used to grow rat hepatoma cells (10^6 cells in 100ml medium for 7 days). The conditioned DMEM10redox was then collected, filtered over a bottle top filter (Filtropur BT50, SARSTEDT) and store at -20^{o}C until use.

2.2.3.3 Generation, expansion and maintenance of parasite larvae.

Upon obtention of parasite homogenate from infected hosts (section 2.2.3.1), and three washing steps in PBS (1 X):

- Protoscoleces were isolated as previously described [196]. Briefly, the parasite homogenate was filtered once through a nylon mesh of 150 μm pore size, thus separating protoscoleces from large pieces of metacestode tissue. The flow through was then filtered through a nylon mesh of 30 μm pore size, retaining the protoscoleces and allowing the single cells and small cell clumps in the flowthrough to be washed away. Protoscoleces were then washed off the nylon mesh with sterile PBS and separated from equally sized metacestodes by microscope-aided, manual picking with a pipette tip. For maintenance, protoscoleces were resuspended in $DMEM10_{Redox}$ and sealed in Nitrogen filled Ziploc freezer bag prior to incubation at 37oC, 5%CO2.The medium was replaced twice per week until subsequent applications.

- Metacestodes were expanded by resuspending the homogenate in DMEM10 with 10^6 host feeder cells (Rat Reuber RH- hepatoma cells, ATCC no. CRL-1600) followed by incubation at 37oC, 5%CO2 with frequent medium and feeder cells replacement. 6-8 weeks post maintenance in co-culture, metacestode vesicles were obtained and thoroughly washed. Broken and tiny cysts (<5mm) were selected out and the remaining cysts were transferred to DMEM10redox for axenic maintenance with medium change twice per week.

- Primary cells were isolated from the germinal layer of axenically maintained metacestode vesicles as previously described [186,190]. Metacestode vesicles of at least 6-8 weeks of age and kept under axenic conditions were thoroughly washed in PBS (1 X). The cysts were then broken and the hydatid fluid released was washed away with PBS. The broken cysts were then digested for 30 minutes in 8 volumes of Trypsin/Ethylenediamine tetraacetic acid (Biochrom, cat. L2143) at 37oC with intermittent agitation to favor germinal layer cells detachment [186]. Following

digestion, the cyst lysates were filtered through a nylon mesh of 150 µm pore size, selectively retaining large pieces of metacestode tissue. The flow through was then filtered through a nylon mesh of 30 µm pore size, separating the diffusing parasite germinal cells from remnants of the cyst carbohydrate rich layer. For maintenance, primary cells were resuspended in $DMEM10_{Redox}$ and sealed in nitrogen filled Ziploc freezer bag prior to incubation at 37oC, 5%CO2.The medium was replaced twice per week until subsequent applications.

2.2.4 PCR-based assessment of host cell remnants.

After one week of axenic cultivation (i.e. in DMEM10redox), the different parasite larvae were analyzed for contamination with jird remnants. In doing so, chromosomal DNA was isolated from each larva as well as from liver tissue of a non-infected Jird using the DNeasy isolation kit (Qiagen). The total amount of DNA was assessed on a UV spectrophotometer (NanoDrop® ND-1000, PEQLAB) and 10ng was used per PCR reaction. Part of the parasite specific gene elp (erzin-radixin-moesin-like; [197]) was amplified using the primers Em10-15 (5´-TCC TTA CCT TGC AGT TTT GT- 3´) and Em10-16 (5´-TTG CTG GTA ATC AGT CGA TC- 3´). To test host-DNA contamination, part of the mongolian jird β-tubulin encoding gene was amplified (137) using primers Tub12-Up (5´-CAA CGT CCA ACA CCG AGT CA-3´) and Tub12-ST (5´-CTG GGC AGT GCG GCA ACC A-3´) [198]. PCRs were performed using Taq Polymerase (NEB) with 5 pmoles of each primer and a protocol of 30 cycles of 30 s at 94°C, 30 s at 58°C, and 2 min at 72°C. PCR-products were then separated on a 1.5% agarose gel and stained with ethidium bromide.

2.2.5 Normalization of *Echinococcus multilocularis* larvae biomasses.

Once qualitatively assessed for purity thus absence of host remnants (section 2.2.4), 40ml of metacestode vesicles were processed for germinal cells isolation. $1/12^{th}$, $1/6^{th}$ and $1/3^{rd}$ of the isolated cells were seeded in axenic culture (section 2.2.3.2.c) so as to monitor the *in vitro* kinetics of regeneration into metacestode vesicles. The amount of cells capable of forming *de novo* metacestodes within a time frame comparable to the *in vivo* situation was determined. This amount of cells was used as reference for the parasite biomass to be used for stimulation assays.

An equivalent biomass of *in vitro* cultivated metacestode vesicles and protoscoleces was determined by comparing their level of beta-actin expression to that of the reference amount of parasite cells. Briefly, upon a week of axenic cultivation, material of all three larval stages was passed through a pipette tip and washed in PBS (1X). The various homogenates were then treated with 50 µl of 2 X STOP mix (2ml 0.5M Tris-HCl pH 6.8, 1.6ml glycerol, 1.6ml 20% SDS, 1.4ml H2O, 0.4ml 0.05% (w/v) bromophenol blue, 7 µl ß-mercaptoethanol per 100 µl) and boiled for 10 min at 100^{o}C. 10µl of each larvae protein mix were separated by SDS-PAGE and analyzed by Western blotting using an antibody directed against ß-actin (Cell signaling technology®, cat. No 4967) of a variety of metazoan organisms. Images were subsequently analyzed for the relative expression of ß-actin using the Image-J program (http://rsb.info.nih.gov/ij/ ; [199]). The relative expressions transcribed as values of area under the curve (AUC) were used to normalize the β-actin content from one sample to another. Additionally, the total protein content from the normalized amount of each larval stage was determined. Briefly, material from each larvae were lysed using a high-speed benchtop homogenizer (FastPrep®-24, MP Biomedicals) and the total amount of protein determined using the bicinchoninic acid assay (Pierce ® BCA Protein Assay Kit, ThermoScientific, prod # 23228) as per the manufacturer instructions.

2.2.6 Preparation of *Echinococcus multilocularis* E/S products.

A comparable amount of axenically cultivated parasite material, normalized for β-actin/protein content, from each of the three larval stages was then extensively washed with PBS (1X) and resuspended in DMEM10redox under axenic conditions (section 2.2.3.3). After 48 h of culture, the supernatant containing the larvae E/S products was collected and filter through a 0.2 µm-membrane (Filtropur S filter, SARSTEDT). The total amount of proteins was then determined using the bicinchoninic acid assay (Pierce ® BCA Protein Assay Kit, ThermoScientific, prod # 23228). The protein concentrations across the collected larval E/S products were normalized using ddH2O then the samples aliquoted and stored at -80^{o}C until use.

2.2.7 Generation of murine bone marrow-derived dendritic cells.

Dendritic cells were obtained by GM-CSF-driven differentiation of mice bone marrow precursor cells as previously described [200]. Briefly, C57BL/6 mice (Charles River/Wiga, Sulzfeld, Germany) aged 6-14 weeks and bred within the animal facility of the Institute of Virology and Immunobiology, University of Würzburg, under specific pathogen-free conditions were sacrificed using CO_2 asphyxiation. Femurs and tibiae were removed and purified from the surrounding muscle tissue. Thereafter the marrow were flushed with PBS (1X), resuspended by gently pipetting and washed once in medium. It should be cautioned here, as previously defined [200], that the medium used was R10 medium composed of RPMI-1640 (GIBCO BRL) supplemented with 100U/ml Penicillin (Sigma), 100µg/ml Streptomycin (Sigma), 2mM L-glutamine (Sigma), 50µM ß-mercaptoethanol (Sigma) and 10% heat-inactivated and filtered (0.22 µm, Millipore) fetal calf serum (FCS, PAA Laboratories). Once resuspended, the bone marrow precursor cells were counted using the trypan blue (Biochrom) exclusion test on a bright-line Neubauer counting chamber. 2 x 10^6 precursor cells were cultured in R10 medium supplemented with 10% GM-CSF-containing supernatant from a previously defined cell line [201] as already performed [200]. At day 8, non-adherent cells representing at a high frequency newly differentiated DC (70-89% CD11c+) were harvested, washed once in R10 medium prior to stimulation assays.

2.2.8 Isolation of murine splenocytes.

Single cell suspensions were obtained from the spleen of C57BL/6 mice by mechanically squeezing the tissue with glass slides in cold PBS (1X) and filtered through a 70µm nylon cell strainer (BD Biosciences). Red blood cells in the filtrate were lysed with 1.4% NH4Cl for 5 minutes at 37^{o}C. The filtrate was then washed in R10 medium and the splenocytes obtained were counted using the trypan blue (Biochrom) exclusion test on a bright-line Neubauer counting chamber.

2.2.9 Human red blood cells preparation.

Blood was drawn from 2 volunteers in (BD Vacutainer® Blood Collection tubes, BD Biosciences) by a physician upon informed consent. The heparinized blood samples were gently spun at 300g for 10 minutes, the plasma discarded and the whole blood cells were resuspended in PBS (1/10) prior to hemolytic tests.

2.2.10 Assessment of the influence of *Echinococcus multilocularis* larvae E/S products on host cells.

2.2.10.1 Dendritic cell stimulation.

A comparable amount of cultivated parasite material from each larval stage was washed thrice in R10 medium then used throughout the stimulation process. Tissue culture inserts (1µm, Greiner Bio-One) with or without larvae were added to DC cultures (10^6 cells /ml) in R10 medium and kept at 37^{o}C under 5% CO2 for different time points before analysis. Alternatively, collected E/S products were used instead of full larvae. In parallel, LPS (0.1µg/ml, *E. coli* 0127:B8) was used to generate a positive control for DC maturation whereas UV-irradiation of DC (9000mW/cm2, 313nm) stood as a reference for apoptosis. For restimulation experiments, inserts containing parasite larvae were removed 24 hours post stimulation, DC were harvested and re-plated at an equal number of living cells ($5x10^5$ cells / ml) in R10 medium with or without LPS (0.1µg/ml, *E. coli* 0127:B8) for additional 48 hours. Upon completion, DC viability was assessed by trypan blue exclusion test (Biochrom) on a bright-line Neubauer counting chamber and the cells stained for flow cytometric analyses.

2.2.10.2 Red Blood cell stimulation (Hemolytic test).

A hemolysis assay was used to determine the potential of the parasite secretions to lyse red blood cells from humans with heparinized red blood cells being suspended in PBS. Erythrocytes were incubated in the presence of collected parasite secretions in comparison to $DMEM10_{redox}$ (negative control) and an equal volume of H_2O (positive control). Then, after 48 hours of incubation at 37^{o}C, the blood samples were centrifuged at 1000g for 5 min, and the supernatants

were separated from the pellet. The absorbance was measured at 540 nm. The hemolytic activity was defined as per Valdez et al [202] by the ratio: Sample absorbance / H_2O absorbance.

2.2.10.3 Splenocyte stimulation.

With reference to DC stimulation assays, a comparable amount of cultivated metacestode vesicles washed thrice in R10 medium was used to stimulate murine splenocytes. Tissue culture inserts (1µm, Greiner Bio-One) with or without the parasite cysts were added to splenocytes cultures (10^6 cells /ml) in R10 medium and kept at 37^oC under 5% CO2 for up to 72 hours. Upon completion, cell viability was assessed by trypan blue exclusion test (Biochrom) on a bright-line Neubauer counting chamber and an equal number of living splenocytes stained for flow cytometric analyses.

2.2.10.4 Flow cytometry.

2.2.10.4.a Dendritic cells.

To assess the level of maturation, DC surface staining was performed using fluorochrome-conjugated antibodies (anti-mouse) directed against the surface lineage marker CD11c (PE-Cy5.5, Caltag Laboratories), the major histocompatibility complex class II molecules MHC II (PE, eBioscience) and the costimulatory CD86 (FITC, eBioscience). After 30 min of incubation at 4^oC in the dark with a cocktail of CD11c, MHC II and CD86 antibodies, DC were washed twice with FACS buffer (1x PBS supplemented with 3% heat-inactivated and filtered FCS and 0.1%NaN3) and acquired on a cytometer (FACSCalibur^{TM}, Beckton Dickinson) equipped with CellQuest software. Results were further analyzed on FlowJo software (Tree Star, USA).

To monitor the level of DC apoptosis, annexin-V (FITC, BD Pharmingen) targeting phosphatidylserine and the nucleic acid stain, 7-AAD (BD Pharmingen) were used. DC were resuspended in 50µl of 1 x annexin-V binding buffer (BD Pharmingen). Next, 5 µl of 7-AAD and 2 µl of Annexin V-FITC were added to the tubes before incubation for 15 minutes at room temperature. DC were then resuspended in 200 µl of 1 x annexin-V binding buffer and acquired

on a cytometer (FACSCalibur^{TM}, Beckton Dickinson) equipped with CellQuest software. Results were further analyzed on FlowJo software (Tree Star, USA).

2.2.10.4.b Splenocytes.

Splenocytes surface staining was performed using fluorochrome-conjugated antibodies (anti-mouse) directed against the B-cell surface lineage marker and T-cell exclusion marker CD19 (PE-Cy5, BD Pharmingen).After 30 min of incubation at 4^{o}C in the dark with CD19 antibody, splenocytes were washed twice with FACS buffer (1x PBS supplemented with 3% heat-inactivated and filtered FCS and 0.1%NaN3) and acquired on a cytometer (FACSCalibur^{TM}, Beckton Dickinson) equipped with CellQuest software. Results were further analyzed on FlowJo software (Tree Star, USA).

2.2.10.5 Measurement of cytokine release by dendritic cells.

Following DC stimulation, the production of IL-6, IL-10 and IL-12p70 was assessed in the culture supernatants using sandwich enzyme-linked immunosorbent assays (ELISA, OptEIA kits, BD Pharmigen) according to the manufacturer´s instructions with the following pre-defined detection limits (39pg/ml, 19pg/ml and 19pg/ml for IL-6, IL-10 and IL-12p70 respectively).

2.2.11 Statistical analysis.

All results were expressed as mean ±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. All statistical analyses were performed with STATISTICA version 8.0.725.0 (Statsoft GmbH).

2.3. Influence of E/S products of *E. multilocularis* larvae on Foxp3+ Treg expansion.

2.3.1 Ethics statement.

All experiments were in compliance with European and German regulations on the protection of animals (*Tierschutzgesetz*). 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).

2.3.2 Parasite material.

For *in vivo* assays, metacestodes were obtained from infected Mongolian jirds (*Meriones unguiculatus*). The recovered parasite homogenates were washed thrice in PBS (1X) then transferred to DMEM10redox for axenic maintenance with medium change twice per week for up to 10 days. The complete removal of host contaminants was assessed by PCR (section 2.2.4). The host-free parasite homogenates were then washed in PBS (1X), separated in aliquots of 5000 acephalic cysts resuspended in a total volume of 500 µl PBS (1X) to be used for intraperitoneal injections. An equal volume (500 µl) of the carrier solution PBS (1X) was used in parallel for mock injections. Parasite preparations from 5 unrelated Jird infections were used so as to include any eventual parasite-related variation in the analysis. For *in vitro* assays, E/S products collected from *in vitro* cultivated metacestode vesicles (section 2.2.6) were used in comparison to those of *in vitro* cultivated primary cells.

2.3.3 Mice.

All mice were bred and maintained at the Institute for Hygiene and Microbiology and the Institute of Virology and Immunobiology of the University of Würzburg according to the Local Ethics committee guidelines. C57BL/6 mice were purchased from Charles River. OT-II mice (TCR transgenic mice with TCR specific for I-Ab:OVA 323–339) were kindly provided by Francis Carbone, Melbourne, Australia. OT-II mice were crossed with RAG-1(-/-) mice (devoid of mature B and T cells), a generous gift from Thomas Winkler, Erlangen, Germany. C57BL/6

wild type mice were separated in groups of five (parasite injection, one animal for each parasite preparation) or fifteen (Mock injection, subdivided in sub-groups of three animals for each parasite preparation).

2.3.4 Peritoneal lavage.

At defined points within a time frame of 42 days post intraperitoneal injection, mice were sacrificed by CO_2 asphyxiation. Ice cold PBS (1x) with 10% heat-inactivated filtered FBS Superior (Biochrom AG) was then used to wash the peritonea and retrieve the peritoneal exudates. In each of the five experimental replicates, i.e. the use of a different parasite biological replicate for i.p injection, the peritoneal cells were harvested from naïve (a pool of 3 mice) or infected (1 mouse) mice at each time point. The suspensions of peritoneal exudates were filtered through a 70µm nylon cell strainer (BD Biosciences). Red blood cells in the filtrates were lysed with 1.4% NH4Cl for 5 minutes at 37^{o}C. The filtrates were then washed in R10 medium and the total numbers of recovered cells determined using the trypan blue (Biochrom) exclusion test on a bright-line Neubauer counting chamber prior to analysis. At the end of the infection follow up (42 days), the parasitic tissue was thoroughly excised from the peritonea of infected mice and the mass was determined.

2.3.5 Flow cytometric analyses.

2 x 10^5 peritoneal exudates were stained with fluorochrome-conjugated antibodies (anti-mouse) directed against the T-cell subset surface marker CD4 (Biotin, Miltenyi Biotec), the alpha chain of the IL-2 receptor CD25 (PE, eBioscience) present on activated T-cells and Foxp3 (APC, Miltenyi Biotec), the intracellular master transcription factor of regulatory T-cells. Biotinylated CD4 antibodies were detected by incubation with either FITC- or Pe-Cy5-conjugated streptavidin (BD Biosciences). As isotype control of activated/regulatory T-cells, Rat IgG1 K Isotype (PE, eBioscience) was used. The staining procedure was executed as per the manufacturer instructions (Treg Detection Kit, Miltenyi Biotec). The cells were resuspended in FACS buffer (1x PBS supplemented with 3% heat-inactivated and filtered FCS and 0.1%NaN3)

and acquired on a cytometer (FACSCalibur™, Beckton Dickinson) equipped with CellQuest software. Results were further analyzed on FlowJo software (Tree Star, USA).

2.3.6 *In vitro* regulatory T-cell suppression assay.

From the spleen of a healthy mouse (section 2.2.8), and peritoneal exudates (section 2.3.4) of mice 7 days post infection (20 mice pooled), CD4+ cells were separately isolated using mouse CD4+ T-cell negative selection protocol (EasySep™ Mouse CD4+ T Cell Enrichment Kit, Stem Cell Technologies) to a purity of > 90% according to the manufacturer´s instructions. Purified CD4+ T-cells were stained with anti-CD4 (Biotin, Miltenyi Biotec) and anti-CD25 (PE, eBioscience) followed by incubation with Pe-Cy5-conjugated streptavidin (BD Biosciences). CD4+CD25- and CD4+CD25+ cells were then sorted on a MoFlo high-speed sorter (Cytomation). Sorted CD4+CD25- splenic cells (responders) were labeled with 2µM CFSE (CFDA SE, Molecular Probes/Invitrogen) at 37°C for 10 min and washed twice in R10 medium before use. For *in vitro* proliferation of the isolated responder cells, total splenocytes from a healthy mouse (section 2.2.8), were labeled with a cocktail of non-APC (antigen-presenting cells) binding antibodies directed against the murine T-cell lineage anti-Thy-1.2 (BD Pharmingen) , anti-CD4 (eBioscience) and anti-CD8 (eBioscience) on Ice for 30 minutes. The cells were then washed in 1x PBS supplemented with 3% heat-inactivated and filtered Fetal Calf Serum (FCS, PAA Laboratories) prior to antibody-mediated complement lysis (Rabbit complement, Cedarlane) at 1/10 dilution for 45 minutes under constant agitation at 37^{o}C. Next, the suspension was filtered through a 70µm nylon cell strainer (BD Biosciences) and the filtrate, representing antigen presenting cells (APC), washed and resuspended in R10 medium (RPMI-1640 from GIBCO BRL supplemented with 100U/ml Penicillin (Sigma), 100µg/ml Streptomycin (Sigma), 2mM L-glutamin (Sigma), 50µM ß-mercaptoethanol (Sigma) and 10% heat-inactivated filtered (0.22 µm, Millipore) fetal calf serum (FCS, PAA Laboratories). The purified antigen-presenting cells were then irradiated on an X-ray unit (Faxitron, CellRad) with 20 Grays and counted using the trypan blue (Biochrom) exclusion test on a bright-line Neubauer counting chamber. A total of 2 X 10^5 irradiated APC were then cultured in anti-CD3 (1ug/ml, eBioscience) pre-coated 96-well round-bottom plates with 2 X 10^4 CFSE-labeled responders (Splenic CD4+CD25- cells) and 1-2 X 10^4 CD4+CD25+ cells for 5 days. The cells were then

harvested, resuspended in FACS buffer (1x PBS supplemented with 3% heat-inactivated and filtered FCS and 0.1%NaN3) prior to acquisition on a cytometer (FACSCalibur^{TM}, Beckton Dickinson) equipped with CellQuest software. Results were further analyzed on FlowJo software (Tree Star, USA).

2.3.7 *In vitro* regulatory T-cell conversion assay.

Spleens and lymph nodes from 6-14 weeks old OT-II and OT-II×RAG-1(-/-) transgenic mice were isolated and cellular suspensions obtained as previously described (section 2.2.8). CD4+ T-cells were isolated using a T-cell negative selection kit (Easy Sep mouse T-cell enrichment kit, Stem Cell Technologies) to a purity >90% as per the manufacturer´s instructions. Unlike OT-II×RAG-1(-/-) mice which have no resting CD4+CD25+Foxp3+ T-cells [203], CD4+ T-cells from OT-II mice were further enriched for CD25- cells using Miltenyi Biotec´s LD columns with a suitable MACS separator achieving > 90% purity as per the manufacturer´s instructions. Murine bone marrow-derived DC at day 8 of cultivation (70-80% CD11c+, section 2.2.7) were incubated with 3-fold higher numbers of CD4+CD25- T-cells (OT-II or OT-II×RAG-1(-/-)) and 200ng/ml OVA peptide (amino acid 323-339, grade V, Sigma) supplemented or not with parasite larvae E/S products (section 2.2.6). Recombinant human/porcine TGF-β1 (1, 2,10ng/ml; R&D Systems) were used as standards. In some experiments, the neutralizing multispecies anti-TGF-β antibody (1D11, R&D Systems) was added at 20µg/ml or 5µM of the TGF-β / Activin / Nodal subfamily - related ALKs (4,5 and 7) inhibitor, SB431245 (146; Sigma) was used. After 5 days of incubation, the cells were harvested and stained using the Treg detection kit (Miltenyi Biotec), resuspended in FACS buffer (1x PBS supplemented with 3% heat-inactivated and filtered FCS and 0.1%NaN3) prior to acquisition on a cytometer (FACSCalibur^{TM}, Beckton Dickinson) equipped with CellQuest software. Results were further analyzed on FlowJo software (Tree Star, USA).

2.3.8 *In vitro* IL-10 secretion assay.

CD4+ T-cells were isolated from healthy C57BL/6 mice using a T-cell negative selection kit (Easy Sep CD4+ T cell enrichment kit, Stem Cell Technologies) to > 90% purity according to

the manufacturer´s instructions. The CD25- fraction was further enriched using Miltenyi Biotec´ LD columns with a suitable MACS separator achieving > 90% purity for CD4+CD25- T-cells. Next, 2 x 10^5 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 µg/ml, eBioscience) overnight at 4^oC in R10 medium. The cells suspension was supplemented with 5µg/ml anti-CD28 (eBioscience). Next, parasite E/S products (section 2.2.6) or unconditioned axenic culture medium ($DMEM10_{redox,}$ section 2.2.3.2.b) was added to the wells and supernatants were collected 72 hours later. The amount of IL-10 in the culture supernatants was determined by ELISA as per the manufacturer´s instructions (BD OptEIA™ - Mouse IL-10 ELISA Set - BD Biosciences).

2.3.9 Statistical analyses.

All results were expressed as mean ±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 GraphPad Prism 6.00 for Windows (GraphPad Prism® Software).

2.4. *E. multilocularis* larvae E/S products and Foxp3+ Tregs.

2.4.1 Animals and Ethics statement.

C57BL/6 and NMRI 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. All animals handling, care and subsequent experimentation were performed in compliance with European and German regulations on the protection of animals (*Tierschutzgesetz*). 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).

2.4.2 Parasite material.

Echinococcus multilocularis larvae, i.e. primary cells, metacestodes and protoscoleces were isolated (section 2.2.3.3), freed from host contaminants as described elsewhere (section 2.2.4). The various larvae secretions were collected as previously detailed (section 2.2.6). The isolated parasite larvae and E/S products were then subsequently used as templates for RNA and protein assays.

2.4.3 *Echinococcus multilocularis* Genome sequencing project.

The nuclear genome of *E. multilocularis* has been produced by whole genome shotgun sequencing using a mixture of capillary sequencing, illumina sequencing and 454 pyrosequencing [191,192]. A profiling of the different larvae transcriptome is currently underway.

2.4.4 Identification of parasite factors, cloning and sequence analysis.

2.4.4.1 Identification of a TGF-beta ligand from E. multilocularis.

The full length of the TGF-β/Activin subfamily member, SmInACT (*Schistosoma mansoni* Inhibin/Activin, A4UAH0) was used as a query to search the *E. multilocularis* protein database ([204], http://www.genedb.org/Homepage/Emultilocularis). A 2 step search was performed in order to identify a true TGF-β/Activin sub-family member. Briefly, SmInACT was first used for a Blastp against *Echinococcus multilocularis* predicted protein database (http://www.genedb.org/Homepage/Emultilocularis). The obtained hits were then used as queries for a Blastp against mammalian proteins to identify the true Inhibin/Activin orthologues from *Echinococcus multilocularis*.

2.4.4.2 Primer list.

EmACT_Dw	5´- ATGACCATTACTACCCCCATGAAG-3´
EmACT_Up	5´- ACTACAACCGCACTCTAGGACAATG-3´
EmACT_DW 1	5´-GTCGTTCGACGAACACGGAG-3´
PBAD_Fwd	5´- ATGCCATAGCATTTTTATCC-3´
PBAD_Rev	5´- GATTTAATCTGTATCAGG-3´
tEcACT_Dw (Hind III)	5´- GTTGGAAGCTTACTCTTTAGACAGCCTGCAATAATG-3´
tEcACT_Up (Not I)	5´- GTGCGGCCGCCGAACAACCGCACTCTAGGAC-3´
T7_Dw	5´- TAATACGACTCACTATAGGG-3´
Psec_Rev	5´- TAGAAGGCACAGTCGAG-3´
EmACT 1	5´- TGAGCTCGAGAATTCAGTCGGCTTTTC-3´
EmACT 2	5´- GAGACCTTTGTGCCGAGTTGT-3´
EmACT 3	5´- GTACCGGAGTCAAAATTACGAC-3´

2.4.4.3 Amplification, Cloning and Sequence analysis of E. multilocularis TGF-beta ligand.

RNA was isolated from in vitro cultivated metacestode vesicles using TRIzol® reagent (Invitrogen) as per the manufacturer´s instructions. Due to the fluid-containing structure of metacestode vesicles, the protocol was slightly modified. Briefly, a maximum of 5 cysts were ruptured using a sterile needle (23G, Neoject®, Dispomed Witt oHG). The released cyst fluid was removed upon centrifugation at 400g 3minutes at RT, and then the pellet was resuspended in 1ml TRIzol® reagent and subsequently processed as per the manufacturer´s guidelines. The isolated RNA was reverse-transcribed into complementary DNA (cDNA) using Omniscript RT Kit (Qiagen, Hilden, Germany) in a total volume of 20 µl as per the manufacturer´s prescriptions. For amplification of the coding sequence, primers were made corresponding to the 5´and 3´coding termini (EmACT_Dw and EmACT_Up for Em*act*).1µl of cDNA was used per 20ul reaction as per the manufacturer´s guidelines (Phusion® High-Fidelity DNA Polymerase, New England Biolabs). 2µl of the amplicon were resolved on a 1.5% agarose gel and stained with Ethidium bromide prior to visualization under a UV transilluminator.

For cloning into PBAD/Thio-TOPO® (Invitrogen), A overhangs were added at the 3´ends of the amplified fragment. For this purpose, the PCR mix containing the fragment was purified over a silica gel column (NucleoSpin® Gel and PCR Clean-up, MACHEREY-NAGEL) as per the user manual. 150-1500ng of the amplicon was incubated at 72oC for 20minutes with 1U DNA polymerase (Taq DNA polymerase, NEB), 0.2mM dATP and 1X Buffer (Taq polymerase buffer). The 3´A-overhanged fragment was then ligated into pBAD/Thio_TOPO and chemically competent *E. coli* (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen) were transformed with the ligation product as per the pBAD/Thio-TOPO manual directives. Using a pair of primers (PBAD_Fwd and EmACT_Up), several transformed *E.coli* colonies growing on an ampicillin-supplemented solid medium were used as templates for PCR. Successful clones (yielding an amplicon) were sub-cultured overnight at 37^{o}C in Luria Bertani medium supplemented with Ampicillin (50-100µg/ml) followed by plasmid isolation (Nucleospin Plasmid preparation, MACHEREY-NAGEL). 400ng of plasmid DNA as determined by spectrophotometry (NanoDrop® ND-1000, PEQLAB), were used for sequencing (3130 Genetic Analyzer, Applied Biosystems) with the primer pair (PBAD_Fwd and PBAD_Rev) and the sequencing readouts assembled into a single coding sequence using BIOEDIT (Version 7.1.3).

2.4.5 Recombinant expression, protein purification and antiserum generation.

2.4.5.1 Bacterial expression of recombinant protein and generation of polyclonal antiserum.

Upon identification of positive *E.coli* transformants, the arabinose-driven induction of Thio tag-fused protein expression was conducted. The cloned full length factor was checked in sillico for antigenicity (tools.Immunoepitope.org) then recombinantly expressed. For the purpose, A single positive *E.coli* colony was grown overnight at 37^{o}C in a 50ml inoculate of LB medium containing ampicillin (50-100ug/ml).The next day, the turbid LB medium was transferred to a 1 Liter LB inoculum and kept on orbital shaker at 37oC until OD600nm =0.5. When the required OD600nm was achieved, a 1ml aliquot was taken and labeled as sample before induction. 2% Arabinose was then added to the broth culture and kept on orbital shaker at 37oC for an additional 4 hours. Another aliquot of 1ml was taken and labeled as sample after induction. The

remaining broth medium was centrifuged for 20 minutes at 6000g and the pellet processed for purification of the recombinant thio-tagged protein over a nickel-charged Sepharose® resin, the ProBond^{TM} resin (Invitrogen) as per the manufacturer instructions. The purified Thio-tagged protein was then diafiltered on Centrifugal Filter Units (Millipore) against sterile PBS (1X) before quantification using the BCA assay (ThermoScientific). An aliquot of the purified thio-tagged protein was also collected and stored for subsequent gel analysis. The stored aliquots were diluted 1:1 with 2 x STOPP mix (2ml 0.5M Tris-HCl pH 6.8, 1.6ml glycerol, 1.6ml 20% SDS, 1.4ml H2O, 0.4ml 0.05% (w/v) bromophenol blue, 7 µl ß-mercaptoethanol per 100 µl) and boiled for 10 min at 100oC. 10µl of each protein sample were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Ponceau S staining (Sigma-Aldrich). After obtention of a clean pattern of the recombinant Thio-tagged protein on the gel, NMRI mice were immunized for generation of antiserum. NMRI mice were injected at two different locations with 100µg of the recombinant Thio-tagged protein resuspended in 100µl Freund Incomplete adjuvant (Sigma). The double injections were repeated four weeks later to boost the mice immune response. Finally, ten days after the boost, the mice were bleeded from the heart and the serum collected and stored at -20^{o}C until use. In parallel, naïve mice were also bleeded and the serum collected and stored as pre-immune serum or normal mouse serum.

2.4.5.2 Recombinant expression in HEK cell line for functional assays.

The identified TGF-β ligand was inserted in frame within the pSecTag2/Hygro expression system. In order to do so, the primer pair tEcACT_Dw and tEcACT_Up were used. The PCR program was designed as per the manufacturer instructions (Phusion High-Fidelity DNA Polymerase, New England Biolabs) generating a fragment flanked at the 5´end by a HindIII cutting site and at the 3´end by a NotI recognition site so as to mediate an in-frame insertion within the multiple cloning site of the vector. The PCR amplicon and the eukaryotic expression vector pSecTag2/Hygro were separately loaded on a 1.5 % agarose gel and the corresponding bands excised and the DNA cleaned up as per the kit directives (NucleoSpin® Gel and PCR Clean-up, MACHEREY-NAGEL). The purified vector and the amplicon were then digested simultaneously with HindIII and NotI as per the manufacturer´s instructions (New Englands Biolabs). The products of digestion were loaded on a 1.5% agarose gel, excised and cleaned

again as previously described (NucleoSpin® Gel and PCR Clean-up, MACHEREY-NAGEL). Using a well established vector-insert stochiometric ratio, the double digested amplicon and pSecTag2/Hygro were ligated using the T4 DNA ligase (New England Biolabs) as per the manufacturer´s instructions. Following ligation, the new plasmid construct was used to transform chemically competent *E.coli* as indicated by the supplier (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen). Using a pair of primers (T7_Fwd and PSec_Rev), several transformed *E. coli* colonies growing on an ampicillin-supplemented solid medium were used as templates for PCR. Successful clones (yielding an amplicon) were sub-cultured overnight at 37^{o}C in Luria Bertani medium supplemented with Ampicillin (50-100µg/ml) followed by plasmid isolation (Nucleospin Plasmid preparation, MACHEREY-NAGEL) and confirmation of the reading frame by sequencing. For this purpose, 400ng of plasmid DNA as determined by spectrophotometry (NanoDrop® ND-1000, PEQLAB), were used for sequencing (3130 Genetic Analyzer, Applied Biosystems) with the primer triplet (EmACT1, EmACT2 and EmACT3) and the sequencing readouts assembled using BIOEDIT (Version 7.1.3) to attest of the proper insertion of the TGF-β ligand within pSecTag2/Hygro. Next, 293T Human Embryonic Kidney cells were used as the vessel for pSecTag2/Hygro-driven expression of the factor. More precisely, HEK 293T cells kept in DMEM10 medium (section 2.2.3.2.a) were grown to 50-70% confluency in a T75 flask (SARSTEDT) and harvested by trypsin treatment (Trypsin/Ethylenediamine tetraacetic acid, Biochrom, cat. L2143) at 37^{o}C for 10 minutes. The cell concentration in the retrieved HEK culture suspension was determined (Trypan Blue, Neuebauer chamber) and 3 x 10^{6} cells resuspended in 10 ml DMEM10 and seeded in a petri dish (92 x 16 mm [Ø x height], SARSTEDT) for 16 hours. The 293T human embryonic kidney cells were transfected with 10µg of either the empty expression vector pSecTag2/Hygro or the expression construct pSecTag2/Hygro-factor using linear polyethyleneimine (25kDa, Sigma). The supernatants were harvested upon 24 hours of incubation, filtered over a bottle top filter (Filtropur BT50, SARSTEDT), normalized for the total protein content (BCA Protein Assay Kit, ThermoScientific) and stored as aliquots at -80^{o}C until use.

2.4.6 Assessment of factor secretion by *in vitro* cultivated metacestodes and transfected HEK cells.

1ml of parasite or transfected HEK cell supernatants was resuspended in 9 volumes of 100% ice-cold ethanol. The mixtures were kept at -80º C for at least 2 hours, then centrifuge for 30 min at 14,000 rpm in a refrigerated centrifuge. The ethanolic supernatants were discarded and the pellets were dried thoroughly at 50^{o}C and resuspended in 50µl of 2 x STOPP mix(2ml 0.5M Tris-HCl pH 6.8, 1.6ml glycerol, 1.6ml 20% SDS, 1.4ml H2O, 0.4ml 0.05% (w/v) bromophenol blue, 7 µl ß-mercaptoethanol per 100 µl) and boiled for 10 min at 100^{o}C. 10µl of each protein sample were separated by SDS-PAGE and transferred to a nitrocellulose membrane for immunodetection with the previously generated antiserum as compared to the pre-immune serum.

2.4.7 Treatment of murine BMDC.

Transiently transfected 293T HEK cell supernatants normalized for the total protein content were used throughout the stimulation process (see section 2.4.5.2 for collection and storage of supernatants). 8 days old cultures of *in vitro* generated BMDC from C57BL/6 mice (section 2.2.7) were used at a concentration of 5 x 10^5 cells /ml of R10 medium and supplemented with either culture medium DMEM10 (see section 2.2.3.2.a), the supernatant of empty vector transfected HEK cells or that of the factor-containing vector transfected HEK cells. The DC were stimulated at 37^{o}C under 5% CO2 for 24 hours before analysis. For restimulation assays, 24 hours post initial treatment, DC were further stimulated with LPS (0.1µg/ml, *E. coli* 0127:B8) for an additional 24 hours. Upon completion, DC were stained for the lineage marker CD11c, the maturation markers MHCII and CD86 as previously described (section 2.2.10.4.a) and analyzed over a Flow cytometer (FACSCalibur^{TM}, Beckton Dickinson) equipped with CellQuest software. Results were further processed on FlowJo software (Tree Star, USA).

2.4.8 Treg conversion assay.

Spleens and lymph nodes from 6-14 weeks OT-II×RAG-1(-/-) transgenic mice were isolated and cellular suspensions obtained as previously described (section 2.2.8). CD4+ T-cells were isolated using a T-cell negative selection kit (Easy Sep mouse T-cell enrichment kit, Stem Cell Technologies) to a purity >90% as per the manufacturer´s instructions. OT-II mice derived CD4+ T-cells were further enriched for CD25- cells using Miltenyi Biotec´s LD columns with a suitable MACS separator achieving > 90% purity as per the manufacturer´s instructions. Murine BMDC at day 8 of cultivation (70-80% CD11c+, section 2.2.7) were incubated with 3-fold higher numbers of CD4+CD25- T cells (OT-II or OT-II×RAG-1(-/-)) and 200ng/ml OVA peptide (323-339, grade V, Sigma) supplemented or not with transfected 293T HEK cell supernatants (empty vector- and factor-transfected cells, see section 2.4.5.2) or unconditioned medium (DMEM10, section 2.2.3.2.a). Recombinant human/porcine TGF-β1 (1, 2,10ng/ml; R&D Systems) were used as standards or in conjunction with the various supernatants. After 5 days of incubation, the cells were harvested and stained using the Treg detection kit (Miltenyi Biotec), resuspended in FACS buffer (1x PBS supplemented with 3% heat-inactivated and filtered FCS and 0.1%NaN3) prior to acquisition on a cytometer (FACSCalibur^{TM}, Beckton Dickinson) equipped with CellQuest software. Results were further analyzed on FlowJo software (Tree Star, USA).

2.4.9 IL-10 secretion assay.

CD4+ T-cells were isolated from healthy C57BL/6 mice (6-8weeks old) using a T-cell negative selection kit (Easy Sep CD4+ T-cell enrichment kit, Stem Cell Technologies) to > 90% purity according to the manufacturer´s instructions. The CD25- fraction was further enriched using Miltenyi Biotec´ LD columns with a suitable MACS separator achieving > 90% purity for CD4+CD25- T cells. Next, 2 x 10^5 CD4+CD25- T-cells were seeded in a 24-well tissue culture plate (Flat bottom, SARSTEDT) that had been coated with anti-CD3 (0.1µg/ml, eBioscience) overnight at 4^oC in R10 medium. The cells suspension was supplemented with 5µg/ml anti-CD28 (eBioscience). Next, 50% of transfected 293T HEK cell supernatants (empty vector and factor-transfected cells, see section 2.4.5.2) or unconditioned culture medium (DMEM10, section 2.2.3.2.a) was added to the wells and supernatants were collected 72 hours later for the amount

of IL-10 to be determined by ELISA as per the manufacturer´s instructions (BD OptEIA™ - Mouse IL-10 ELISA Set - BD Biosciences with a detection limit of 19pg/ml).

2.4.10 Statistical analyses.

All results were expressed as mean ±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 GraphPad Prism 6.00 for Windows (GraphPad Prism® Software).

2.5 *E. multilocularis* larvae E/S products and Th1 immune responses.

2.5.1 Animals and Ethics statement.

See 2.4.1

2.5.2 Parasite material.

See 2.4.2

2.5.3 Identification of parasite factors, cloning and sequence analysis.

2.5.3.1 Identification of a T-cell immunomodulatory protein from E. multilocularis.

A hit with significant similarity to Human T-cell Immunomodulatory protein [205] was identified within a previously established *E. multilocularis* trans-spliced cDNA library [206] and termed Em*tip* (*Echinococcus multilocularis* T-cell Immunomodulatory Protein).

2.5.3.2 Primer list.

EmTIP_Dw	5´-CCTTGCAGTTTTGTATGAAAATG-3´
EmTIP_Up	5´-GATCATTCGACCTTCTACATTGC-3´
FG-GAP_Dw	5´-GAAACGTTGAAACAGATCG-3´
FG-GAP_Up	5´-GCTTATGTTTCCGAGCTTG-3´
AbTIP_Dw	5´-GCTGATTTGGCCGCTTTTG-3´
AbTIP_Up	5´-CTGAGATTCAACTCCAGGCAAAAG-3´
tEmTIP_Dw (EcoRV)	5´-GTTGGGATATCGGTGGCCATC-3´
tEmTIP_Up (NotI)	5´-GTGCGGCCGCCTACATTGCATC-3´
EmTIP1	5´-GAATGTAACCCGTTCTGTTG-3´
EmTIP2	5´-CATCATTGGACTCTCTTTAG-3´
EmTIP3	5´-GAAAATGTACCCGTTTCCGTTC-3´

2.5.3.3 Amplification, Cloning and Sequence analysis of Emtip.

In order to verify the Em*tip* open reading frame, RNA was isolated from the parasite primary cells, metacestode vesicles and protoscoleces (section 2.4.4.3). More precisely, RNA was isolated using TRIzol® reagent (Invitrogen) as per the manufacturer´s instructions. Due to the fluid-containing structure of metacestode vesicles, the protocol was slightly modified for the latter. Briefly, a maximum of 5 cysts were ruptured using a sterile needle (23G, Neoject®, Dispomed Witt oHG). The released cyst fluid was removed upon centrifugation at 400g 3minutes at RT, and then the pellet was resuspended in 1ml TRIzol® reagent and subsequently processed as per the manufacturer´s guidelines. The isolated RNA was retro-transcribed into complementary DNA (cDNA) using Omniscript RT Kit (Qiagen, Hilden, Germany) in a total volume of 20 µl as per the manufacturer´s prescriptions. For amplification of Em*tip* coding sequence, primers (EmTIP_Dw and EmTIP_Up) were designed corresponding to the 5´and 3´flanking regions of the corresponding nucleotide sequence as retrieved online and aligned to *E.*

multilocularis genomic DNA (147, http://www.genedb.org/Homepage/Emultilocularis). 1µl of cDNA was used per 20ul reaction as per the manufacturer´s guidelines (Phusion® High-Fidelity DNA Polymerase, New England Biolabs). 2µl of the amplicons were resolved on a 1.5% agarose gel and stained with Ethidium bromide prior to visualization under a UV transilluminator. EmTIP epitopes were predicted *in sillico* (tools.Immunoepitope.org). The antigenic N-terminal portion of the protein was chosen for immunization. A nested PCR was performed using as template 1µl of the full-length Em*tip* amplicon according to the enzyme manufacturer´s directives (Phusion® High-Fidelity DNA Polymerase, New England Biolabs) with the primers pair AbTIP_Dw / AbTIP_Up. Again, 2µl of the nested PCR amplicons , destined to insertion within the bacterial expression vector pBAD/Thio-TOPO® (Invitrogen) for sequence analysis and recombinant expression, were resolved on a 1.5% agarose gel and stained with Ethidium bromide prior to visualization under a UV transilluminator. A overhangs were then added at the 3´ends of the amplified fragment. For this purpose, the nested PCR mix containing the fragment was purified over a silica gel column (NucleoSpin® Gel and PCR Clean-up, MACHEREY-NAGEL) as per the user manual. 150-1500ng of the amplicon was incubated at 72oC for 20minutes with 1U DNA polymerase (Taq DNA polymerase, NEB), 0.2mM dATP and 1X Buffer (Taq polymerase buffer). The 3´A-overhanged fragment was then ligated into pBAD/Thio_TOPO and chemically competent *E. coli* (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen) were transformed with the ligation product as per the pBAD/Thio-TOPO manual directives. Using a pair of primers (PBAD_Fwd and AbTIP_Up), several transformed *E. coli* colonies growing on an ampicillin-supplemented solid medium were used as templates for PCR. Successful clones (yielding an amplicon of expected size) were sub-cultured overnight at 37^{o}C in Luria Bertani medium supplemented with Ampicillin (50-100µg/ml) followed by plasmid isolation (Nucleospin Plasmid preparation, MACHEREY-NAGEL). 400ng of plasmid DNA as determined by spectrophotometry (NanoDrop® ND-1000, PEQLAB), were used for sequencing (3130 Genetic Analyzer, Applied Biosystems) with the primer pair (PBAD_Fwd and PBAD_Rev) and the sequencing readouts assembled into the corresponding thio tag-fused Em*tip*-coding fragment using BIOEDIT (Version 7.1.3).

2.5.4 Recombinant expression of EmTIP and generation of antiserum.

2.5.4.1 Bacterial expression of Thio tag-fused EmTIP and generation of polyclonal antiserum.

Upon identification of Em*tip*-containing pBAD/Thio-TOPO positive *E. coli* transformants, the arabinose-driven induction of bacterial Thio-EmTIP expression was conducted. For the purpose, a single Thio-EmTIP expressing *E. coli* colony was grown overnight at 37oC in a 50ml inoculate of LB medium containing ampicillin (50-100ug/ml).The next day, the turbid LB medium was transferred to a 1 Liter LB inoculum and kept on orbital shaker at 37oC until OD600nm =0.5. When the required OD600nm was achieved, a 1ml aliquot was taken and labeled "Thio-EmTIP before induction". 2% Arabinose was then added to the broth culture and kept on orbital shaker at 37oC for an additional 4 hours. Another aliquot of 1ml was taken and labeled "Thio-EmTIP after induction". The remaining broth medium was centrifuged for 20 minutes at 6000g and the pellet processed for recombinant Thio-EmTIP purification over a nickel-charged Sepharose® resin, the ProBond^{TM} resin (Invitrogen) as per the manufacturer instructions. The purified Thio-EmTIP was then diafiltered on Centrifugal Filter Units (Millipore) against sterile PBS (1X) before quantification using the BCA assay (ThermoScientific). An aliquot of purified Thio-EmTIP was also collected and stored for subsequent gel analysis. The stored aliquots were diluted 1:1 with 2 x STOPP mix(2ml 0.5M Tris-HCl pH 6.8, 1.6ml glycerol, 1.6ml 20% SDS, 1.4ml H2O, 0.4ml 0.05% (w/v) bromophenol blue, 7 µl ß-mercaptoethanol per 100 µl) and boiled for 10 min at 100oC. 10µl of each protein sample were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Ponceau S staining (Sigma-Aldrich).

After obtention of a clean pattern for recombinant Thio-EmTIP on the gel (i.e. a single band visible), Inbred rabbits (1 year old) were intradermally immunized repetitively with the Thio-EmTIP antigen suspensions in collaboration with the Biotechnology company ImmunoGlobe Antikörpertechnik GmbH (D-63762 Großostheim) generating 50 ml of polyclonal antiserum. An aliquot of serum collected prior to the immunization scheme was termed pre-immune serum and used as specificity reference for the polyclonal antibodies generated. For affinity purification of the antiserum, 200-500µg of purified Thio-EmTIP antigen was run on SDS PAGE 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. 5g of skimmed milk in 100ml

of 1 xTris Buffer Saline supplemented with 0.1% Tween 20) for 1 hour at room temperature on a rocking plate. The nitrocellulose stripe was then incubated overnight at 4^{o}C with 500ul of anti-Thio-EmTIP antiserum on a rotating wheel. Next, the stripe was sequentially washed in 0.15M NaCl, 1 X PBS then the bound antibodies eluted with 0.2M glycin solution supplemented with 1mM EGTA at pH 2.5. The eluted anti-EmTIP antibody solution was neutralized to pH 7 with Tris solution (1M), then diafiltered on Centrifugal Filter Units (Millipore) against sterile PBS (1X) before quantification for the protein content using BCA assay (ThermoScientific) as per the manufacturer´s instructions then stored at -20^{o}C until use. In parallel, whole IgG solution from naïve rabbit (Santa Cruz Biotechnology, inc.) was similarly eluted and diafiltered before storage at -20^{o}C.

2.5.4.2 Eukaryotic expression and collection of recombinant EmTIP.

Metacestode cDNA was used as a template to amplify Em*tip*. The amplified amplicon was inserted in frame within the pSecTag2/Hygro expression system. In order to do so, the primer pair tEmTIP_Dw and tEmTIP_Up were used. The PCR program was designed as per the manufacturer instructions (Phusion High-Fidelity DNA Polymerase, New England Biolabs) generating an Em*tip* fragment flanked at the 5´end by a EcoRV cutting site and at the 3´end by a NotI recognition site so as to mediate the in-frame fragment insertion within the multiple cloning site of the vector. The PCR amplicon and the eukaryotic expression vector pSecTag2/Hygro were separately loaded on a 1.5 % agarose gel and the corresponding bands excised and the DNA cleaned up as per the kit directives (NucleoSpin® Gel and PCR Clean-up, MACHEREY-NAGEL). The purified vector and Em*tip* amplicon were then digested simultaneously with EcoRV and NotI as per the manufacturer´s instructions (New Englands Biolabs). The products of digestion were loaded on a 1.5% agarose gel, excised and cleaned again as previously described (NucleoSpin® Gel and PCR Clean-up, MACHEREY-NAGEL). Using a well established vector-insert stochiometric ratio, the double digested Em*tip* and pSecTag2/Hygro were ligated using the T4 DNA ligase (New England Biolabs) as per the manufacturer´s instructions. Following ligation, the Em*tip*-pSecTag2/Hygro plasmid was used to transform chemically competent *E. coli* as indicated by the supplier (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen). Using a pair of primers (T7_Fwd and PSec_Rev), several transformed *E. coli* colonies growing

on an ampicillin-supplemented solid medium were used as templates for PCR. Positive clones (yielding an amplicon) were sub-cultured overnight at 37^{o}C in Luria Bertani medium supplemented with Ampicillin (50-100µg/ml) followed by plasmid isolation (Nucleospin Plasmid preparation, MACHEREY-NAGEL) and confirmation of the reading frame by sequencing. For this purpose, 400ng of plasmid DNA as determined by spectrophotometry (NanoDrop® ND-1000, PEQLAB), were used for sequencing (3130 Genetic Analyzer, Applied Biosystems) with the primer triplet (EmTIP1, EmTIP2 and EmTIP3) and the sequencing readouts assembled using BIOEDIT (Version 7.1.3) to attest of the proper insertion of Em*tip* within pSecTag2/Hygro. Next, 293T Human Embryonic Kidney cells were transfected. More precisely, HEK 293T cells kept in DMEM10 medium (section 2.2.3.2.a) were grown to 50-70% confluency in a T75 flask (SARSTEDT) and harvested by trypsin treatment (Trypsin/Ethylenediamine tetraacetic acid, Biochrom, cat. L2143) at 37^{o}C for 10 minutes. The cell concentration in the retrieved HEK culture suspension was determined (Trypan Blue, Neuebauer chamber) and 3 x 10^{6} cells resuspended in 10 ml DMEM10 and seeded in a petri dish (92 x 16 mm [Ø x height], SARSTEDT) for 16 hours. The 293T human embryonic kidney cells were transfected with 10µg of either the empty expression vector pSecTag2/Hygro or the expression construct pSecTag2/Hygro-Em*tip* using linear polyethyleneimine (25kDa, Sigma). The supernatants were harvested upon 24 hours of incubation, filtered over a bottle top filter (Filtropur BT50, SARSTEDT), normalized for the total protein content (BCA Protein Assay Kit, ThermoScientific) and stored as aliquots at -80^{o}C until use.

2.5.5 Expression profile of EmTIP.

The expression of EmTIP in larvae of *E. multilocularis* and as recombinantly expressed protein in 293T HEK cells was analyzed by RT-PCR, Immunoprecipitation and Immunodetection. First, 1µl of the different parasite larvae cDNA were used for PCR amplification using the TAQ polymerase (NEB) with 5 pmoles of each primer (FG-GAP_Dw/FG-GAP_Up) and a protocol of 30 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C. PCR-products were then separated on a 1.5% agarose gel and stained with ethidium bromide for visualization. Next, *E. multilocularis* larvae at quantities previously defined (section 2.2.5) were passed through a pipette tip and washed in PBS (1X). The various homogenates were then treated with 50 µl of 2 X STOPP mix

(2ml 0.5M Tris-HCl pH 6.8, 1.6ml glycerol, 1.6ml 20% SDS, 1.4ml H2O, 0.4ml 0.05% (w/v) bromophenol blue, 7 µl ß-mercaptoethanol per 100 µl) and boiled for 10 min at 100^{o}C. The larvae protein mixes were separated by SDS-PAGE and analyzed by Western blotting using polyclonal rabbit anti-EmTIP antibodies. Similarly, the 293T human embryonic kidney cells that were transfected with 10µg of either the expression vector pSecTag2/Hygro or the expression construct pSectag2-Em*tip* (section 2.5.4.2) were harvested, washed thrice in 1 X PBS, treated with 50ul 2 x STOPP mix, resolved on a SDS gel, blotted then probed against anti-EmTIP antibodies.

Finally, secretions of *E. multilocularis* larvae normalized as described elsewhere (section 2.2.6) as well as pSecTag2/Hygro- and pSecTag2/Hygro-Em*tip*-transfected 293T HEK cell supernatants (section 2.5.4.2) were immunoprecipitated and probed with anti-EmTIP antibody. More precisely, 20µg of anti-EmTIP antibody was captured on Protein G agarose as per the manufacturer´s guidelines (Protein G Agarose, Fast Flow, Upstate cell signaling solutions). The anti-EmTIP coated Protein G agarose beads were packed into a column and used to capture EmTIP from the above mentioned supernatants. The beads were then resuspended in 2 x STOPP mix (2ml 0.5M Tris-HCl pH 6.8, 1.6ml glycerol, 1.6ml 20% SDS, 1.4ml H2O, 0.4ml 0.05% (w/v) bromophenol blue, 7 µl ß-mercaptoethanol per 100 µl) and boiled for 10 min at 100^{o}C. The protein mixes were separated by SDS-PAGE and analyzed by Western blotting using polyclonal rabbit anti-EmTIP antibodies.

2.5.6 Immunohistochemical localization of EmTIP within *Echinococcus multilocularis* larvae.

Samples of *in vitro* maintained parasite larvae (section 2.2.3.3), *in vivo* retrieved parasitic material within jird liver (2.2.3.1) and liver tissue of a non infected jird were fixed in 2% paraformaldehyde (in 1 x PBS) and washed at 4^{o}C overnight in 1 x PBS containing 6.8% sucrose. The tissues were then dehydrated in 100% acetone for 1 hour at 4oC prior to embedding into Technovit 8100 as per the manufacturer´s instructions (Technovit® 8100, Heraeus Kulzer Technik). Next, using a rotary microtome, 4µm sections were prepared and stained sequentially with purified anti-EmTIP antibody then peroxydase-coupled anti-rabbit-IgG (Jackson

ImmunoResearch) against haematoxilin counterstain as recommended (Technovit® 8100, Heraeus Kulzer Technik).

2.5.7 Neutralization of EmTIP during Echinococcus primary cells cultivation.

The purified antibodies solutions (anti-EmTIP and IgG) prepared as described previously (section 2.5.4.1) were used for the following neutralization assays.

2.5.7.1 Isolation of parasite primary cells and quantification.

E. multilocularis metacestodes were maintained in DMEM10 with 10^6 rat hepatoma cells as feeder cells (Rat Reuber RH- hepatoma cells, ATCC no. CRL-1600) for 2-3 months with weekly medium and RH- cells replacement. When the cysts achieved a diameter > 5mm (2-3 months of culture), the latter were extensively washed with 1 X PBS and positively selected for integral laminated layer where the broken or compromised ones were gently aspired using a 10ml serological pipette (SARSTEDT) and discarded . 20ml of uncompromised cysts were then cultured in 50 ml of DMEM10redox for 96 hours without medium change under axenic conditions (i.e. under nitrogen phase, 130). Next, the cysts were washed thrice with 1 X PBS and the parasite germinal layer/primary/stem cells were isolated as extensively described elsewhere (130).The isolated cells were resuspended in 2 ml of DMEM10redox and the absorbance of a 1:80 dilution of the suspension measured at 600nm.An OD600nm of 0.02/ml of the diluted suspension was taken as a reference unit for parasite cells quantification.

2.5.7.2 Monitoring parasite primary cells proliferation in vitro

7-10 units of isolated parasite germinal layer cells were seeded in a total volume of 100µl DMEM10redox supplemented or not with different concentrations of antibodies solution (purified rabbit IgG or anti-EmTIP) in flat-bottom 96 well plates (Nunc, Thermo Scientific). The plates were sealed in ziploc freezer bags filled with nitrogen before incubation in a CO2 incubator at 37oC for 36 hours. Next, 10µM BrdU solution (BrdU Cell proliferation Elisa kit; Roche Applied Science) was added to the cultures and the cells were reincubated for 4 additional

hours. The cells were subsequently fixed and the amount of incorporated BrdU revealed after cell DNA denaturation, probing with a peroxydase conjugated anti-BrdU monoclonal antibody and finally colorimetric detection with tetra methyl-benzidine (peroxydase substrate) as per the BrdU Cell proliferation Elisa kit manufacturer´s directives (Roche Applied Science). In parallel, 0.1 x 10^4 Rat hepatoma cells were seeded in a total volume of 100µl DMEM10 with similar stimuli (purified rabbit IgG or anti-EmTIP) and the proliferation rate also assessed by BrdU incorporation.

2.5.7.3 Monitoring parasite primary cells differentiation into metacestode vesicles in vitro

70 units of isolated parasite germinal layer cells were seeded in a total volume of 500µl DMEM10redox supplemented or not with a 30µg/ml of anti-EmTIP in flat-bottom 48 well plates (Nunc, Thermo Scientific). The plates were sealed in ziploc freezer bags filled with nitrogen before incubation in a CO2 incubator at 37oC. The medium was replaced with anti-EmTIP supplemented-DMEM10redox every 48 hours. At day 8, the DMEM10redox medium was completely aspired and fully replaced with A4 medium (see section 2.2.3.2.c) to fasten the regeneration process. The cultures were then further kept with frequent A4 medium change (twice per week). After 14 days, when cyst generation was distinctively noticeable, aggregates of parasitic primary cells were dissociated by gently pipetting up and down with a 1-mL pipette to loosen the aggregates and release newly formed cysts hitherto hidden. The total numbers of cysts per well were then determined by microscope-aided counting.

2.5.8 *In vitro* stimulation of CD4+ T-cells.

CD4+ T-cells were isolated from healthy C57BL/6 mice (6-8weeks old) using a T-cell negative selection kit (Easy Sep CD4+ T cell enrichment kit, Stem Cell Technologies) to > 90% purity according to the manufacturer´s instructions. The CD25- fraction was further enriched using Miltenyi Biotec´ LD columns with a suitable MACS separator achieving > 90% purity for CD4+CD25- T cells. Next, 2 X 10^5 CD4+CD25- T-cells were seeded in a 24-well tissue culture plate (Flat bottom, SARSTEDT) that had been coated with anti-CD3 (0.1µg/ml, eBioscience)

overnight at 4^{o}C in R10 medium. The cells suspension was supplemented with 5µg/ml anti-CD28 (eBioscience). Next, transfected 293T HEK cell supernatants (empty vector and Em*tip*-construct transfected cells, see section 2.5.4.2), unconditioned culture medium (DMEM10, section 2.2.3.2.a) or parasite larvae E/S (section 2.2.6) was added to the wells and supernatants were collected 72 hours later for the amounts of IL-10 and IFN-γ to be determined by ELISA as per the manufacturer´s instructions (BD OptEIA™ - Mouse IL-10 ELISA Set; Mouse IFN-γ ELISA Set - BD Biosciences with detection limits of 19pg/ml and 0.762pg/ml respectively). In another set of experiments, Brefeldin A (5µg/ml; Sigma) was added during the final 6h of CD4+ T-cell treatment 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 FACScalibur flow cytometer (BD) and data were analyzed with FlowJo software (TreeStar).

2.5.9 Statistical analyses.

See 2.4.10

3. RESULTS

3.1 Isolation and normalization of *E. multilocularis* larval material.

In order to assess the influence of E/S products of *E. multilocularis* larvae on the host immune system, early (primary cells), mid- (metacestodes) and late (protoscoleces) *E. multilocularis* larval stages devoid of any host contaminants were isolated according to Spiliotis and Brehm [186]. Briefly, *E. multilocularis* material was excised from intraperitoneally infected jirds 2-3 months post infection. After extensive washing, the material was axenically cultivated to remove host contaminants [186]. Host-free primary cells (Fig. 10A), metacestode vesicles (Fig. 10B) and protoscoleces (Fig. 10C) could thus be obtained as shown by polymerase chain amplification of a jird (host)-specific β-tubulin gene and the parasite specific *elp* gene (Fig. 11A). A series of experiments revealed that approximately one sixth of *E. multilocularis* primary cells isolated from 40 ml of metacestodes could regenerate into metacestode vesicles (Fig. 11B) within a time window similar to that reported for the *in vivo* transition of oncosphere into metacestode; i.e. 2-4 weeks [207]. Since this amount of primary cells did display an aggregation of cells into cavities but failed to show any budding metacestode structure a week after *in vitro* cultivation, the latter was used as a reference for primary cell material for the following stimulation assays and thereby as the reference amount to be used for the normalization of *E. multilocularis* material (Fig. 11C). Hence, 4 metacestode vesicles (2 months of age) of a diameter of 5 mm and 2000 protoscoleces were used in comparison since the latter were shown to contain a similar amount of protein to that of the defined primary cells reference (Fig. 11C). E/S products could then be generated by incubation of an equivalent amount of each larvae material in serum-containing DMEM10redox medium (Fig. 10). The collected E/S products were further adjusted for the total protein content (Fig. 11D) prior to use in the subsequent stimulation assays.

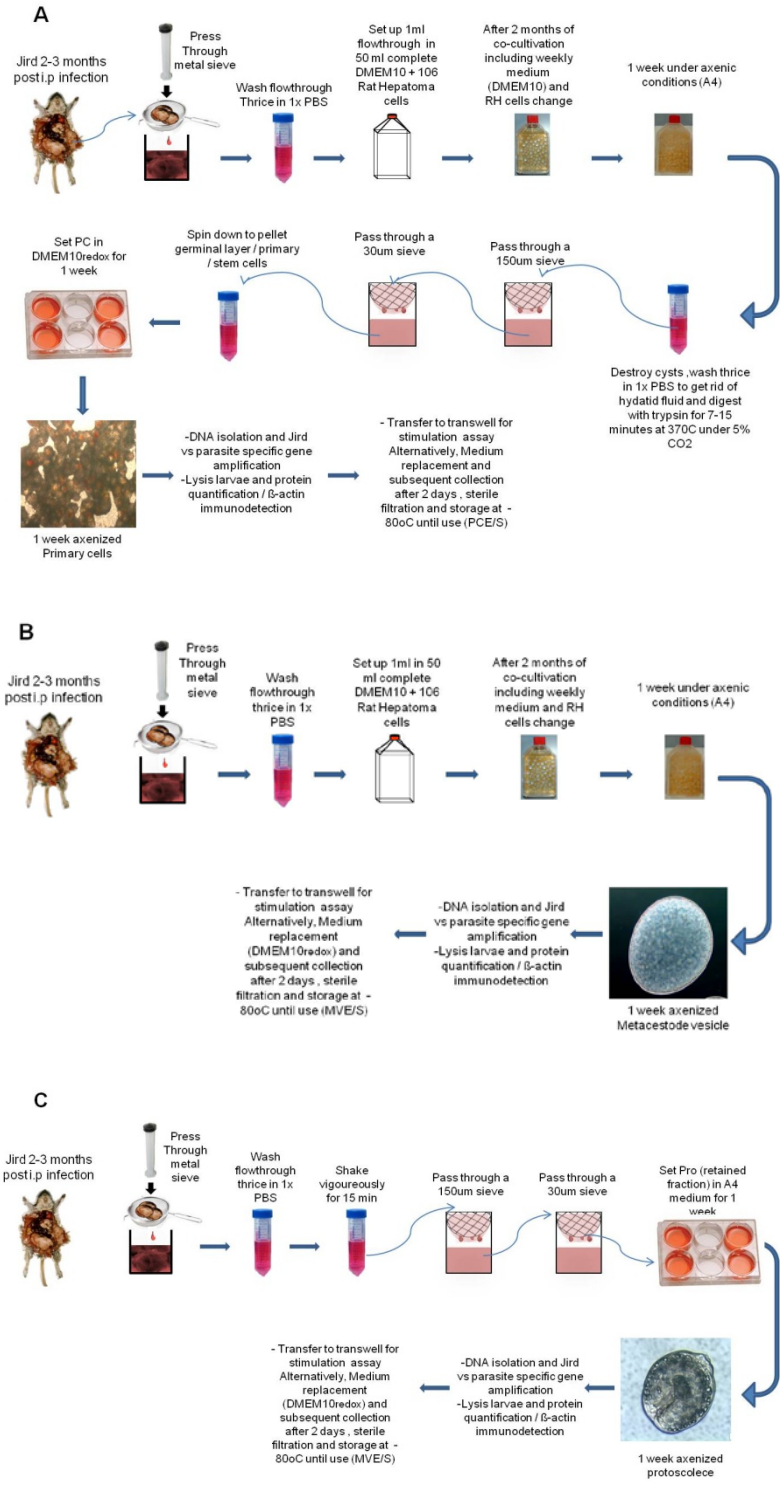
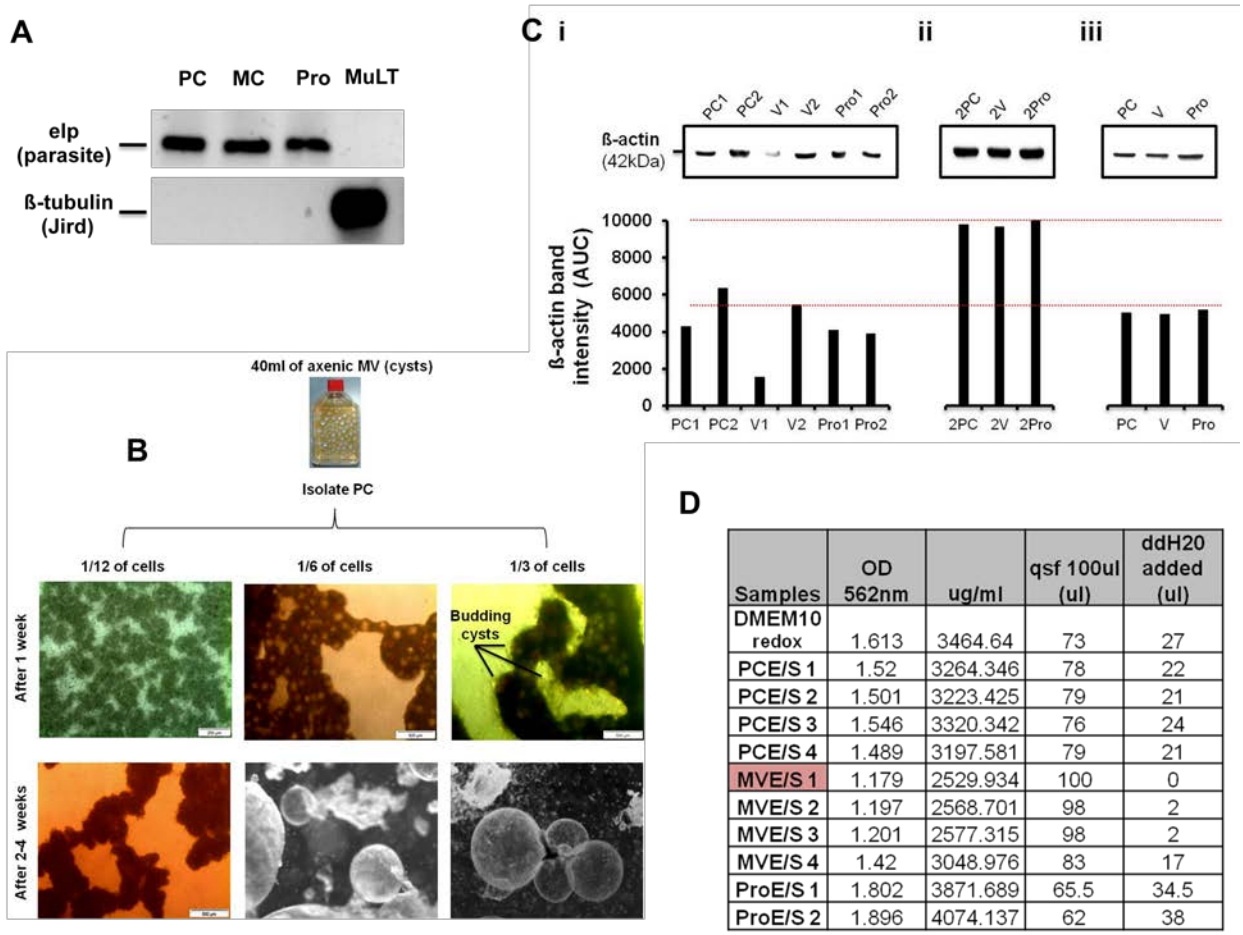


Figure 10: Preparation of *E. multilocularis* larval material. Isolation, maintenance and collection of E/S products from *E. multilocularis* primary cells (**A**), metacestode vesicles (**B**) and protoscoleces (**C**).



D

Samples	OD 562nm	ug/ml	qsf 100ul (ul)	ddH20 added (ul)
DMEM10 redox	1.613	3464.64	73	27
PCE/S 1	1.52	3264.346	78	22
PCE/S 2	1.501	3223.425	79	21
PCE/S 3	1.546	3320.342	76	24
PCE/S 4	1.489	3197.581	79	21
MVE/S 1	1.179	2529.934	100	0
MVE/S 2	1.197	2568.701	98	2
MVE/S 3	1.201	2577.315	98	2
MVE/S 4	1.42	3048.976	83	17
ProE/S 1	1.802	3871.689	65.5	34.5
ProE/S 2	1.896	4074.137	62	38

Figure 11: Qualitative and quantitative assessment of *E. multilocularis* larval material. (A) Isolated and axenically maintained larvae of *E. multilocularis* are free of host contaminants. Genomic DNA was isolated from parasite larvae (PC: primary cells, MV: metacestode vesicles, Pro: protoscoleces) 1 week post-axenic maintenance, or liver tissue from *M. unguiculatus* (MuLT). Each DNA sample was used as PCR template for amplification of parasite (*elp*) and jird (beta-tubulin) specific genes. (**B**) Determination of a reference amount of parasite primary cells for stimulation assays *in vitro*. Series of experiments on different amounts of primary cells kept in axenic culture. Note the development of 1/6 of the total amount of primary cells isolated from 40 ml of metacestode vesicles in vitro herein defined as reference quantity for larvae isolation. No budding cysts could be observed after a week but regeneration into metacestode vesicles was achieved within 2-4 weeks as reported *in vivo* with oncospheres [207]. (**C**) Normalization of parasite material. Various quantities of *E. multilocularis* larvae free from host cells were probed for β-actin content and the blots analyzed by ImageJ (**i**). Based on the values obtained, as a mean of quantification of the β-actin content, the starting material of larvae were adjusted (**ii**) and the reliability of such system of inference tested on half of the normalized quantities obtained from each larval stage (**iii**). The reference value defined here (2PC) was obtained from 7-days-old-cultured primary cells (1/6 of total) obtained from 40 ml of vesicles as previously defined. **(D)** Normalization of parasite excretory/secretory products. The total protein content of E/S products collected from *E. multilocularis* larvae were determined using BCA assay. The lowest value (highlighted) was used as a baseline for normalization by determining the amount necessary to give an equal final concentration in a total volume of 100ul. The solutions were topped to 100ul using double distilled sterile water as reported.

3.2 Excretory/secretory-products of *Echinococcus multilocularis* larvae induce apoptosis and tolerogenic properties in dendritic cells *in vitro*.

3.2.1 E/S products of *E. multilocularis* primary cells and metacestodes but not those of protoscoleces kill DC *in vitro*.

To compare the influence of E/S products from each of *E. multilocularis* larvae on DC, BMDC were exposed for 48 h to an equal biomass of each larvae through thin well inserts allowing uniquely fluid exchange but not cell-to-cell contact. BMDC viability appeared considerably reduced after exposure to primary cells and metacestode vesicles but largely unchanged with protoscoleces (Fig. 12A). Since metabolically active larvae could be depriving BMDC of nutrients thus inducing death by starvation, collected E/S products were used instead of live larvae. A similar reduction of BMDC viability was observed with primary cell secretions (Fig. 12) indicating that *E. multilocularis* primary cells and metacestodes release a factor (or factors) capable of inducing BMDC death.

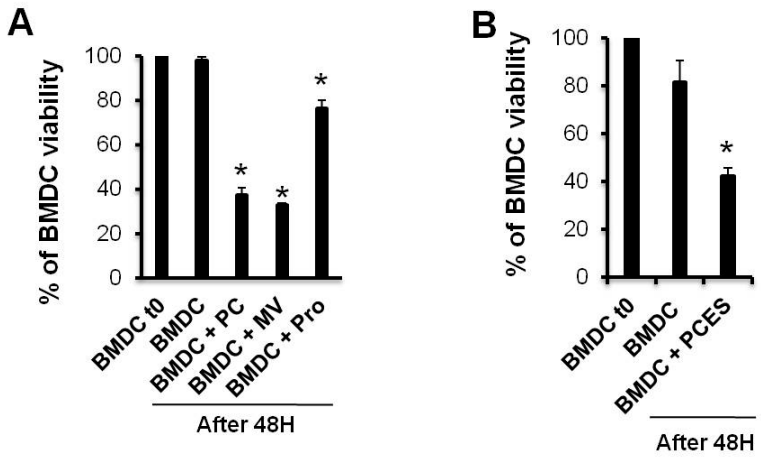


Figure 12: E/S products of *E. multilocularis* larvae kill BMDC *in vitro*. BMDC were either exposed to live larvae, separated by tissue culture inserts (A), or to collected larvae E/S products (B). 48 hours later, cells were harvested and viability assessed by trypan blue. Viability is expressed as a percentage of the number of living cells harvested to the number of living cells seeded. The results represent the mean ± SD from 4 independent experiments. *:$p<0.05$.

Since *Echinococcus* spp. hydatid fluid has been shown to lyse peritoneal macrophages, splenocytes, primary T-cells or T-cell lines [156-159], I reasonably hypothesized that the

cytotoxic factor (s) of *Echinococcus* hydatid fluid could be released in culture and thus mediate the observed BMDC death. To examine this hypothesis, mouse splenocytes were exposed to *E. multilocularis* metacestodes through transwells for up to 72 hours. Interestingly, metacestode secretions failed to affect mouse splenocyte viability in general (Fig. 13A) as well as splenic B-cells and non B-cells in particular (Fig. 13B). Furthermore, I found that human red blood cells exposed to any of the parasite larvae E/S products were also not lysed (Fig. 13C) suggesting that the cytotoxic factor(s) released by *E. multilocularis* primary cells and metacestodes do(es) not mediate BMDC death by cell lysis and functionally differ(s) from those previously described within the hydatid fluid of *Echinococcus* spp. [208–211].

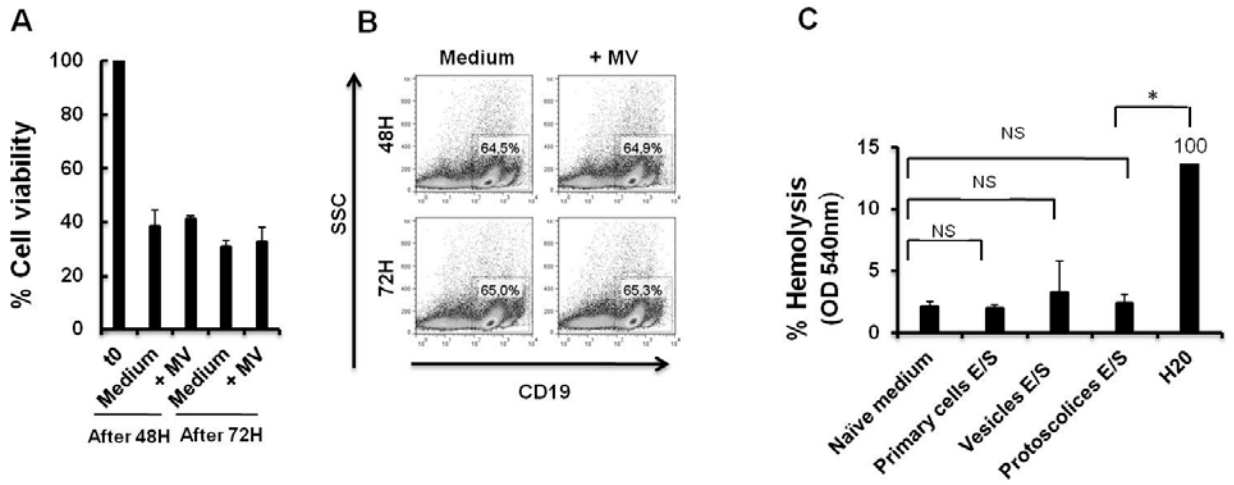


Figure 13: E/S products of *E. multilocularis* metacestode vesicles larvae do not kill murine splenocytes and fail to lyse human red blood cells *in vitro*. (**A**) Splenocytes from female C57BL/6 mice were exposed to metacestode vesicles (MV), separated through a transwell system, and viability was assessed using the Trypan Blue exclusion test after 48 h and 72 hours. (**B**) Within the splenic lymphocytes, as defined with respect to the forward and side scatter, CD19+ lymphocytes were stained and CD19− lymphocytes deduced by flow cytometry and the proportions of both cell populations (CD19− and CD19+) were monitored for changes upon exposure to metacestode vesicles. The scatter plots are representatives of the two experiments depicting similar results. (**C**) E/S products of *E. multilocularis* larvae fail to lyse human red blood cells. Human blood was collected from 2 healthy donors in heparinized tubes and for each donor, an equal volume of red blood cell suspension was seeded in parasite culture medium. Red blood cells were exposed to comparable amounts of primary cells, metacestode vesicles and protoscoleces through a transwell system and were maintained in culture. Culture medium (DMEM10redox) was used as negative control whereas an equal volume of water was used as positive control. After 48 hours, the transwells were removed, the plate centrifuged and the supernatant collected for measurement of hemoglobin content as a marker of red blood cell lysis. The percentage of hemolysis is expressed as a ratio of the sample absorbance over that of water (540nm) fixed as the 100% reference. (**A, C**)Bars are means ± SD from 2 independent experiments. (**C**) Results shown are means +- SD obtained with 2 healthy donors. *NS, not significant*; *, $p<0.05$.

3.2.2 E/S products of *E. multilocularis* primary cells and metacestodes induce DC death via apoptosis

A previous study has indicated that the expression of pro-apoptotic markers was significantly higher in lymphocytes following treatment with hydatid fluid from *Echinococcus* spp. [212]. I thus reasonably suggested that the death of BMDC following exposure to E/S products of *E. multilocularis* larvae could be mediated by apoptosis. To examine this, BMDC were exposed to the larvae E/S products for 48 h and the levels of Annexin V expression (a maker of apoptosis) and 7-AAD (a marker of necrosis for exclusion of necrotic events) expression were measured. My analyses revealed that BMDC considerably underwent apoptosis following exposure to E/S products of primary cells (early larva) and metacestode vesicles (mid-larva) where as those exposed to protoscolex E/S products did not. These findings indicate that *E. multilocularis* early and mid larval stages secrete (a) factor(s) that induce(s) BMDC death via apoptosis whereas protoscoleces failed to do so.

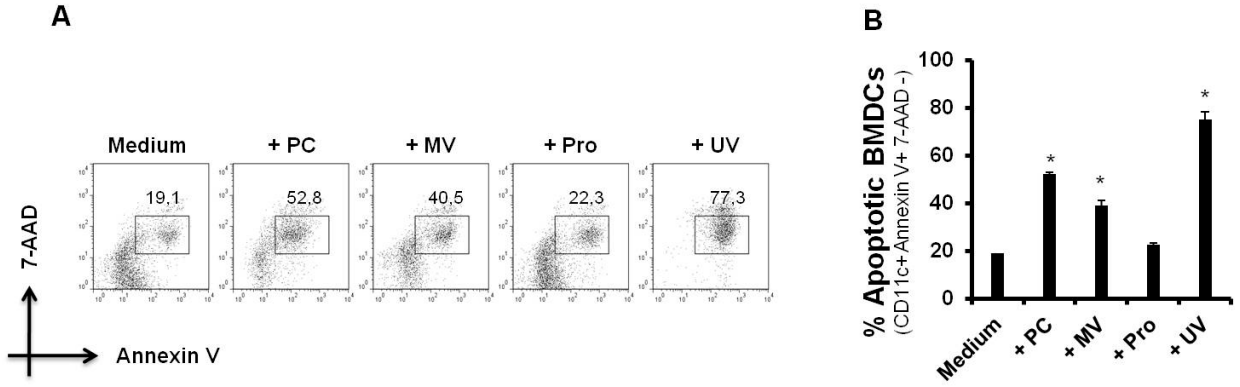


Figure 14: E/S-products of *E. multilocularis* larvae induce DC death via apoptosis. DC were exposed to comparable amounts of *E. multilocularis* larvae (primary cell aggregates, PC; metacestode vesicles, MV; protoscoleces, Pro), separated by a transwell system, for 24 hours. DC were then analyzed by flow cytometry for the expression of Cd11c, AnnexinV and 7-AAD. The proportion of apoptotic DC (Cd11c+AnnexinV+7-AAD−) was then determined. UV-treatment of DC was used as positive control for apoptosis-inducing conditions. (**A**) Representative plots of the proportion of AnnexinV+ 7-AAD− cells, gated on CD11c+ cells. (**B**) Mean percentage ± SD of apoptotic DC (Apo-DC) of 4 independent experiments. *=$p<0,05$.

3.2.3 E/S products of *E. multilocularis* primay cells and metacestodes but not those of protoscoleces impair DC maturation and responsiveness *in vitro*.

Crude extracts of *E. multilocularis* larvae have been shown not to mediate DC maturation *in vitro* [182]. To test whether the parasite E/S products influence the host DC, BMDC were treated *in vitro* with E/S products from each of the parasite larvae for 72 hours. Neither E/S products of primary cells nor those of metacestode vesicles could elicit BMDC maturation with respect to MHCII and CD86 expression after this time (Fig. 15A,B). DC exposed to E/S products of protoscoleces rather showed an intermediate maturation state when compared to fully matured LPS-pulsed DC (Fig. 15A,B). Furthermore, *E. multilocularis*-treated DC showed a differential cytokine secretion profile depending on the larval stage E/S products that were used (Fig. 15C). From an equal number of surviving *E. multilocularis*-treated DC, no detectable amount of IL-12p70 was secreted in the culture supernatants upon treatment with E/S products of any of the parasite larvae (Fig. 15C). Of interest, E/S products of primary cells triggered the release of IL-10 by DC. DC exposed to protoscolex E/S products only appeared to secrete IL-6 whereas metacestode vesicle E/S products could not elicit the release of detectable amounts of any of the cytokines (Fig. 15C). Collectively, these findings showed that the influence of *E. multilocularis* E/S products on DC is larva-specific. Whereas E/S products of primary cells render DC tolerogenic, protoscolex E/S products are rather immunogenic.

To investigate whether E/S products of *E. multilocularis* larvae could impair DC activation as observed during experimental AE [213], BMDC first exposed to each of the *E. multilocularis* larvae E/S products for 24 hours were harvested and an equal number of surviving cells were restimulated with LPS (0.1ug/ml) for an additional 48 hours. Interestingly, DC exposed to E/S products of primary cells or metacestode vesicles had an impaired maturation in response to LPS (Fig. 16A,B). DC exposure to protoscolex E/S products failed to show such an effect (Fig. 16A,B). Furthermore, DC treated with primary cell or metacestode vesicle E/S products showed a reduced level of IL-12p70 secretion in response to LPS (Fig.16C). Interestingly, LPS-driven release of IL-12p70 was completely abrogated in DC treated with protoscolex E/S products (Fig.16C). These data show that E/S products of early (primary cells) and mid (metacestode vesicles) but not late (protoscoleces) *E. multilocularis* larvae impair classical DC maturation and responsiveness *in vitro*.

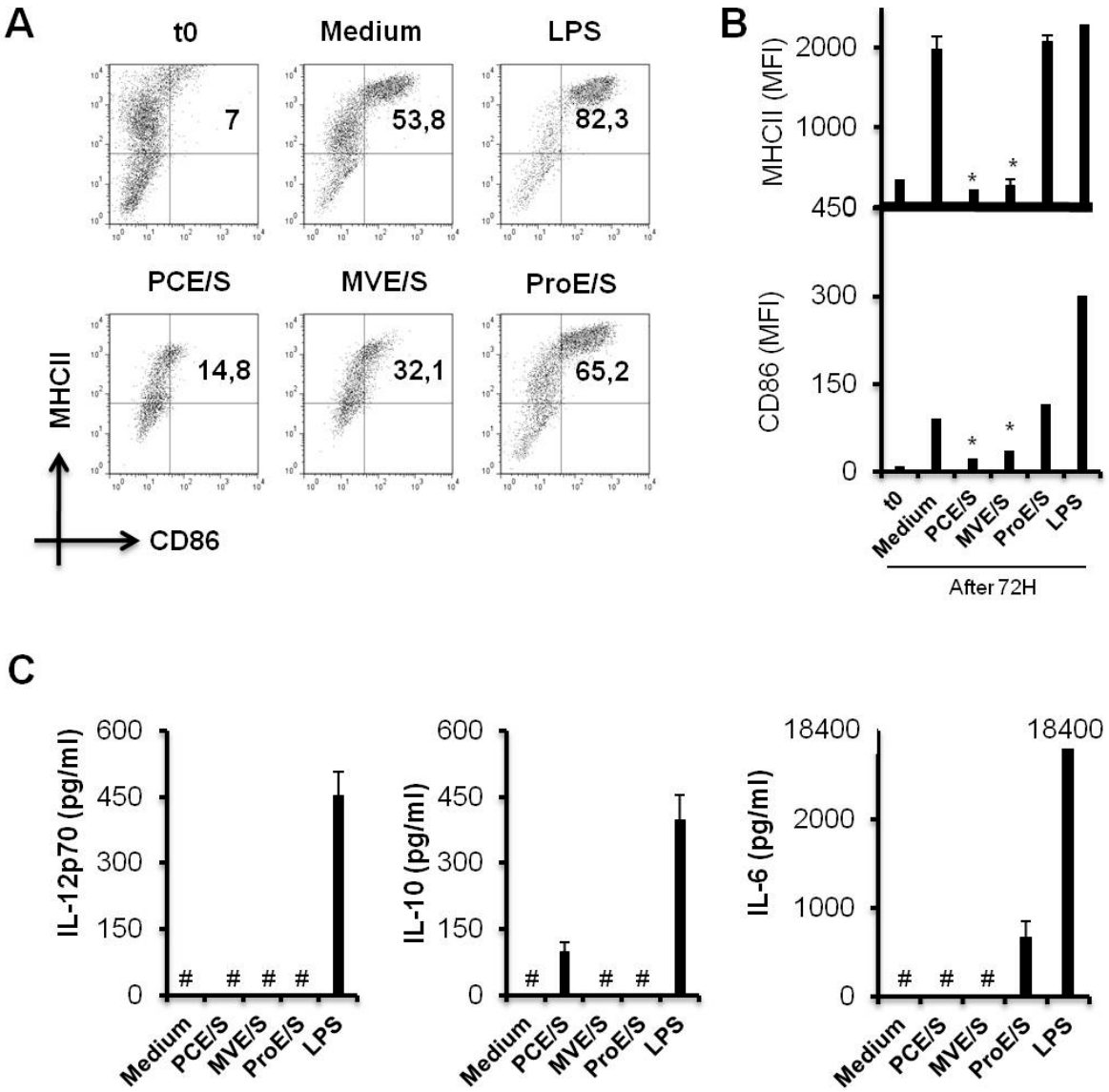


Figure 15: E/S-products of *E. multilocularis* larvae differentially affect DC maturation and cytokine profile. DC were exposed to *E. multilocularis* larvae (primary cell aggregates, PC; metacestode vesicles, MV; protoscoleces, Pro), separated through a transwell system, for 72 hours and their maturation was assessed by flow cytometry after staining for surface markers CD11c, MHCII and CD86. (A) Representative scatter plots showing the proportion of matured DC (upper right quadrant) with respect to MHCII and CD86, gated on CD11c cells. t0 indicates maturation at the beginning of the experiment. (B) Mean fluorescence intensities of MHCII and CD86 on DC after 72 hours of treatment. (C) DC supernatants were collected and analyzed for the presence of IL-12p70, IL-10 and IL-6 by ELISA. Data shown are the mean ± SD of 4 independent experiments. # : below the ELISA kit detection limit.

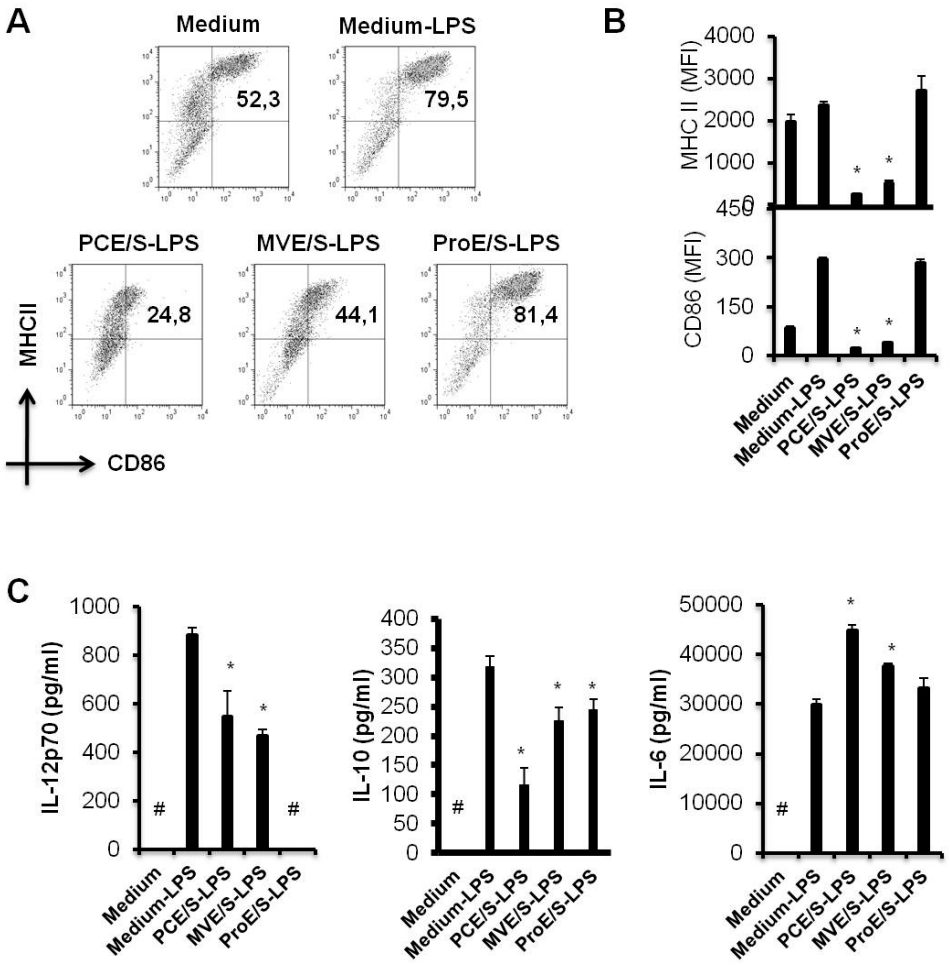


Figure 16: E/S-products of *E. multilocularis* differentially alter DC responsiveness to LPS.DC were first exposed to *E. multilocularis* larvae (primary cell aggregates, PC; metacestode vesicles, MV; protoscoleces, Pro), separated through a transwell system, for 24 hours. DC were then harvested, counted and seeded at equal numbers of living cells in medium containing LPS for 48 h, prior to staining and flow cytometric detection for surface markers CD11c, MHCII and CD86. (**A**) Representative scatter plots showing the proportion of matured DC (upper right quadrant) with respect to MHCII and CD86, gated on CD11c cells. (**B**) Mean fluorescence intensities of MHCII and CD86 on DC. (**C**) DC supernatants were collected and analyzed for the presence of IL-12p70, IL-10 and IL-6 by ELISA. Data shown are the mean ± SD of 4 independent experiments. *=p<0,05. # : below the ELISA kit detection limit

Taken together, these findings indicated that E/S products of *E. multilocularis* larvae directly infecting the intermediate host (rodents, humans), i.e. primary cells and metacestodes, but not those of the larva released within the definitive carnivorous host (foxes, dogs), i.e. protoscoleces, induce apoptosis and tolerogenic properties in DC *in vitro*.

3.3 Expansion of regulatory T-cells (Treg) by *E. multilocularis* metacestodes

3.3.1 E/S products of *E. multilocularis* metacestode vesicles promote Foxp3 expression and the production of the anti-inflammatory cytokine IL-10 by CD4+ T-cells *in vitro*.

Maturation-resistant DC have been defined as tolerogenic DC [214]. Those DC share common characteristics, such as moderate or high expression of surface MHC molecules, in association with a low ratio of costimulatory to inhibitory signal and an impaired ability to secrete Th1 cell-driving cytokines, such as IL-12p70 [214]. Furthermore, tolerogenic DC constantly present antigens to T-cells in a fashion that promotes tolerance, at least in part, through the control of regulatory T-cells [215] of which Foxp3+ regulatory T-cells are by far the best studied [216,217]. With regards to my previous observations, E/S products of *E. multilocularis* primary cells and metacestode vesicles evoked a tolerogenic profile in BMDC. It would therefore be reasonably expected that E/S products of primary cells and metacestode vesicles condition DC to expand regulatory T-cells as a result of their tolerogenic properties. To examine this hypothesis, OVA-loaded BMDC were co-cultured with OT-II CD4+CD25- T-cells for 5 days in the presence of E/S products of primary cells, metacestode vesicles or protoscoleces as compared to recombinant TGF-β (positive control since shown to induce Foxp3+ regulatory T-cell conversion [218]). CD4+ T-cells were then analyzed by flow cytometry for expression of IL-2 receptor alpha (CD25) and Foxp3 (Fig.17A,B). No expansion of CD4+CD25+Foxp3+ Tregs could be observed with primary cell or protoscolex E/S products (Fig.17A,B). Strikingly, metacestode vesicle E/S products induced a considerable expansion of Foxp3+ Tregs, up to 3 fold more than the untreated controls (Fig. 17A,B).

Mechanistically, Foxp3+ Treg expansion in this experimental setting can either be due to the *de novo* conversion of Foxp3-CD4+ T-cells and/or the proliferation of already present (contaminating) Foxp3+CD4+ T-cells. In order to discriminate between these mechanisms, naïve CD4+ T-cells from OT-II×RAG-1(-/-) mice, devoid of contaminating Foxp3+CD4+ T-cells (Fig. 17C), were used for the Treg conversion assays in place of naïve CD4+ T-cells from OT-II mice, to specifically monitor the rate of *de novo* Treg conversion from naïve T-cells. Interestingly, co-

cultures of DC and OT-II×RAG-1(-/-) CD4+ T-cells exposed to E/S products of metacestode vesicles showed a higher rate of *de novo* Foxp3+ Treg conversion (Fig. 17D,E). These findings indicated that E/S products of *E. multilocularis* metacestode vesicles promote Foxp3+ Treg conversion *in vitro*.

Foxp3+ Treg functions have been tightly linked to the anti-inflammatory cytokine IL-10 [219–221]. Furthermore, infections with *E. multilocularis* larvae were reported to provoke a higher production of IL-10 by the host CD4+ T-cells [142,143,151,163,164]. Since E/S products *of E. multilocularis* metacestodes were able to expand Foxp3+ Tregs *in vitro*, their effect on IL-10 release by CD4+ T-cells was investigated. Interestingly, IL-10 release by CD4+ T-cells was considerably triggered if the latter were activated in the presence of metacestode vesicle E/S products (Fig. 17F).

Collectively, these findings indicate that *E. multilocularis* potentiate the regulatory arm of the host CD4+ T-cell response *in vitro*.

3.3.2 *E. multilocularis* metacestodes expand bona fide Foxp3+ regulatory T-cells in mice.

Having shown that metacestodes of *E. multilocularis* excrete/secrete factor(s) that drive regulatory CD4+ T-cell expansion *in vitro*, I sought to determine whether these parasite larvae can expand such cells in the course of an infection. Since, the intraperitoneal injection of *E. multilocularis* larvae is an accepted experimental model for secondary echinococcosis [222], metacestodes were injected in the peritoneum of mice and the peritoneal exudates were analyzed over time for CD4+CD25+Foxp3+ Tregs (Fig. 18).

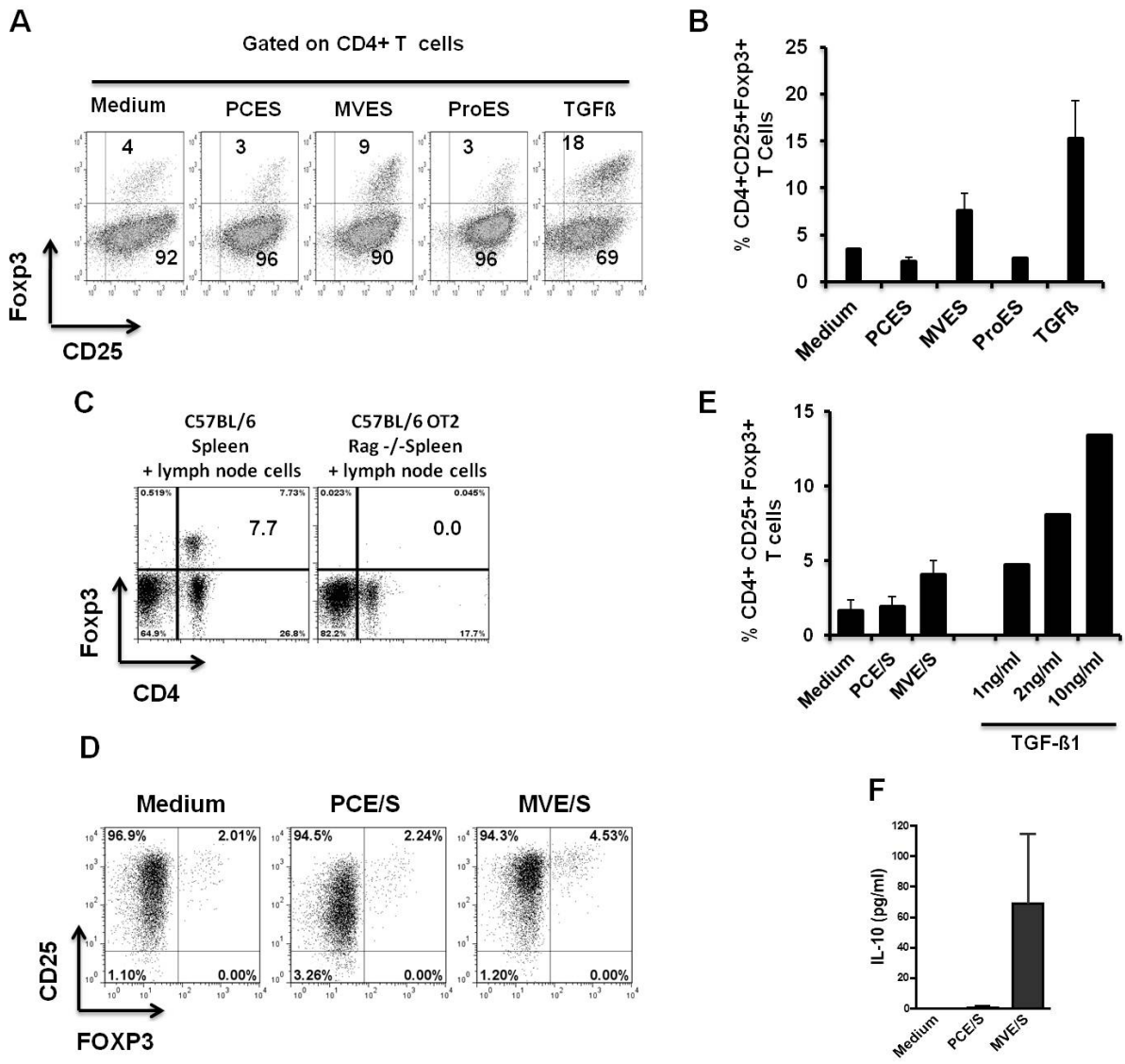


Figure 17: E/S-products of *E. multilocularis* metacestode vesicles induce *de novo* CD4+CD25+Foxp3+ T-cell conversion *in vitro*. Freshly generated DC (Day 8) were co-cultured with naïve CD4+CD25− OT2 T cells at a DC:T-cell ratio of 1:3 in R10 medium supplemented with OVA peptide (200ng/ml) in the presence of medium conditioned with primary cell aggregates (PCE/S), metacestode vesicles (MVE/S) or protoscoleces (ProE/S). After 5 days of incubation, cells were harvested and stained for CD4, CD25 and Foxp3 prior to flow cytometry analysis. As a positive control for CD4+CD25+Foxp3+ T cell conversion, recombinant Transforming Growth Factor-β was used (TGF-β). (**A**) Representative plots showing the proportion of CD4+CD25+Foxp3+ T-cells (upper right quadrant) of pooled duplicates from 2 independent experiments, summarized in (**B**). (**C**) Treg (CD4+ Foxp3+) staining of total spleen and lymph node cells from wild type C57BL/6 or C57BL/6 OT2 Rag -/- mice over a C57BL/6 background. (**D**) Reperesentative plots of Treg conversion assay performed with OT2 Rag -/- CD4+ T cells as in **A** and summarized in (**E**). (**F**) E/S products of *E. multilocularis* metacestode vesicles prompt IL-10 release by CD4+ T cells. Freshly isolated CD4+CD25- T cells (2 X 10^5 /ml) were stimulated with α-CD3 (0.1µg/ml) and α-CD28 (5µg/ml) in the presence of fresh medium or supplemented at 50% with E.multilocularis larvae products (MVE/S or PCE/S as control). Three days later, the T-cell supernatants were collected and probed for IL-10 concentration by Elisa. (**B,E,F**) The bars represent the mean ± SD from two independent experiments.

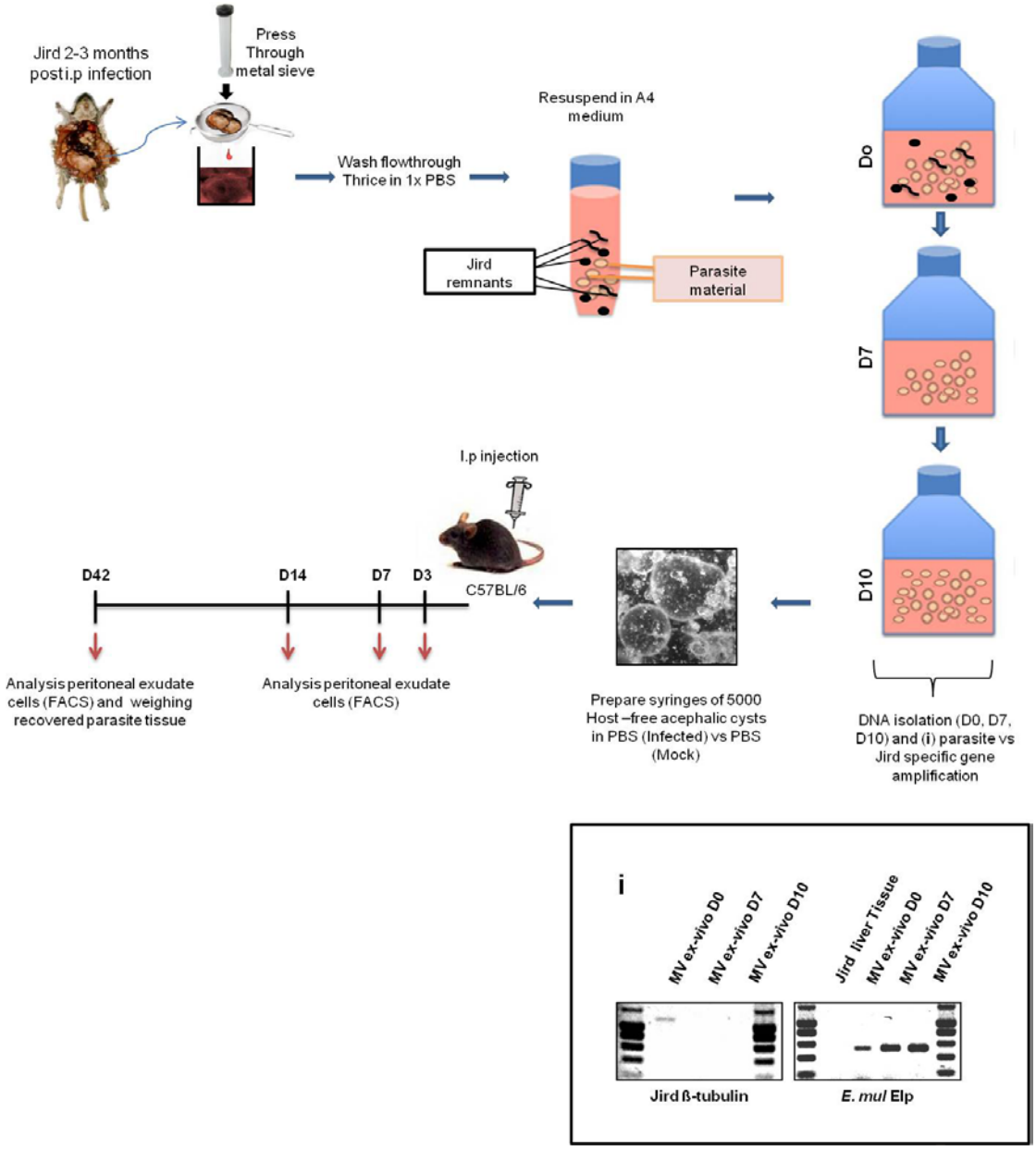


Figure 18: Experimental set up for the follow up of mice intraperitoneally infected with *E. multilocularis* metacestodes. Parasite homogenate was excised from infected jirds and thoroughly washed. Host contaminants were removed by axenic cultivation (see material and methods) and the axenization procedure assessed using host versus parasite gene specific amplification. 5000 acephalic cysts (metacestode vesicles) resuspended in PBS or PBS alone was injected in the peritoneum of C57BL/6 mice. Peritoneal exudates were then recovered and analyzed by FACS over a 6 weeks period. Finally, the mass of parasite material recovered was determined at the end of the study period as a measure of disease progression. **(i)** Qualitative assessment of *E. multilocularis* cyst material**.** Chromosomal DNA was isolated from liver tissue of healthy Jirds and parasite metacestode tissue harvested from infected jirds and kept for 0, 7 or 10 days under axenic conditions. The presence of host contaminants was assessed by organism-specific PCR, namely the host-specific β-tubulin (Left) and the parasite-specific elp (Right) genes were amplified.

An increase of total peritoneal cell numbers was observed as early as day 3 post injection and gradually rose throughout the study time (42 days) following injection of metacestodes. In naïve mice, peritoneal cell numbers remain relatively constant during this study period. (Fig. 19A). CD4+ T-cells showed a similar kinetics over time, increasing steadily in infected mice but remaining constant in naïve mice (Fig. 19B). Interestingly, despite the central role of CD4+ T-cells in restraining *E. multilocularis* metacestode proliferation [161], about 10 fold more parasite tissue was recovered from infected mice autopsied at the end of the study time (Fig. 19C). Altogether, these observations show that intraperitoneal injection of *E. multilocularis* metacestodes in mice results in parasite proliferation and the expansion/recruitment of CD4+ T-cells (Fig. 19) within/to the peritoneum.

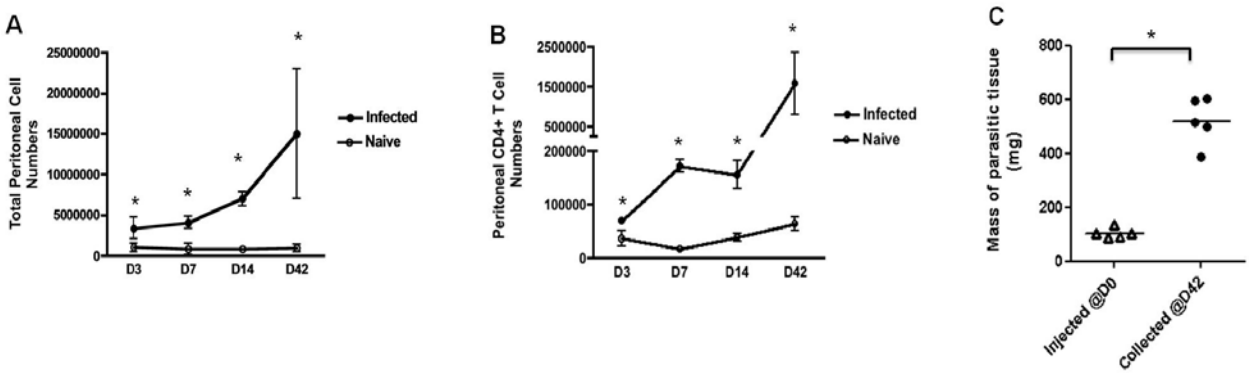


Fig 19: Intraperitoneally injected *E. multilocularis* cysts proliferate in the face of accumulating peritoneal CD4+ cells. (**A, B**) Peritoneal exudates recovered from naive (PBS) or infected (live cysts) mice at different time points were counted and analyzed by flow cytometry for CD4 expression. Parasite-driven accumulation of total (**A**) or CD4+ cells (**B**) is shown for D3-42 post injection. (**C**) Masses of parasitic tissue injected and recovered after 42 days. Horizontal bars stand for mean levels. Data represent means ± SD from groups of five mice for each time point assayed individually (Infected). Naive mice were clustered in sub-groups of 3 mice pooled as one per assay (15 mice for each time point).*, $p < 0.05$.

To determine whether *E. multilocularis* metacestodes induce regulatory T-cell expansion *in vivo*, peritoneal CD4+ T-cells were analyzed by flow cytometry for expression of CD25 and Foxp3. Infected mice displayed a significantly higher number of CD4+CD25+Foxp3+ regulatory T-cells when compared to naïve mice at every time point during the study (Fig. 20A). Startlingly, a higher frequency of CD4+CD25+Foxp3+ regulatory T-cells could be observed 7 days post

injection in infected mice when compared to naïve mice (Fig. 20B). To substantiate this finding, the ratios of CD4+CD25+Foxp3+ regulatory T-cells to CD4+CD25+Foxp3- effector T-cells were determined (Fig. 20C). Supportively, at day 7 post injection, infected mice showed a higher ratio of regulatory T-cells per effector T-cells than naïve mice (Fig. 20D) indicating that *E. multilocularis* metacestodes expand Foxp3+ regulatory T-cells *in vivo*.

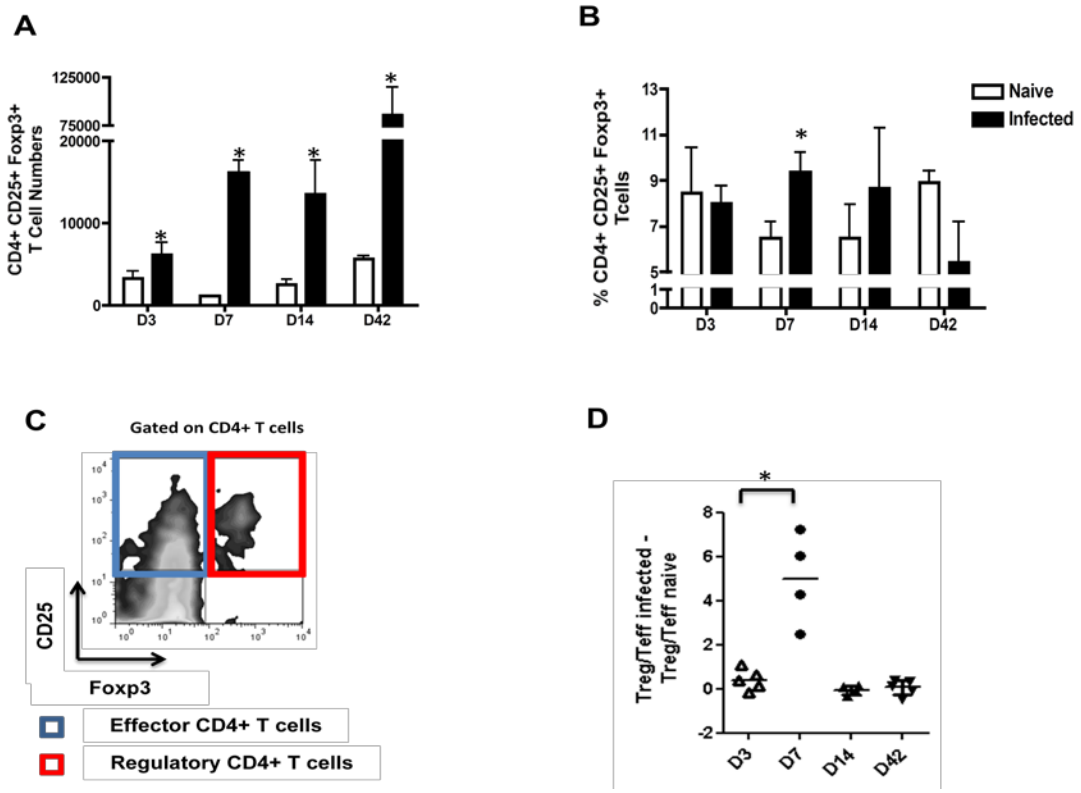


Fig 20: Initial CD4+ T-cell responses to *E. multilocularis* cysts are biased towards Foxp3+ regulatory T-cells. Analysis of peritoneal cells harvested at days 3, 7, 14 and 42 postinfection respectively by flow cytometry over the Treg markers CD4, CD25 and Foxp3. (**A,B**) Kinetics of CD4+CD25+Foxp3+ Treg numbers and frequencies is displayed. (**C**) Representative cluster of CD4+CD25+ population into effector or regulatory T-cells with regards to the level of Foxp3 expression. (**D**) kinetics of CD4+CD25+Foxp3+(Treg) / CD4+CD25+Foxp3- (Teff) ratio over time. Each ratio obtained for given infected mice was substracted of the corresponding naive mice ratio. Data represent means ± SD from groups of five mice for each time point assayed individually (Infected). Naive mice were clustered in sub-groups of 3 mice pooled as one per assay (15 mice for each time point).*, $p < 0.05$.

To investigate whether peritoneal CD4+CD25+T-cells expanded by *E. multilocularis* metacestodes are functional Tregs in terms of suppressive capacity, cells were purified from the peritoneal lavage of metacestode-infected mice 7 days post injection, and from naive spleens. These were then used in a suppression assay, with naive splenic CD4+CD25− cells that had been CFSE-labeled as responder cells, and were stimulated with irradiated APCs and anti-CD3. As can be seen in Fig. 21A, infected peritoneal lavage Tregs are as suppressive as naive splenic Tregs in this assay, with similar proportions of labeled cells in each division in the presence of CD4+CD25+ cells from either source (represented graphically in Fig. 21B).

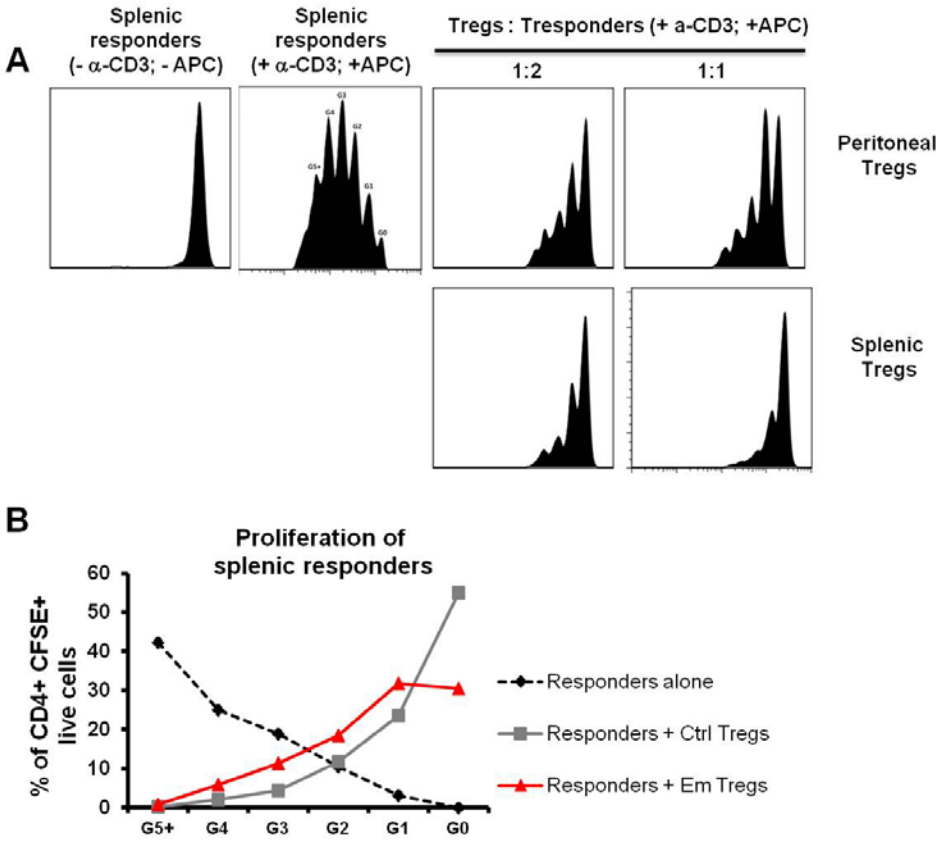


Fig 21: Intraperitoneal injection of *E. multilocularis* metacestodes promotes the expansion of CD4+CD25+ Tregs that are functionally suppressive *in vitro*. Peritoneal exudates from mice infected for 7 days with 5000 acephalic *E. multilocularis* cysts, and naive splenocytes from control mice, were prepared by CD4+T cell magnetic selection, then FACS-sorted into CD4+CD25+ and CD4+CD25- populations. Splenic naive CD4+CD25- cells (responders) were then polyclonally stimulated in the presence or absence of CD4+CD25+ cells from either control or *E.multilocularis*-infected mice. (**A**) Representative plots of CFSE-labeled responder cells proliferation with increasing amounts of regulatory T cells. (**B**) Proportions of labeled live CD4+ cells in each generation of assay conducted at 1:1 ratio, as gated by CFSE dilution. Similar results were obtained in an additional experiment.

3.3.3 *E. multilocularis* metacestodes expand regulatory T-cells through the host TGF-β signaling loop.

Since Foxp3+ regulatory T-cell fate is tightly regulated by the TGF-β signaling loop [103,218,223], TGF-β signaling antagonists were used to unravel the role of this signaling cascade in the ability of *E. multilocularis* metacestode products to promote Foxp3+ regulatory T-cell conversion. Addition of anti-TGF-β neutralizing antibodies, which bind to all host isoforms [224], or a TGF-β type I receptor (ALK 5) inhibitor, SB431245 [225], abrogated the ability of metacestode E/S products to promote Treg conversion *in vitro* (Fig. 22). These results show that E/S products of *E. multilocularis* metacestodes promote *in vitro* Treg conversion through the TGF-β pathway and in a TGF-β-dependent manner.

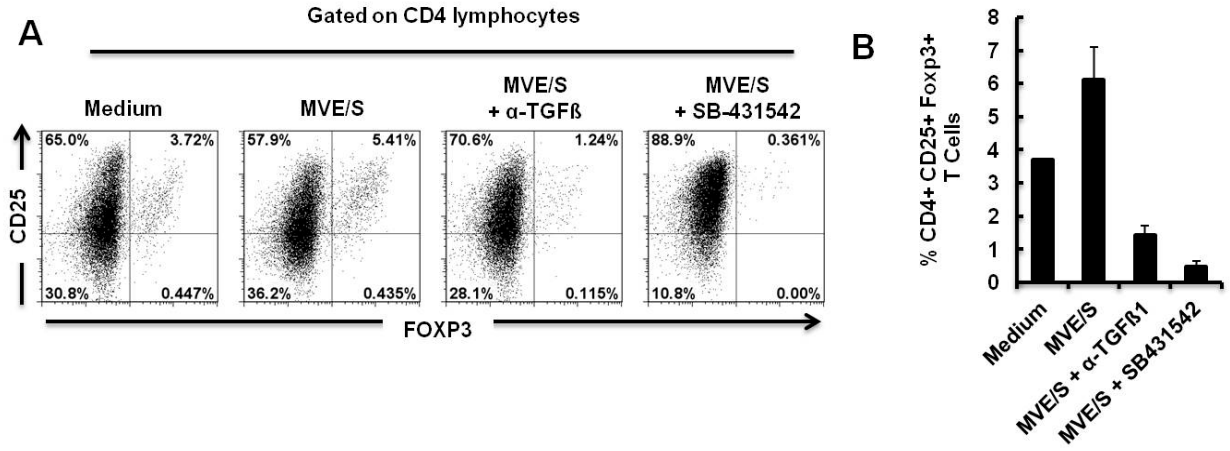


Fig 22: Blockade of the host TGF-β signaling loop prevents *E. multilocularis* cyst-driven Treg conversion *in vitro*. Freshly generated DC (day 8) were co-cultured with naive CD4+CD25- OT-II T cells at a DC:T cell ratio of 1:3 in medium supplemented with OVA peptide in the presence of medium conditioned with *E.multilocularis* cysts (MVE/S). In some instances blockade of soluble TGF-β (20ug/ml, pan-TGF-β antibody 1D11) or of the TGF-β signaling (5uM, ALK4,5,7 inhibitor SB431542) was performed simultaneously with MVE/S addition. 5 days later, the cells were stained for CD4, CD25 and Foxp3 prior to flow cytometry. Recombinant porcine TGF-β1 was used as standard. (**A**) Representative plots showing the proportion of CD4+CD25+Foxp3+ T cells (Upper right quadrant) of 2 experiments, summarized in (**B**). Data presented include experiments conducted elsewhere (Fig. 17A).

3.4 An Activin-ortholog secreted by *Echinococcus multilocularis* metacestodes promotes regulatory T-cell conversion *in vitro.*

3.4.1 EmACT is a TGF-β superfamily member secreted by *in vitro* cultivated *E. multilocularis* metacestodes.

Having shown that E/S products of *E. multilocularis* metacestodes promote TGF-β-driven Foxp3+ Treg conversion, I sought to identify parasite factors mediating this effect. Since Freitas and collaborators identified a TGF-β ligand homologue, SmInact, in the genome of the related flatworm *Schistosoma mansoni* [226], a tblastn search of the Welcome Trust's Sanger Institute's *E. multilocularis* protein database using the C-terminal region of the *S. mansoni* Inhibin/Activin (SmInACT) sequence was performed. Three hits (two Bone Morphogenetic Proteins and one Inhibin beta A) were identified and further studied elsewhere [227]. In the present work, EmuJ_000178100, the Inhibin beta chain A homologue was characterized given the reported ability of mammalian inhibin beta A homodimers or activin A to promote TGF-β-induced regulatory T-cell conversion [228,229]. In mammals, heterodimers of inhibin α and β chains are denominated 'Inhibin' while homodimers of Inhibin β chains form 'Activin' [230]. Since there is only one β-chain orthologue present in *E. multilocularis* and no α-chain ortholog, I named the respective factor EmACT (*E. multilocularis* activin) since only β-chain homodimers of EmACT can be formed.

The 1.5-kb, full-length Em*act* transcript was amplified from cDNA of the parasite metacestodes (Fig. 23A) with a 506 residues long deduced amino acids sequence (Fig. 23B). The deduced amino acid sequence of EmACT contains a signal sequence and three putative N-linked glycosylation sites at position 133, 257 and 382 (Fig. 23B) indicative of its potential secretion by the parasite metacestodes either into hydatid fluid and/or to the extracystic environment. EmACT contains the molecular hallmarks for a TGF-β superfamily member, including a putative basic proteolytic cleavage site [231] located at position 374 (RTRR) where the bioactive, C-terminal domain (130 amino acids) is usually enzymatically separated from the N-terminal pro-domain (Fig. 23B). Within the bioactive domain, EmACT is 49% identical to SmInAct from *Schistosoma mansoni*, 34% identical to human Inhibin beta A (subunit of activin A), 25% identical to DAF-7 from *Caenorhabditis elegans*, 26% identical to dActivin from *D. melanogaster*, and 28%

identical to human TGF-β1 (Fig. 23C). Nine invariant cysteine residues (Fig. 23B), essential for proper dimerization and tertiary structure formation of TGF-β superfamily members [232,233], of which two cysteines (Fig 23. C) are characteristic of TGF-β/Activin subfamily members [232,233], are all present in EmACT. Moreover, phylogenetic analysis of EmACT among other TGF-β superfamily members grouped this homolog with members of the TGF-β/Activin subfamily (Fig. 23D), and further clusters EmACT with TGF-β homologs from the parasitic flatworm *Schistosoma mansoni* (SmInAct), the free-living nematode *C. elegans* (DAF-7) and the parasitic nematodes *Brugia malayi* (Bm-TGH-2) and *Strongyloides stercoralis* (Ss-TGH-1).

To determine the expression of Em*act* at the transcript level, reverse transcriptase–polymerase chain reaction (RT-PCR) was performed on cDNA from primary cells, metacestodes and protoscoleces. Em*act* appeared to be transcribed by all the parasite larvae [227]. Western analyses using mouse polyclonal antibodies raised against recombinant EmACT (as depicted in Fig. 24A,B,C) recognized four distinct bands (14 kDa, 18kDa, 25kDa and 28 kDa) in *E. multilocularis* metacestode secretions (Fig. 24D).

3.4.2 EmACT promotes IL-10 production and expression of the master regulatory transcription factor Foxp3 in CD4+ T-cells *in vitro*.

For functional characterization of the EmACT polypeptide, the protein-coding region spanning amino acid 30 to the stop codon (EmACT without signal peptide) was expressed in HEK 293T (Human Embryonic Kidney) cells fused at the N-terminal to the murine Ig kappa chain leader sequence for optimal protein secretion (pSecTag2/Hygro, Invitrogen) under the control of the human cytomegalovirus promoter (see section 2.4.5.2 for construct design). As shown by Western analysis of the supernatant of Em*act*-transfected HEK cells, recombinant EmACT (rEmACT) was secreted to the medium as 14-28 kDa polypeptides (Fig. 25A) similar to the previously observed secretion pattern of EmACT by *in vitro* cultivated *E. multilocularis* metacestodes (Fig. 24D).

To assess the effect of EmACT on immune cells, BMDC were stimulated for 24 hours with normalized supernatants (Fig. 25B) from HEK cells transfected with either empty pSecTag2/Hygro vector or pSecTag2/Hygro-Em*act* construct (see section 2.4.5.2).

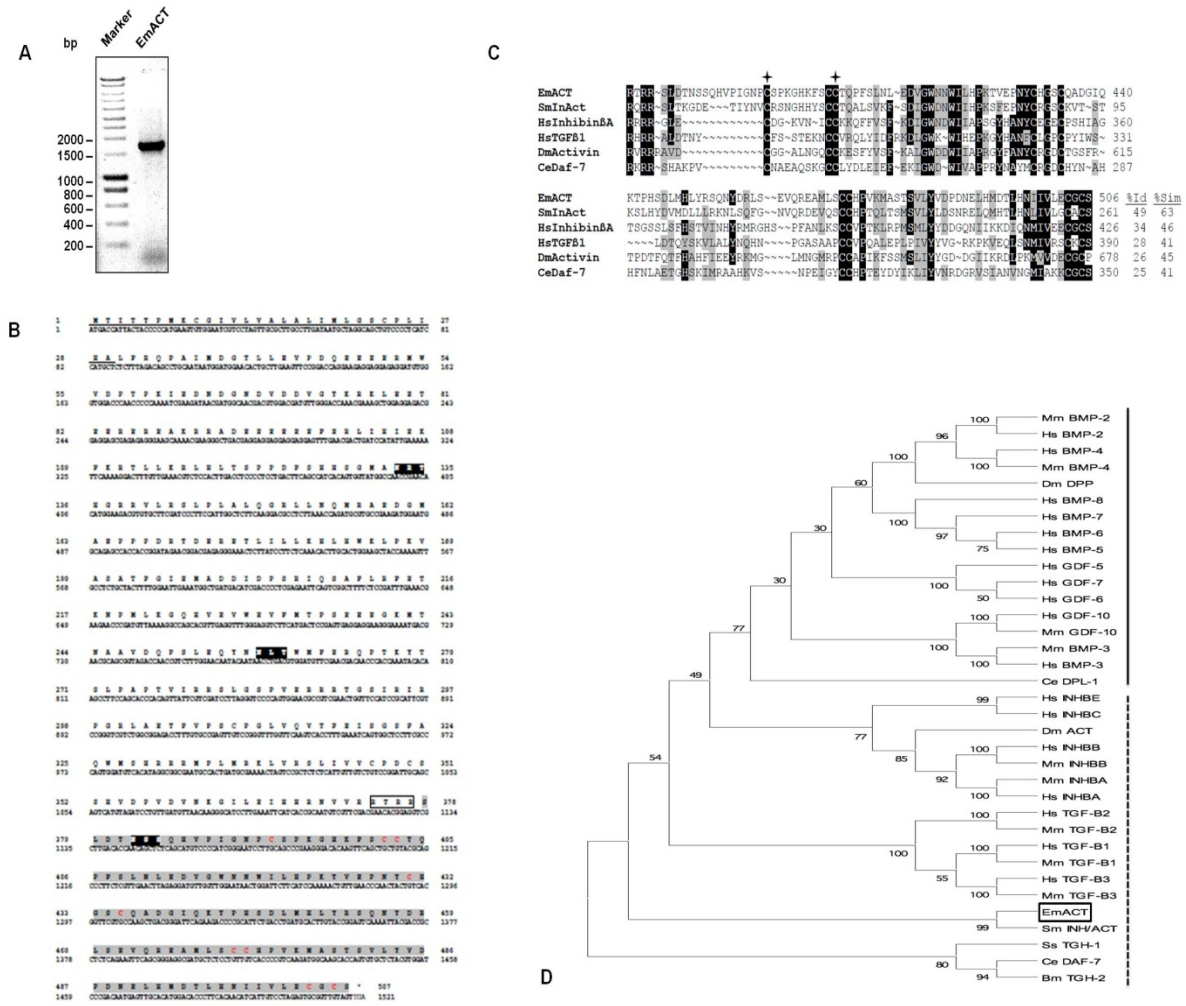


Figure 23: Em*act*, amplification and sequence features. (**A**) Full-length amplification of the Em*act* coding sequence using primers corresponding to the 5´and 3´coding termini (EmACT_Dw and EmACT_Up). 1µl of metacestode cDNA was used as template for PCR with a high fidelity DNA polymerase (Phusion® High-Fidelity DNA Polymerase, New England Biolabs). 2µl of the amplicon were resolved on a 1.5% agarose gel and stained with Ethidium bromide prior to visualization under a UV transilluminator. (**B**) Nucleotide and deduced amino acid sequence of the *E.multilocularis* Em*act* cDNA. The TGF-β superfamily homology region located at the C-terminus, is shaded in grey. The potential proteolytic cleavage cleavage site is an open box. The potential glycosylation sites (NRT, NLT and NSS) are shown in solid boxes. Nine cysteine residues found in invariant positions in the active domain are displayed in red. The 5´signal sequence is underlined. **(C)** Alignment of the C-terminal amino acid sequences of EmACT and five other representatives of the TGF-β/Activin subfamily of the TGF-β superfamily. Residues that are identical are displayed in black, similarities in grey. Gaps introduced to maximize the alignment are represented by tildes. Two conserved cysteines found only in TGF-β/Activin subfamily are shown with asterisks. Numbers at the start and finish of each line correspond to the amino acid numbers in each respective sequence. The percentages of identities (% Id) and similarities (%Sim) to EmACT are given for each sequence. Accession numbers for the sequences shown are as follows: Em*act*, HF912278; S. mansoni InAct, A4UAH0 ;Human Inhibin beta A, P08476 ; Human TGF-β1, P01137 ;*D. melanogaster* Activin, O61643; *C. elegans* DAF-7, P92172. **(D)** Phylogenetic representation of the sequence relationship between active domains of members of the TGF-β superfamily. Numbers represent bootstrap values. EmACT is displayed within an open box. Solid line represents the BMP subfamily, dashed line clusters the TGF-β/Activin subfamily.

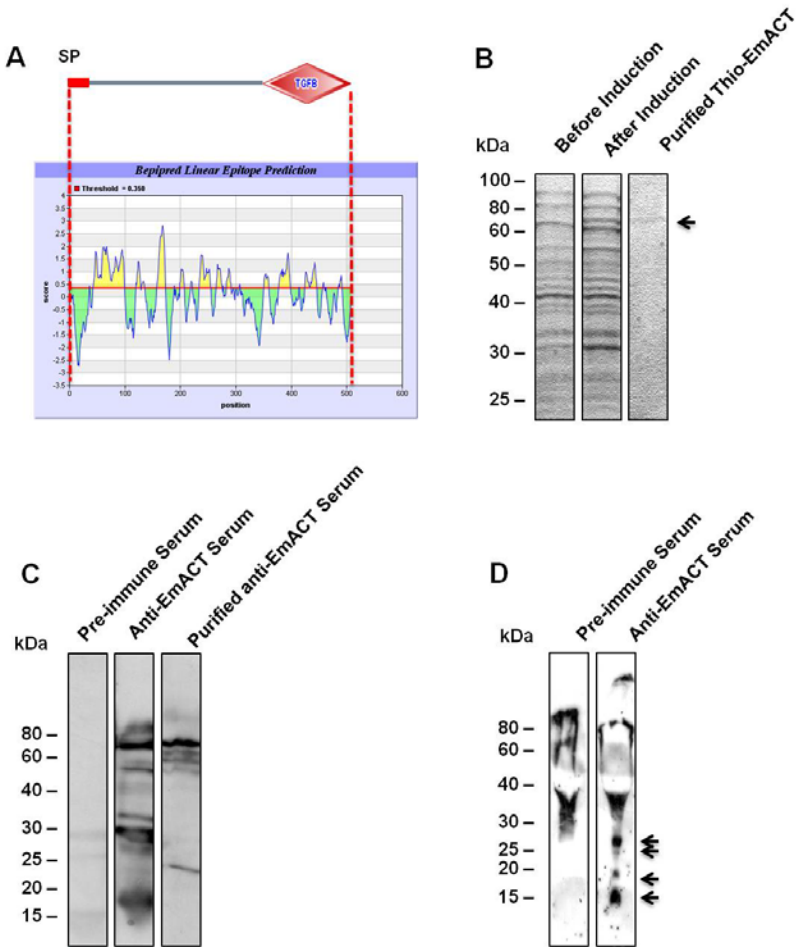


Figure 24: Secretion of EmACT by metacestode vesicles of *E. multilocularis* in culture. (**A**) *In sillico* prediction of EmACT epitopes. An epitope prediction was conducted online with the full EmACT sequence (tools.Immunoepitope.org). The portion retained for immunization and recombinantly expressed as a fusion protein is encompassed within the dashed red lines. A representation of EmACT (SMART analysis) showing the N-terminal signal peptide (SP) and the TGF-β superfamily domain has been aligned to the epitopes prediction. The region corresponding to yellow peaks above the default threshold line (horizontal solid red line) are potential EmACT epitopes. (**B**) Expression and purification of thio-EmACT fusion protein. EmACT was cloned into the bacterial expression vector pBADThio/TOPO. Competent *E. coli* (Top 10) bacteria were transformed with the Thio-EmACT plasmid and induced to express the fusion Thio-EmACT protein under arabinose control. A C-terminal histidine repeats fused to the expressed Thio-EmACT fusion protein by the pBADThio/TOPO expression vector was used as target tag for protein purification over Nickel-supplemented beads. Lysates of pBADThio/TOPO-EmACT transformed *E. coli* before and after arabinose-driven protein expression as well as purified Thio-EmACT were separated by SDS PAGE, blotted over a nitrocellulose membrane and the protein detected by Ponceau S staining. The arrow indicates the recombinant Thio-EmACT. **(C)** Profile of anti-Thio-EmACT antibodies. Western blot of a similar amount of Thio-EmACT containing bacterial lysates obtained after arabinose driven induction. 1:50 dilutions of normal mouse (pre-immune) serum and crude/purified mouse anti-Thio-EmACT sera were used for detection followed by ECL detection and autoradiography. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (**D**) EmACT is present in metacestode E/S products. Shown is a Western blotting of metacestode vesicle E/S products (MVE/S) probed with normal (pre-immune) mouse serum or mouse anti-Thio-EmACT antibodies followed by ECL detection and autoradiography. The positions of the molecular mass markers (in kilodaltons) are shown on the left. The arrows indicate secreted EmACT variants.

rEmACT failed to induce DC maturation as judged by MHC II and CD86 expression (Fig. 25C). When treated with rEmACT, there was no significant impairment of DC maturation in response to subsequent LPS stimulation (Fig. 25D). These results show that EmACT fails to activate host DC *in vitro*.

Human (mammalian) activin A has been shown to promote the TGF-β-induced differentiation of Foxp3+ regulatory T-cells [228,229]. To examine EmACT in this regard, OVA-loaded BMDC were co-cultured with OT-II×RAG-1(-/-) CD4+CD25- T-cells in the presence of normalized supernatants (Fig. 25B) from HEK cells transfected with either pSecTag2/Hygro vector or pSecTag2/Hygro-Em*act* vector (see material and methods). CD4+CD25+Foxp3+ Treg cells were analyzed 5 days later by flow cytometry. Although rEmACT failed to induce *de novo* Treg cell conversion (Fig.26A), it significantly increased the rate of TGF-β-induced Treg conversion (Fig. 26B). This finding indicates that, similarly to *E. multilocularis* metacestode E/S products (Fig. 22), EmACT is able to expand Foxp3+Tregs but requires TGF-β to do so.

Since activin A triggers IL-10 release by CD4+ T cells (200), EmACT was also investigated with respect to IL-10 production by CD4+ T cells. CD4+CD25- T cells (C57BL/6) showed a higher propensity to secrete IL-10 when stimulated in the presence of rEmACT (Fig. 26C), an effect above-reported with *E. multilocularis* E/S products (Fig. 17F).

Collectively, these data show that EmACT, a TGF-ß ligand secreted by *E. multilocularis* metacestodes, failed to activate host DC, promoted TGF-beta-driven Foxp3+ Treg conversion and triggered IL-10 release by CD4+ T-cells similarly to *E. multilocularis* metacestode secretions. This indicates that *E. multilocularis* metacestodes might elude DC recognition and expand regulatory T-cells by the release of evolutionary conserved cytokine like EmACT.

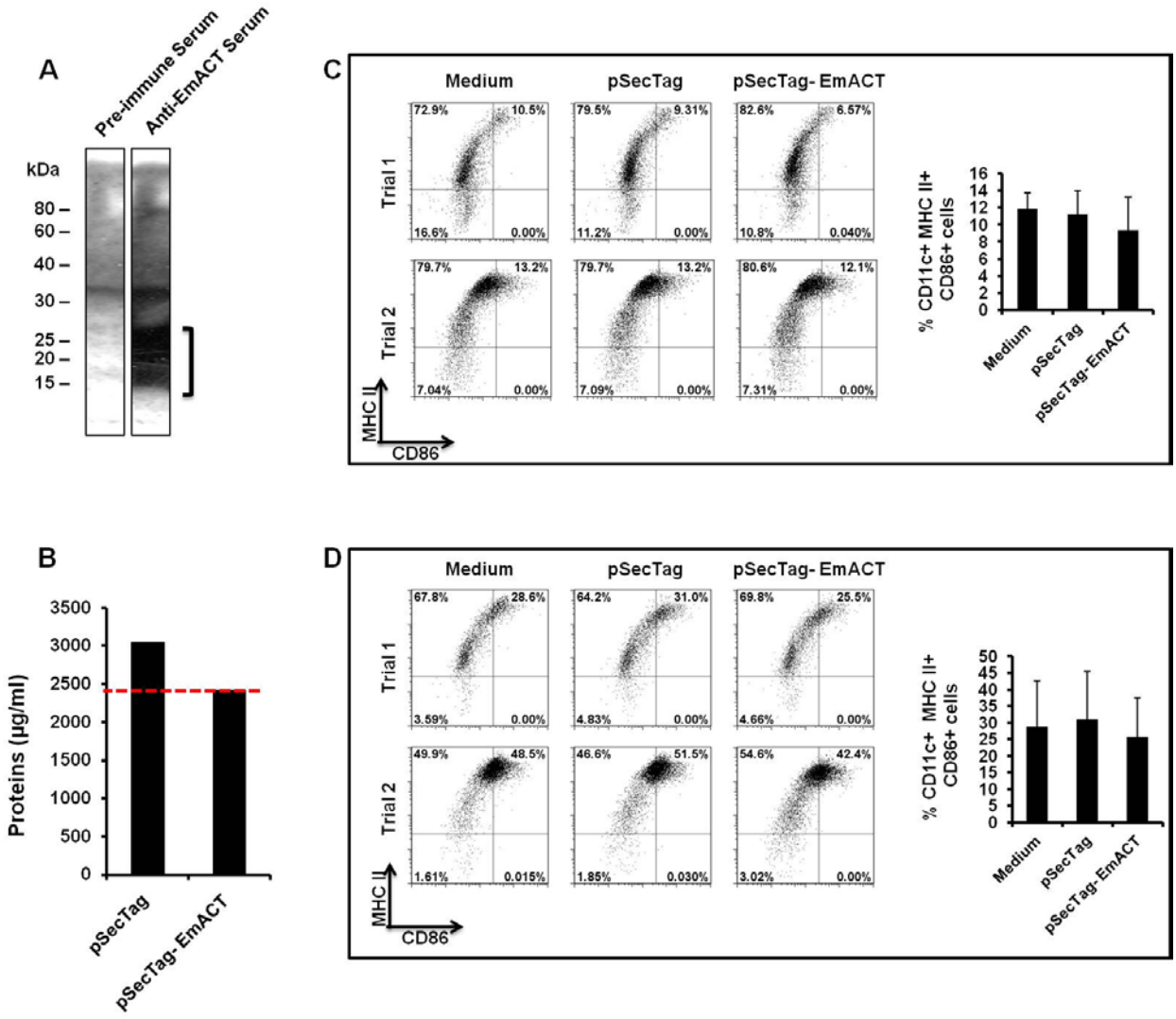


Figure 25: EmACT do not induce BMDC maturation. (**A**) Secretion of recombinant EmACT by pSecTag2/Hygro-Em*act* transfected HEK cells. Shown is a Western blot of the Ethanol-precipitated supernatant of pSecTag2/Hygro-Em*act* transfected HEK cells probed with either normal (pre-immune) mouse serum or mouse anti-Thio-EmACT antibodies followed by ECL detection and autoradiography. The positions of the molecular mass markers (in kilodaltons) are shown on the left. The bracket delimitates -the location of recombinant EmACT variants. (**B**) Normalization of pSecTag2/Hygro- and pSecTag2/Hygro-Em*act*-transfected HEK cell supernatants. The total protein contents of E/S collected from transfected HEK cells were determined using BCA assay. The lowest value (dashed red line) was used as a baseline for normalization by determining the amount necessary to give an equal final concentration in a total volume of 100ul. The solutions were accordingly topped to 100ul using double distilled sterile water. (**C, D**) Recombinant EmACT failed to induce or promote DC maturation. Flow cytometric analyses of BMDC surface maturation markers CD11c, MHCII and CD86 following treatment with recombinant EmACT. BMDC were treated with DMEM10 (medium), the supernatant of empty vector-transfected HEK cells (pSecTag2/Hygro) or the supernatant of full Em*act*-vector construct transfected HEK cells (pSecTag2/Hygro-Em*act*) for 24 hours (C) then supplemented for an additional 24 hours with 0.1µg/ml LPS (D) for a total of 48 hours, prior to staining and flow cytometric detection. (C,D) Shown are detailed scatter plots of two independent experiments summarized as means ± SD.

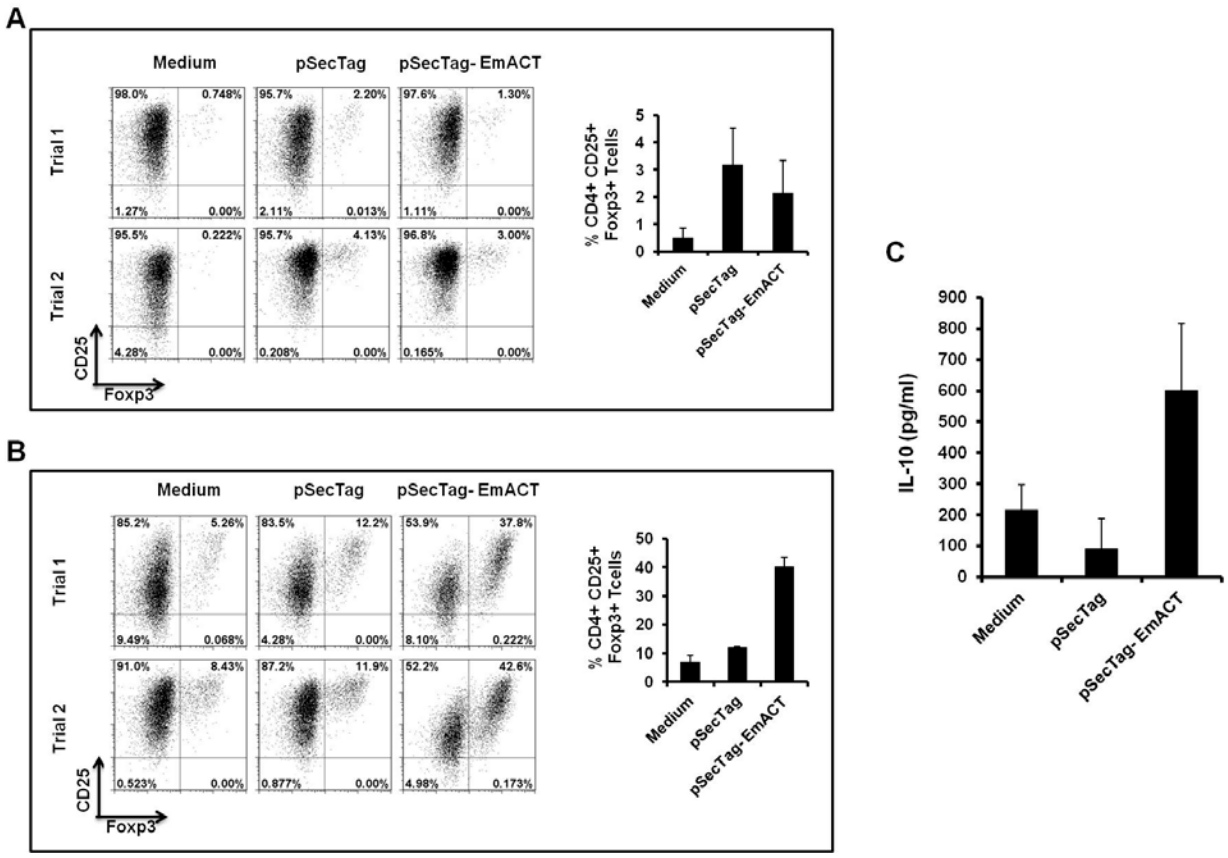


Figure 26: EmACT promotes *de novo* TGF-β- mediated Foxp3+Treg conversion and triggers IL-10 release by CD4+ T-cells *in vitro*. Freshly generated DC (Day 8) were co-cultured with naïve (CD25-) OT-II×RAG-1(-/-) CD4+ T cells at a DC:T-cell ratio of 1:3 in R10 medium supplemented with OVA peptide (200ng/ml) in the presence of DMEM10 (medium), the supernatant of empty vector-transfected HEK cells (pSecTag2/Hygro) or the supernatant of full Em*act*-vector construct-transfected HEK cells (pSecTag2/Hygro-Em*act*) in the absence (**A**) or presence (**B**) of 1ng/ml of recombinant TGF-β1. After 5 days of incubation, cells were harvested and stained for CD4, CD25 and Foxp3 prior to flow cytometry analysis. (**A,B**) Shown are detailed plots of two independently performed Treg conversion assay summarized as means (bars) ± SD. (**C**) Recombinant EmACT prompts IL-10 release by CD4+ T cells. Freshly isolated CD4+CD25- T-cells (2 X 10^5 /ml) were stimulated with α-CD3 (0.1µg/ml) and α-CD28 (5µg/ml) in the presence of DMEM10 (medium), the supernatant of empty vector-transfected HEK cells (pSecTag2/Hygro) or the supernatant of full Em*act*-vector construct-transfected HEK cells (pSecTag2/Hygro-Em*act*). Three days later, the T-cell supernatants were collected and probed for IL-10 concentration by Elisa. The results represent the mean ± SD from two independent experiments.

3.5 EmTIP, a T-cell immunomodulatory protein secreted by *E. multilocularis* is important for early larval development.

3.5.1 Identification of EmTIP, a T-cell immunomodulatory protein homologue from *E. multilocularis.*

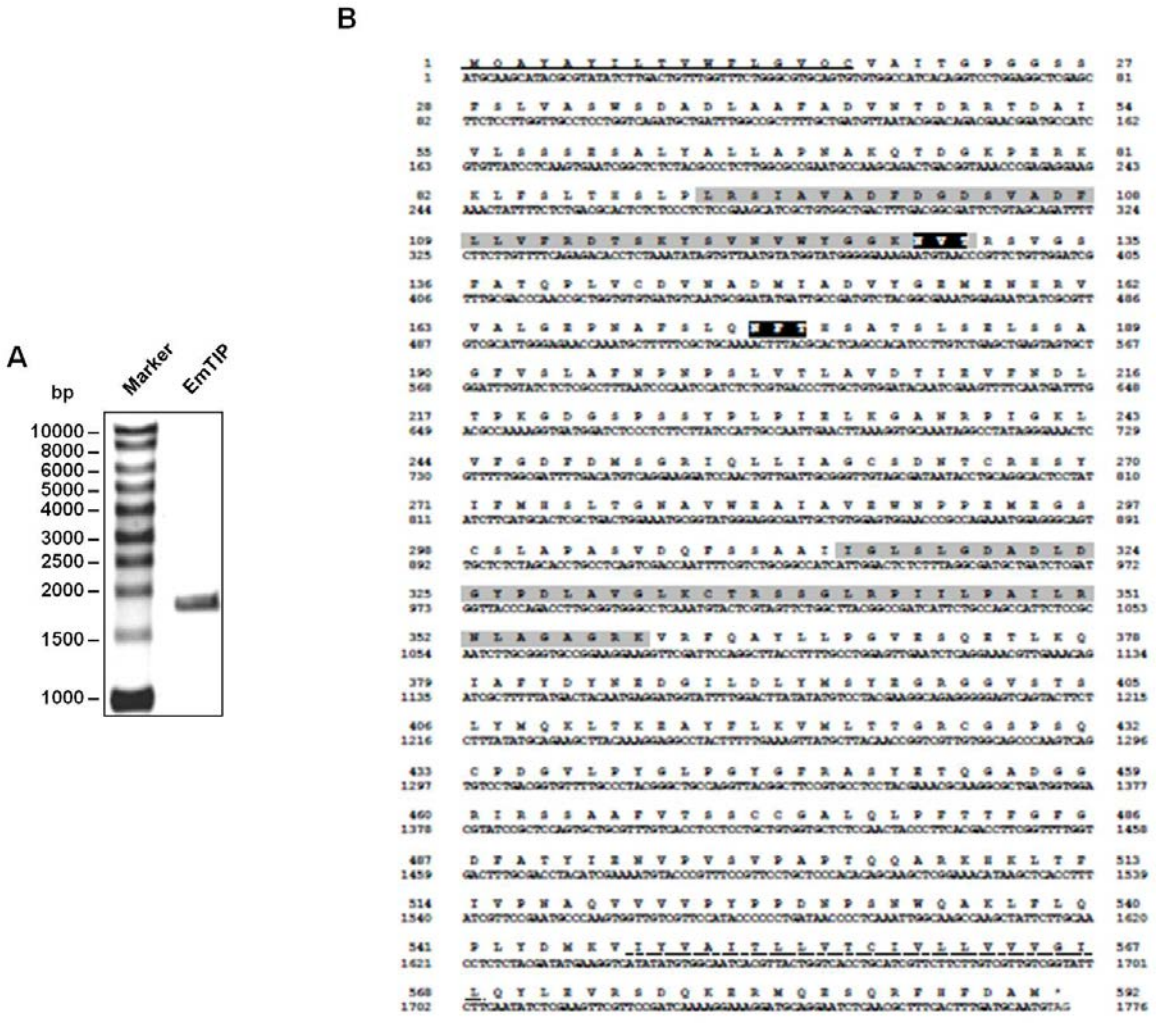


Figure 27: EmTIP sequence features. (**A**) Full-length amplification of Em*tip* Coding sequence using primers corresponding to the 5´and 3´coding termini (EmTIP_Dw and EmTIP_Up). 1µl of Metacestode cDNA was used as template for PCR with a high fidelity DNA polymerase (Phusion® High-Fidelity DNA Polymerase, New England Biolabs). 2µl of the amplicon were resolved on a 1.5% agarose gel and stained with Ethidium bromide prior to visualization under a UV transilluminator. (**B**) Nucleotide and deduced amino acid sequence of the *E.multilocularis* Em*tip* cDNA. The 5´signal sequence is underlined. The FG-GAP repeats located at the N-terminal end of Integrin-alpha FG-GAP repeat-containing proteins family, are shaded in grey. The potential glycosylation sites (NVT and NFT) are shown in solid boxes. The transmembrane region is delimitated with dashed underlines.

In previous investigations on the mechanism of trans-splicing in *E. multuilocularis* Brehm *et al* [206] were able to isolate a cDNA clone that apparently encoded a protein with significant homology to human T-cell Immunomodulatory Protein (TIP) [205].

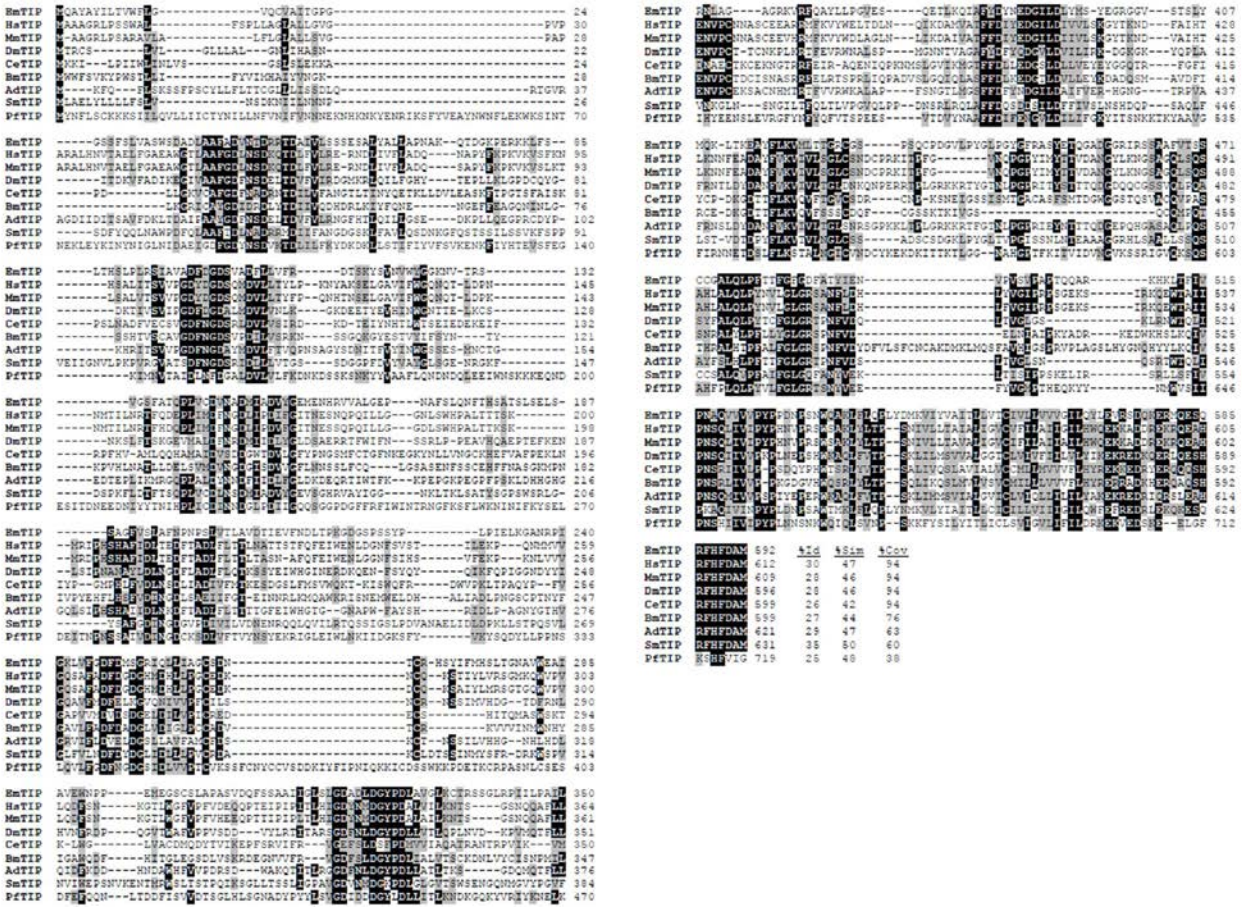


Figure 28: Alignment of the amino acid sequences of EmTIP and eight other representatives of the T-cell Immunomodulatory Protein family. Residues that are identical are displayed in black, similarities in grey. Gaps introduced to maximize the alignment are represented by dashes. Numbers at the start and finish of each line correspond to the amino acid numbers in each respective sequence. The percentages of identities (% Id) , similarities (%Sim) and alignment coverage (%Cov) to EmTIP are given for each sequence. Accession numbers for the sequences shown are as follows: Em*tip*, HF912277; Human TIP, Q8TB96; Mouse TIP, Q99KW9; *D. Melanogaster* predicted TIP, NP648776; *C. elegans* predicted TIP, NP498963; *B. Malayi* predicted TIP, EDP36995; *A. Darlingi* predicted TIP, EFR24550; *S. mansoni* predicted TIP, G4VRP9; *P. Falciparum* predicted TIP, Q8I3H7.

Since human TIP has been shown to influence cytokine production in T-cells [205], and considering T-cell immunomodulatory activities of *E. multilocularis* larvae [130,141,143,161,164,234,235], I further investigated the parasite factor and named it EmTIP (*E. multilocularis* T-cell Immunomodulatory Protein). The 1.76-kb, full-length Em*tip* transcript was amplified from cDNA of the parasite metacestode vesicles (Fig. 27A) with a 592 residues long deduced amino acids sequence (Fig. 27B). The deduced amino acid sequence of EmTIP contains a signal sequence and two putative N-linked glycosylation sites at position 128 and 175 (Fig. 27B) indicative of the possible secretion of EmTIP by the parasite. As shown in Fig. 27B, EmTIP contains atypical FG-GAP repeats (position 92-130 and 314-359), molecular hallmarks of Integrin-alpha FG-GAP repeat-containing proteins [236]. EmTIP also includes a predicted transmembrane domain at its C-terminus (position 548-568).

EmTIP is 35% identical to the predicted SmTIP from *Schistosoma mansoni*, 30% identical to Human TIP, 29% to *Anopheles darlingi* predicted TIP, 28% identical to both mouse and *Drosphila melanogaster* predicted TIP, 27% identical to *Brugia malayi* predicted TIP, 26% identical to *Caenorhabditis elegans* predicted TIP and 25% identical to *Plasmodium falciparum* predicted TIP (Fig. 28).

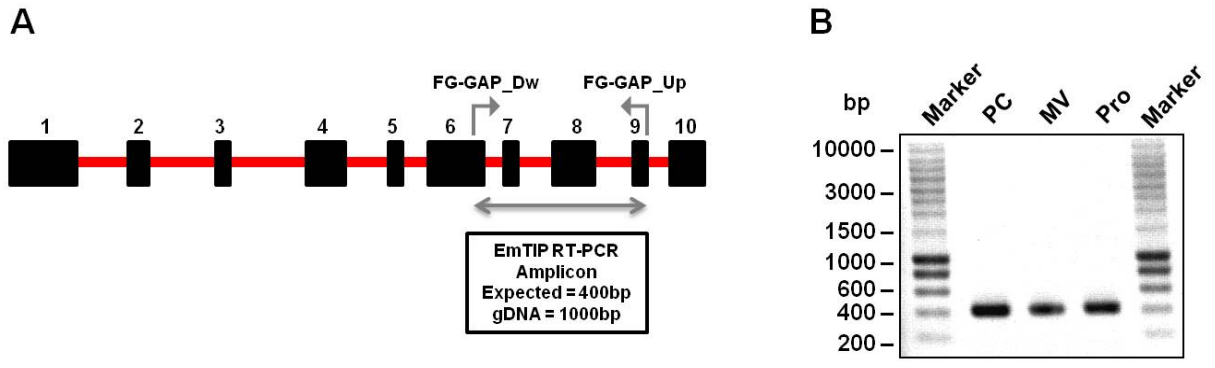


Figure 29: Em*tip* is transcribed throughout *E.multilocularis* larval development. (**A**) RT-PCR strategy for unequivocal amplification of Em*tip* transcript. Shown is an intron (red line)-exon (black boxes) arrangement of the Em*tip* genomic locus. A 400bp product specific for Em*tip* transcript was amplified using the primers FG-GAP_Dw and FG-GAP_Up spanning from exons 6-9. (B) Each *E. multilocularis* larvae (PC, MV and Pro) were separately used for qualitative assessment of Em*tip* transcription. 1µl of each larvae cDNA was used as template for PCR with a high fidelity DNA polymerase (Phusion® High-Fidelity DNA Polymerase, New England Biolabs). 2µl of each PCR amplicon were resolved on a 1.5% agarose gel and stained with Ethidium bromide prior to visualization under a UV transilluminator. gDNA: genomic DNA.

3.5.2 Expression and localization of EmTIP during larval development.

To study the transcription of Em*tip* by *E. multilocularis* larvae, a RT-PCR of an intron flanking portion of the gene (Fig. 29A) was conducted on cDNA from primary cells, metacestode vesicles and protoscoleces. As seen in Figure 29B, Em*tip* is transcribed by all *E. multilocularis* larval stages.

Next, to assess the expression of EmTIP by *E. multilocularis* larvae, Western analyses using rabbit polyclonal antibodies raised against a recombinantly expressed portion of EmTIP (Fig. 30, see section 2.5.4.2 for procedure) were used to investigate the production profile of EmTIP by *E. multilocularis* larvae.

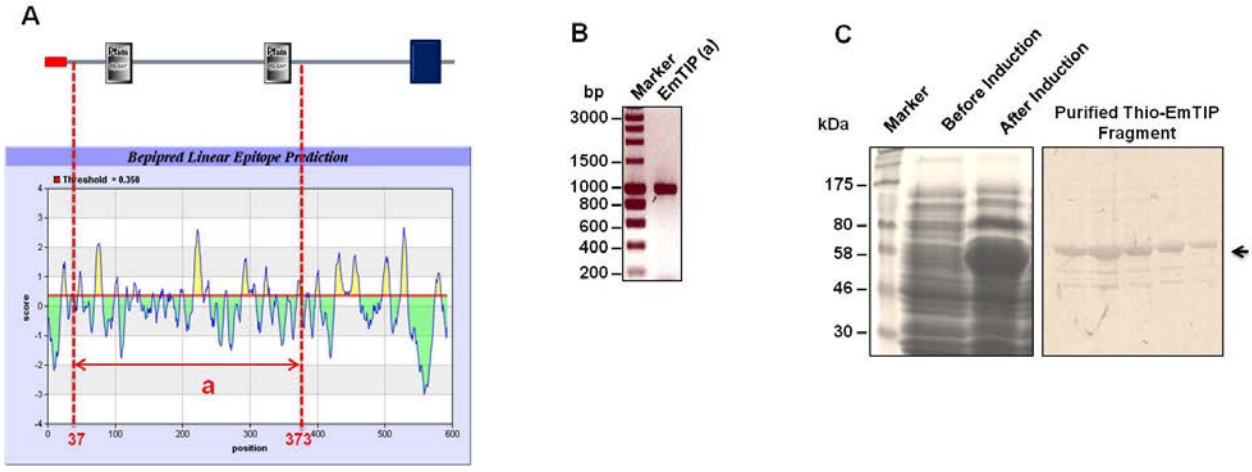


Figure 30: Recombinant expression and purification of a Thio tag-fused EmTIP for immunization. (**A**) *In sillico* prediction and amplification of EmTIP epitopes. An epitope prediction was conducted online with the full EmTIP sequence (tools.Immunoepitope.org). The N-terminal portion retained for immunization (a) is encompassed within the dashed red lines (amino acid 37 to 373). The signal sequence is depicted as a red box, the grey boxes represent the FG-GAP repeats and the blue box shows the transmembrane domain. (**B**) Amplification of the Em*tip* coding sequence portion to be used for immunization (a). 1µl of full-length Em*tip* amplicon was used as template for a nested PCR with a high fidelity DNA polymerase (Phusion® High-Fidelity DNA Polymerase, New England Biolabs) using the primers AbTIP_Dw and AbTIP_Up. 2µl of the new amplicon Em*tip* (a) were resolved on a 1.5% agarose gel and stained with Ethidium bromide prior to visualization under a UV transilluminator. (**C**) Recombinant expression and purification of Thio-tagged truncated EmTIP for antibody production. The truncated Em*tip* (a) was cloned into the bacterial expression vector pBADThio/TOPO. Competent *E. coli* (Top 10) bacteria were transformed with the Thio-Em*tip* (a) plasmid and prompted to express the fusion Thio-EmTIP protein under the arabinose-driven induction of bacterial expression. The C-terminal histidine repeats conferred to the expressed fusion protein by the pBADThio/TOPO expression vector were used as target tag to column-purify the Thio-EmTIP fusion protein over Nickel-supplemented beads. Lysates of pBADThio/TOPO-Em*tip*-transformed *E. coli* before and after arabinose driven protein expression as well as purified Thio-EmTIP were separated by SDS PAGE, blotted over a nitrocellulose membrane and the protein detected by Ponceau S staining. The arrow indicates the recombinant Thio-EmTIP fragment migration close to 58 kDa.

E. multilocularis larvae lysates (Fig. 11C) and E/S products (Fig. 11D), normalized for the protein content, were probed with rabbit polyclonal antibodies against EmTIP (Fig. 31). The anti-EmTIP antibodies specifically recognized a 58kDa protein in all lysates tested (Fig. 31A). Interestingly, a 58kDa band could only be detected in the E/S products of primary cells and protoscoleces whereas metacestode vesicle secretions did not show any reactivity to anti-EmTIP antibodies (Fig. 31B). Additionally, protoscolex secretions revealed a higher molecular weight band (~125kDa, Fig. 31B) when probed with anti-EmTIP antibodies.

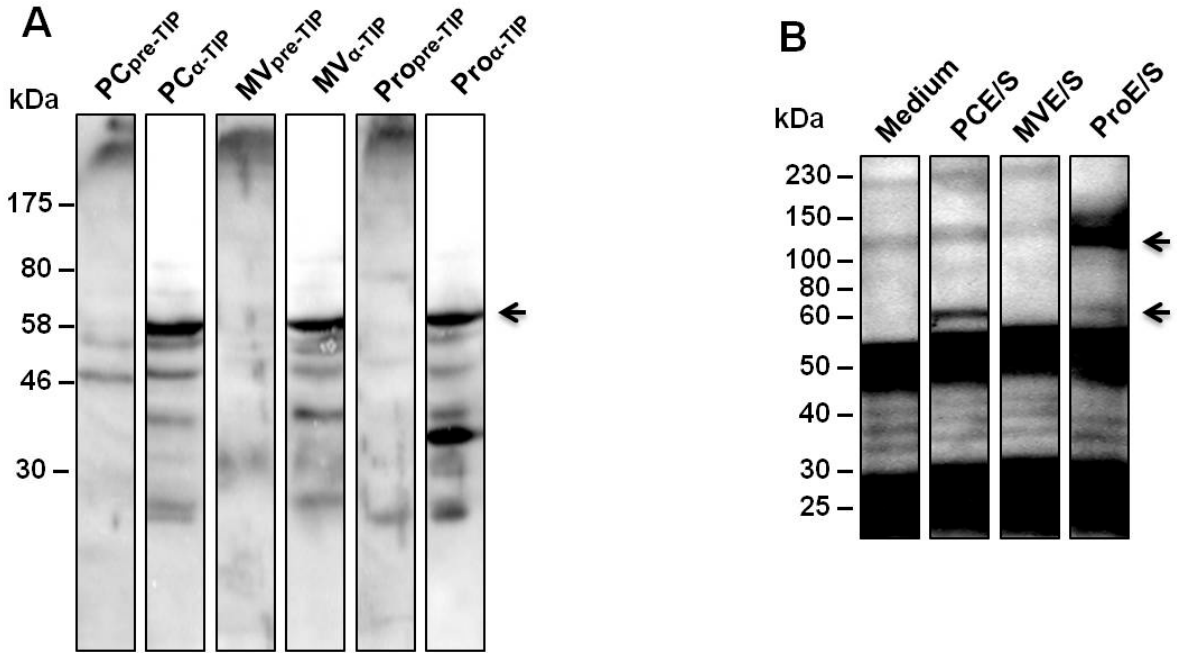


Figure 31: Differential expression of EmTIP protein by *E.multilocularis* larvae. Western blots of *E. multilocularis* larvae lysates (**A**) or E/S products (**B**) probed with pre-immune rabbit (pre-TIP) serum and/or rabbit anti-thio-EmTIP (α-TIP) antibodies followed by ECL detection and autoradiography. (**A**) Whole larvae lysates were probed with Normal rabbit (pre-TIP) serum and/or rabbit anti-thio-EmTIP (α-TIP) antibodies followed by ECL detection and autoradiography. (**B**) Western blot of E/S products of *E. multilocularis* larvae first immunoprecipitated and later on probed with rabbit anti-thio-EmTIP (α-TIP) antibodies followed by ECL detection and autoradiography. The arrows indicate EmTIP position.

In order to localize EmTIP within the parasite larvae, anti-EmTIP antibodies were affinity-purified (see material and methods for procedure) and checked for eventual cross-reactivity (Fig.

32). Although crude anti-EmTIP antibodies non-specifically detected additional bands in Western blots of tag-purified recombinant EmTIP (Fig. 32A) and AE-infected Jird liver tissue (Fig. 32B), affinity purified anti-EmTIP antibodies recognized in a highly specific manner a single EmTIP band (Fig. 32) as required for unambiguous immunolocalization of EmTIP within the parasite larvae tissue.

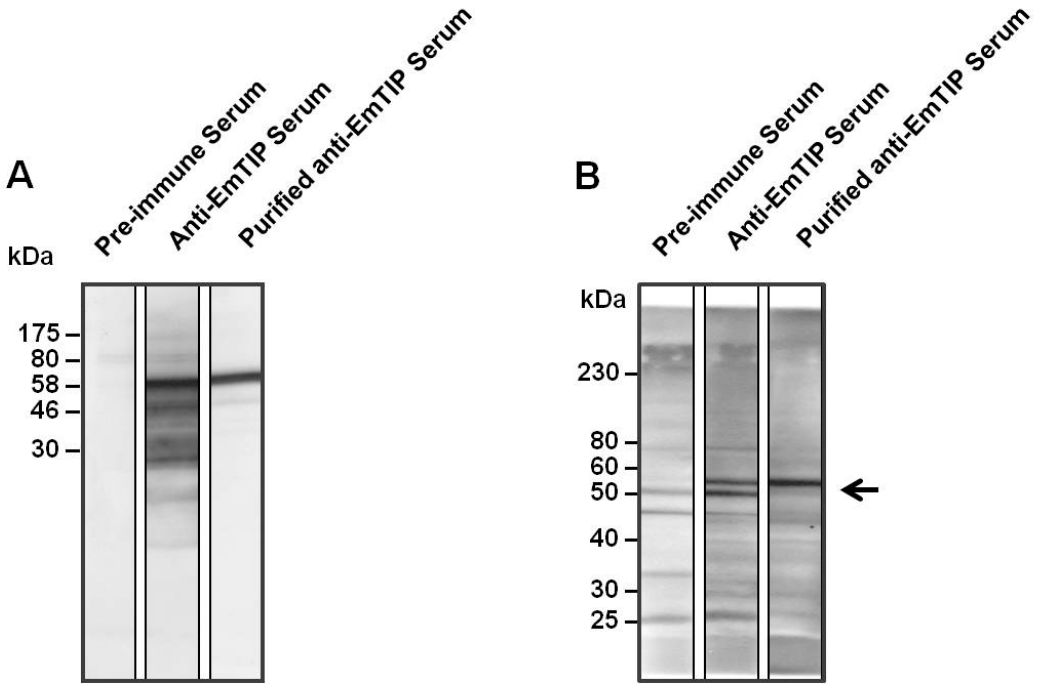


Figure 32: Profile of purified anti-Thio-EmTIP antibodies. Western blot of a similar amount of purified Thio-EmTIP (**A**) or parasite-containing liver tissue from infected jirds (**B**). 1:500 dilutions of normal rabbit (pre-immune) serum or that of crude/purified rabbit anti-Thio-EmTIP sera were used 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 natural EmTIP in parasite lysates between 50-60 kDa.

Affinity-purified anti-EmTIP antibodies also failed to show a signal on liver tissue from a healthy jird as judged by immunohistochemistry (Fig. 33A). Further immunohistochemical analyzes localized EmTIP to the inter-cellular space and the surface of cells within all *E. multilocularis* larvae. (Fig. 33B,C,D,E).

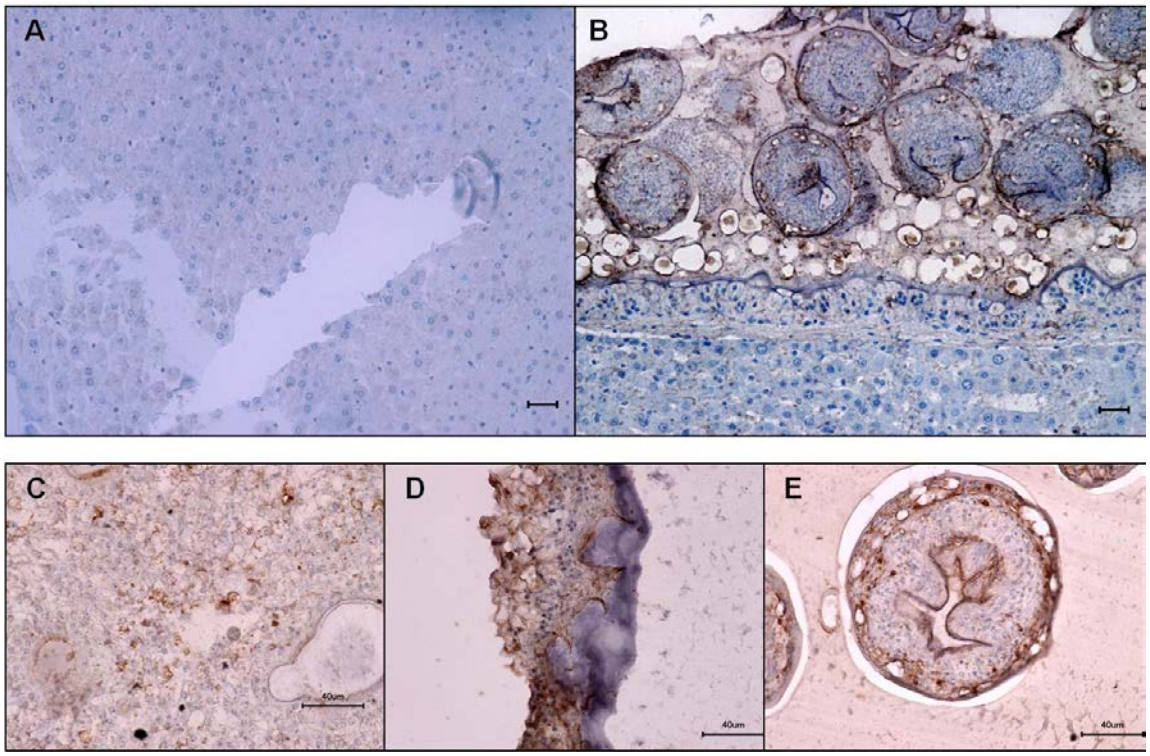


Figure 33: Immunohistochemical localization of EmTIP within *E. multilocularis* larvae. Dual staining (anti-EmTIP/peroxydase-coupled anti-rabbit-IgG (Jackson ImmunoResearch) against haematoxilin counterstain) of representative tissue sections of healthy Jird ´s liver (**A**), infected Jird´s liver (**B**) and *in vitro* maintained *E. multilocularis* larvae (section 2.2.3.3) namely primary cells (**C**), Metacestode vesicles (**D**) and protoscoleces (**E**). Scale bars: 40 µm.

3.5.3 EmTIP triggers IFN-γ secretion by CD4+ T cells.

Since human TIP has been shown to induce IFN-γ, IL-10 and TNF release by T-cells, resulting in a protective effect in graft versus host disease model [205], the influence of EmTIP on CD4+ T-cells was investigated. The supernatant of rEmTIP-expressing HEK cells (see section 2.5.4.2 for recombinant expression, Fig. 34A,B,C) was added to CD4+CD25- T-cells in the presence of anti-CD3 / anti-CD28 activating antibodies. After 3 days in culture, CD4+ T-cell culture supernatants were tested by ELISA for IFN-γ and IL-10 release. When compared to the controls, EmTIP induced a higher release of IFN-γ but failed to trigger IL-10 secretion in CD4+ T-cell cultures (Fig. 34D). Further analyses at a single cell level by intracellular FACS analysis revealed that CD4+ T-cells and not contaminating CD4- cells (< 5%), like NK cells, were the true source of the elevated IFN- γ production in those cultures (Fig. 35). Interestingly, primary cell E/S products evoked a stronger IFN- γ release by CD4+ T-cells than those observed with E/S

products of metacestode vesicles or protoscoleces indicating that *E. multilocularis* primary cells secrete EmTIP which promote IFN- γ release by CD4+ T-cells *in vitro*.

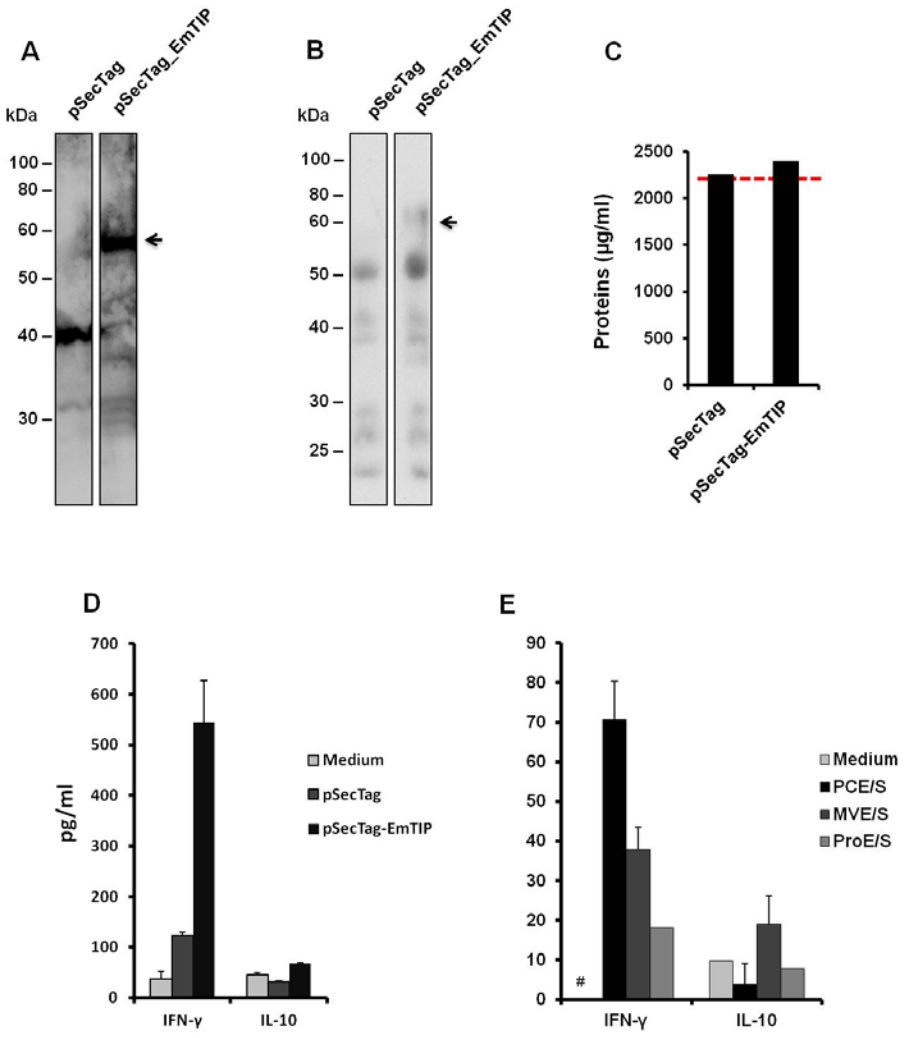


Figure 34: EmTIP augments IFN-γ release by CD4+ T cells. **(A)** Western blots of pSecTag2/Hygro vs. pSecTag2/Hygro-Em*tip* transfected 293T HEK cells lysates probed with purified rabbit anti-EmTIP antibodies followed by ECL detection and autoradiography. (**B**) Western blot of normalized supernatants of pSecTag2/Hygro- and pSecTag2/Hygro-Em*tip*-transfected 293T HEK cells first immunoprecipitated and later on probed with purified rabbit anti-EmTIP antibodies followed by ECL detection and autoradiography. The arrows indicate recombinant EmTIP position on the blots. (**C**) Normalization scheme for collected supernatants of pSecTag2/Hygro- and pSecTag2/Hygro-Em*tip*-transfected 293T HEK cells. The red dashed line represents the reference for protein content normalization. **(D)** Freshly isolated CD4+CD25- T cells (2 X 10^5 /ml) were stimulated with α-CD3 (0.1µg/ml) and α-CD28 (5µg/ml) in the presence of DMEM10 (medium), the supernatant of empty vector-transfected HEK cells (pSecTag2/Hygro) or the supernatant of full Em*tip*-vector construct-transfected HEK cells (pSecTag2/Hygro-Em*tip*) or alternatively (**E**) supplemented with conditioned medium of primary cells (PCE/S), metacestode vesicles (MVE/S) or protoscoleces (ProE/S). Three days later, the T-cell supernatants were collected and probed for IL-10 concentration by Elisa. The results represent the mean ± SD from two independent experiments.

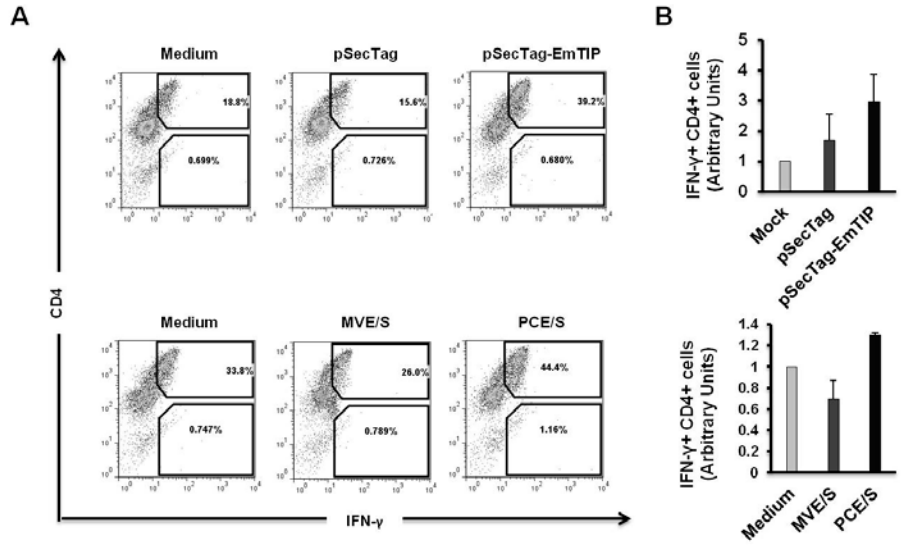


Figure 35: CD4+ T-cells are the true producers of EmTIP-driven IFN-γ. (**A**) Freshly isolated CD4+CD25- T cells (2 X 105 /ml) were stimulated with α-CD3 (0.1µg/ml) and α-CD28 (5µg/ml) in medium supplemented with normalized supernatants of parasite larvae (PCE/S or MVE/S) or transfected HEK cells (pSecTag or pSecTag-EmTIP). At day 3 of culture, 5µg/ml Brefeldin A was added for 6 additional hours then the cells were harvested and 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 2 conducted with similar results is shown. (**B**) Percentages of IFN-γ+ CD4+ cells are summarized by bar graphs with values of each experimental repeat normalized to the "medium" samples and displayed as mean ± SD from the two independent experiments.

3.5.4 Anti-EmTIP antibodies block *E. multilocularis* primary cell proliferation and the generation of metacestode vesicles.

EmTIP, as secreted by *E. multilocularis* primary cells, associates with a higher release of IFN-γ by CD4+ T-cell. Since IFN-γ-driven immune effector functions have been shown to be detrimental for *E. multilocularis* larvae [133,136,137,237], I hypothesized that the release of EmTIP by the parasite might be important for development. To examine this hypothesis, anti-EmTIP antibodies were added to *E. multilocularis* primary cell cultures.

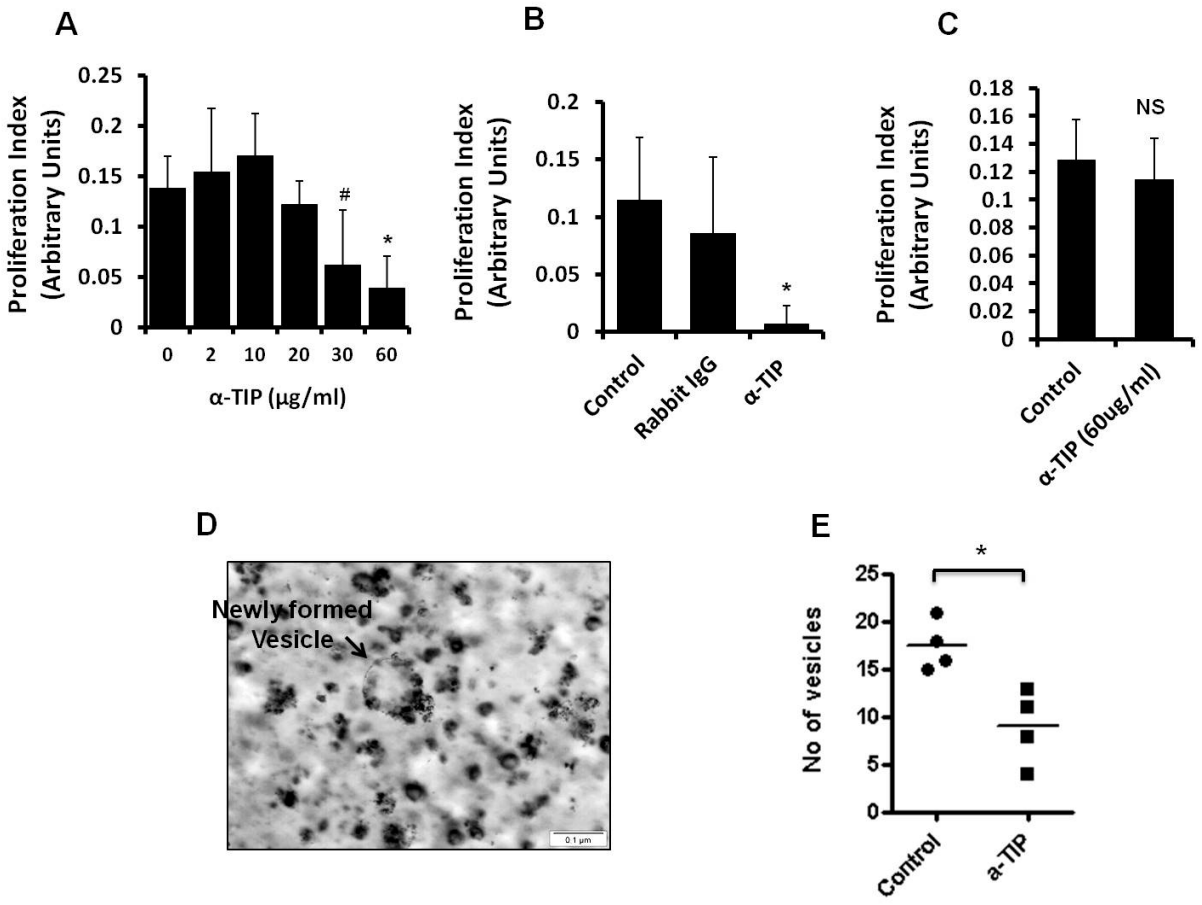


Figure 36: Anti-EmTIP antibody inhibits *E. multilocularis* primary cell proliferation and regeneration into metacestode vesicles. (**A**) Dose-response curve of the antiproliferative effect of purified rabbit anti-EmTIP on E.multilocularis primary cells. (**B**) 60ug/ml of purified rabbit anti-EmTIP antibodies but not pre-immune total rabbit IgG limit *E. multilocularis* primary cells proliferation. (**C**) Effect of purified rabbit anti-EmTIP antibodies (60ug/ml) on Rat hepatoma cell line proliferation. (**D**) Representation of regenerated metacestode vesicle following *in vitro* primary cells cultivation. (**E**) Effect of purified rabbit anti-EmTIP antibodies on *E. multilocularis* metacestode vesicles formation from primary cells. (**A,B,C**) Bars represent mean ± SD for 4-6 replicates of two independent experiments. (**E**) Horizontal bars stand for mean levels. Data represent four biological primary cells isolates assayed individually. #, statistical trend $p<0.1$; *, statistical significance $p<0.05$; NS, not significant.

A considerable inhibition of primary cell proliferation was observed with anti-EmTIP antibodies (Fig. 36A) but not total rabbit IgG (Fig. 36B) as judged by BrdU incorporation. Of note, rat hepatoma cells treated with anti-EmTIP antibodies did not show such an impaired proliferation (Fig. 36C) ruling out general cytotoxicity as mediating the negative effect of anti-EmTIP antibodies on *E. multilocularis* primary cell proliferation.

In vitro cultivation of *E. multilocularis* primary cells enables the *de novo* generation of metacestode vesicles ([190], Fig36D), a unique system to study the post-oncospheral transition into metacestodes as it occurs *in vivo* [191]. To further assess the role of EmTIP in parasite biology, primary cell cultures supplemented with anti-EmTIP antibodies were microscopically monitored over time for metacestode formation. Interestingly, incubation of primary cell cultures with anti-EmTIP antibody led to diminished vesicle formation (Fig. 36E).

Taken together, these findings showed that *E. multilocularis* primary cells secrete a T-cell immunomodulatory protein, EmTIP, that is important for parasite development and also evoke a parasiticidal IFN-γ-rich response from the host CD4+ T-cell.

4. DISCUSSION

4.1 Modulation of the host immune system by *E. multilocularis* larvae E/S products: larvae-specific effects on host immune cells.

The present study demonstrates a larva-specific influence of *E. multilocularis* on host immune effector cells. Notably, taking advantage of an *in vitro* cultivation system for axenic maintenance of larvae from the early (primary cells), mid (metacestode vesicles) and late (protoscoleces) phases of the fox-tapeworm *E. multilocularis*-driven infections [186] and collection of their E/S products, I show that, (i) E/S products of primary cells and metacestode vesicles, but not protoscoleces, induce apoptosis in murine BMDC *in vitro*; (ii) E/S products of primary cells and metacestode vesicles, but not protoscoleces, restrain the ability of BMDC to mature even in the presence of the TLR ligand LPS; and (iii) all *E. multilocularis* larvae E/S products limit the production of the Th1- associated cytokine IL-12 by BMDC, with a unique cytokine profile elicited by each larvae E/S products, where primary cell E/S products induce IL-10 but not IL-6 release, metacestode vesicle E/S products fail to induce any of these cytokines, and protoscolex E/S products only triggering IL-6 release by BMDC.

One problem of previous studies on the influece of parasite tissue on host immune cells [150,213] was the presence of contaminating host products, which could not be entirtely removed through (even extensive) washing [186]. In the present work, I used the method described by Spiliotis *et al.* for the production of parasite material [186]. In this system, contaminating host cells are effectively removed through the application of reducing conditions (axenization), thus allowing investigations under conditions that are (largely) free of host contaminants [186]. This was verified by RT-PCR analyses on the cultured material, demonstrating that cells of the laboratory host are no longer present in these cultures after one week of incubation.

E. multilocularis larvae are morphologically highly distinct. Primary cells in *in vitro* cultivation are of few µm of diameter and form aggregates that represent the post-oncospheral, or early *Echinococcus* larval stage in the infection of the intermediate host [186,190,191]. These aggregates are meshes of intermingled cells within a diffused inter-cellular matrix [190]. After *in vitro* maintenance for several weeks, the aggregates will form central cavities and progressively release buds of nascent vesicles: the metacestode vesicles [190]. Metacestodes, used at 2-5 mm

of diameter, do have a cystic appearance *in vitro*, are fluid-filled with an inner cellular or germinal layer and an outer accelular, carbohydrate-rich layer (or laminated layer) [186]. The inner germinal layer will internally give rise to brood capsules that will later form the head region of the adult worm, the protoscoleces. Protoscoleces are covered by a tegument, have an average 100µm of diameter with a pair of suckers and hooks [11]. The approach used in this work to compare an equivalent amount of each of these larvae, based on the normalization of their biomasses with respect to the total protein content or the level of expression the housekeeping ß-actin gene [238], minimizes the bias introduced by the distinct morphology of the parasite larval stages. As a result, an equal biomass of each parasite larval stages was used throughout the transwell stimulation assays. The differential effects observed with each of the larvae could therefore be interpretated in terms of a qualitative difference between one larval stage and the other considering that a similar quantity of each of the latters was used.

In this work, E/S products used were from normalized and biologically relevant amounts of *E. multilocularis* larvae. Indeed, the amount of parasite used throughout were normalized to the protein content in primary cells capable of regenerating into metacestode vesicles in 2-4 weeks, as reported *in vivo* for the oncosphere-metacestode transition [207,239,240]. This approach is more representative of the *in vivo* situation than the common use of excessively concentrated parasite E/S products to achieve significant effects [241–244].

When exposed to *E. multilocularis* larvae, E/S products of primary cells and those of metacestode vesicles induce DC death whereas protoscolex E/S products largely failed to show this effect. The interference of *Echinococcus* spp. with immune cell proliferation/viability has been suggested in early studies using parasitic cyst fluid [211,245]. It is interesting to note the inability of E/S products of the protoscoleces to mediate DC killing indicating that the factor (s) responsible (s) for this effect is / are poorly, if at all, released by this larval stage. This finding suggests that *E. multilocularis* protoscoleces behave differently from primary cells and metacestode vesicles. Therefore when considering previous studies on the interactions between *E. multilocularis* and its intermediate hosts where protoscoleces, although not in direct contact with the host immune system, were used in place of the tissue-dwelling metacestodes to infer for events governing the chronic phase of the disease [183–185,246–249], our present observation of their dissimilar secretome challenges the pertinence of the latter studies [183–185,246–249].

One of the most important findings of this study was the ability of larval E/S products to induce apoptosis in host DC. A similar activity has previously been reported for E/S products from the parasitic nematode *Brugia malayi* on human DC [250], where they acted through the TNFR pathway [251]. In this respect, it is noteworthy that I have identified a homologue to mammalian TNFR within the *E. multilocularis* genome (Suppl. Fig. 1), indicating that the parasite might also express/secrete a cognate ligand that possibly acts on host TNF receptors. Although *in silico* analyses to identify a gene encoding a respective TNF ligand on the *E. multilocularis* genome were unsuccessful, this does not necessarily mean that they are fully absent since TNF ligands of different organisms are usually highly diverse [252]. Among other TNFR superfamily members that could be involved in BMDC apoptosis driven by *E. multilocularis* larvae is the Fas death receptor [253–255] which is likely to mediate apoptosis in immature DC when exposed to TNF ligands [255]. Corticosteroids have also been shown to be involved in DC apoptosis [256–258]. Interestingly, the tapeworm *Taenia crassiceps* has been shown to produce sex steroids [259,260] and it is reasonably likely that *E. multilocularis* is doing it as well. Whether these hormones are really produced by the parasite has to be shown. Neverthles, the depletion of DC by primary cells and metacestodes would be beneficial to the parasite establishment and persistence in the course of an infection, unveiling here a so far unknown mechanism likely to mediate the subversion of the host immune system during Alveolar echinococcosis.

E. multilocularis larvae secretions also affect the function of surviving DC, modifying their ability to mature and altering their cytokine profile. In this respect, IL-12 is a highly potent inflammatory cytokine released by host immune cells upon encountering invading agents like bacteria. This cytokine is important for the onset of Th1 immune responses which properly control some tissue-dwelling pathogens [133]. Parasitic helminths and their products generally fail to promote the release of this cytokine by cells like DC [250,261–264]. Accordingly, E/S products from larvae of the early, mid- or late phases of *E. multilocularis*-driven infections did not elicit the release of detectable amounts of IL-12 by DC *in vitro*. Additionally, IL-10, a cytokine shown to set a tolerogenic environment around host immune cells l [265] was released by DC in response to primary cell E/S products. This would clearly promote a more permissive environment for the parasite. However, E/S products of *E. multilocularis* metacestodes did not show such an effect although this larval stage still needs to impair the host immune responses. The presence of a protective carbohydrate-rich laminated layer surrounding the germinal layer of

the parasite metacestode suggests that this larval stage is probably less vulnerable to the host immune attacks when compared to primary cells thus the absence of IL-10 production by DC exposed to metacestode products. It stands therefore to reason to propose that the induction of IL-10 secretion in DC by *E. multilocularis* primary cells is a parasite early mechanism to help temper the host response to the vulnerable post-oncospheral stage and thereby facilitates the host colonization. This observation brings more support to the immunoregulatory role of IL-10 during AE, a role already suggested by murine and human studies where an anticytotoxic role was proposed [152,153]. Again, the induction of IL-10 release by DC is no novelty for helminth parasites in general. DC exposed to antigens from the cestodes *Echinococcus granulosus* [266,267] and *Taenia crassiceps* [263] or the nematode *Trichuris muris* [268] had shown an enhanced production of IL-10. "What role would these IL-10-secreting DC play if elicited *in vivo* during AE?" and "which factor(s) within primary cell E/S products prompt IL-10 release by DC?" are questions yet to be answered. Such answers would provide more understanding of the molecular means of host colonization by *E. multilocularis*. As potential factors in nematode infections, filarial cystatins have been associated with IL-10 secretion in APC, including immature DC [269]. Interestingly, ongoing analyses on the *E. multilocularis* genome (http://www.sanger.ac.uk/resources/down-loads/helminths/echinococcus-multilocularis.html) indicate that related molecules are also encoded by the cestode (Suppl. Fig. 2). Further experimentation is therefore needed to assess the possible secretion of these molecules by primary cells and whether they can exert immunomodulatory activities comparable to those of filarial cystatins. Another possible explanation for the observed IL-10 secretion by surviving DC after exposure to primary cell E/S products is the likely uptake of apoptotic DC by the surviving DC. It is indeed conceivable that the uptake of apoptotic bodies by DC would trigger the release of IL-10, as previously reported in the case of apoptotic macrophages [270]. However, DC exposed to metacestode E/S products, of which a fraction also died by apoptosis, failed to release IL-10, therefore challenging this line of interpretation. The likeliest scenario would therefore be that of a differential, yet overlapping, composition of the E/S products from *E. multilocularis* primary cells and metacestodes. While the E/S products of both contain factors that induce apoptosis in DC, only those of primary cells contain an IL-10 inducing compound.

DC exposed to metacestode E/S products as well as primary cell E/S products failed to upregulate DC activation markers with no detectable release of proinflammatory cytokines (IL-6

or IL-12) whereas protoscolex E/S products elicited DC maturation and IL-6 release. This observation further supports the sketched scenario of an overlapping composition of primary cell and metacestode E/S products and strengthens the idea of a different profile for protoscolex E/S products. The absence of DC maturation following exposure to primary cell or metacestode E/S products is consistent with a previous study reporting the inability of *E. multilocularis* metacestode extracts to induce classical DC activation or any apparent sign of activation for that matter [182]. This is a rather common feature of helminth-derived products [63,65,271]. Given the central role of DC activation in the onset of host immune responses [54], I reasonably hypothesize that *E. multilocularis* primary cells and metacestodes, by failing to alert host DC, would most efficiently elude the host immune responses in the course of an infection. In contrast, protoscolex E/S products as potent activators of DC therefore point at a reduced ability of this larval stage to avoid immune recognition by the host DC. This corroborates the previous observation that *E. multilocularis* primary cells and metacestodes could more efficiently establish secondary alveolar AE upon intravenous injection in rodents whereas protoscoleces displayed no infectivity [272].

When further investigating the influence of the parasite E/S products on DC, I found that BMDC pretreated with either primary cell or metacestode E/S products appeared diminished in their ability to respond to subsequent LPS challenge as judged by the lack of surface markers upregulation and the reduction of IL-12p70 release. This indicates that the previously reported, impaired secretion of IL-12 by LPS-pulsed PBMC from AE patients [169], could be a direct consequence of the influence of the parasite E/S products on the host immune cells. TGF-β, a cytokine associated with the impaired responsiveness of a wide range of immune cells [273–275], has been detected in DC of *E. multilocularis*-infected hosts [213]. Accordingly, several studies suggested an important role for the TGF-β pathway during host colonization by *E. multilocularis* [213,235]. It is therefore reasonable to assume that *E. multilocularis* primary cell and metacestode E/S products would not only fail to activate DC but might additionally restrain their ability to respond to any bystander inflammatory stimuli. This does not simply indicate that *E. multilocularis* would avoid a parasiticidal host inflammatory response in the eventuality of bystander immune stimulation but might additionaly limit the host response to unrelated antigens. Indeed, this hypothesis is supported by the recent observations that *E. multilocularis*-infected animals enable a prolonged survival of transplanted organs as a result of a suppressed

immune-mediated rejection [276,277]. It therefore stands to reason to look at *E. multilocularis* products as a novel source of immunomodulatory factors with potential application as immunotherapeutics against host deleterious inflammatory responses [278]. This reconciles with the regulatory component of the hygiene hypothesis which proposes a key role for pathogens to keep host deleterious immune responses in check [279]. Consistent with *E. multilocularis*, several helminths have also been reported to release products with the ability to impair DC responsiveness [63]. The glycoprotein ES-62 of *Acanthocheilonema viteae* [280,281], the E/S products of the rodent nematode *Heligmosomoides polygyrus* [262,282], the egg antigens of the trematode *Schistosoma mansoni* [283,284] or the E/S products from the cestode *Teania crassiceps* [263] have all failed to promote a classical DC maturation and could each block subsequent PAMP-driven pro-inflammatory cytokine secretion by these DC. In this study, the identification of the factor (s) and mechanisms used by *E. multilocularis* primary cells and metacestodes to mediate DC impairment has not been achieved but would clearly provide a valuable wealth of informations to understand the basis of the host immunomodulation by *E. multilocularis*. *In silico* analyses have identified a TGF-β/Activin subfamily member within *E. multilocularis* genome. With respect to circumstancial evidences on the likelihood of true TGF-ß ligands in parasitic helminths [285,286], this factor represents a potential mediator of the DC impairment caused by *E. multilocularis* E/S products. Future experimentation in this regards are currently underway. As a possible mechanism, *Echinococcus multilocularis* larvae E/S-products might negatively regulate the TLR signaling through interaction with non-TLR pattern recognitions receptors such as C-type lectin receptor MGL [263] DC-SIGN and /or Dectin family members [63] as reported for E/S products of the closely related tapeworm *Taenia crassiceps* [263]. Equally likely is the induction of apoptosis by *E. multilocularis* larvae secretions which would explain the limited responsiveness of the surviving DC. In fact, since DC loaded with apoptotic bodies are known to display such an impaired responsiveness to subsequent TLR stimulation [270,287,288], it is reasonable to believe that apoptotic DC bodies induced by *E. multilocularis* larvae products if and when taken up by the surviving DC would most likely lead to the onset in these cells of a tolerogenic program.

E/S products of the pro-adult stage, the protoscoleces failed to inhibit DC responses but rather promoted an intermediate maturation of the latter associated with the sole release of the inflammatory cytokine IL-6. Such a profile resembles that of DC exposed to hydatid fluid or

antigen B of the closely related tapeworm *Echinococcus granulosus* [266,267] but does not fully match it. This discrepancy could simply be due to a differential spectrum of factors between the hydatid fluid content and the secretions of protoscoleces. This differential composition of the parasite preparations is further illustrated by the rate of AgB secretion which is only weakly expressed by protoscoleces [289,290] but considerably produced within the hydatid cyst fluid [291]. Interestingly, the phenotype and cytokine profile of DC exposed to protoscoleces in this study is also similar to those of *Trypanosoma*- treated DC [68] which have been closely related to the induction of Th2 response *in vitro* and *in vivo* [68]. Furthermore, the complete abrogation of LPS-pulsed IL-12 release in protoscolex-treated DC in spite of their ability to still mature in response to LPS, provides yet another supportive evidence for the Th2-inducing profile of these DC given that such a profile has previously been associated with Th2-inducing DC [292–294]. Again, this observation highlights the inability of protoscolex E/S products in impairing the activation of the intermediate host's immune system as reported for primary cell and metacestode products. Moreover, Th2-dominated immune responses might not be entirely relying on protoscoleces since such an immune profile can also be observed in chronic AE under conditions in which no protoscoleces are produced [18]. However, since intestinal luminal infections by adult cestodes are associated with Th2 immune responses [2], the phenotype I observed in this study for DC treated with E/S products of *E. multilocularis* protoscoleces could rather be associated with events that are relevant for an infection of the definitive host [2]. It should therefore be recalled that the interpretation of results obtained when investigating the immune response to *E. multilocularis* larvae during an infection of the intermediate host by exposing host cells to *Echinococcus* protoscoleces [183–185,246–249] or by employing the mouse model of peritoneal, protoscolex-induced secondary alveolar echinococcosis for short-term infections [179], with regards to the evidences herein provided, is highly subjective and probably biologically impertinent.

4.2 Utilizing evolutionarily conserved cytokines to subvert the host immune response: EmACT, an *Echinococcus multilocularis* activin that expands host regulatory T-cells.

Following the observation that *E. multilocularis* primary cell and metacestode E/S products induce tolerogenic DC, which might reasonably expand regulatory T-cells [214], I demonstrated that *Echinococcus multilocularis* metacestode vesicle E/S products induce *de novo* Foxp3+ Treg conversion from naïve CD4+ T-cells in a DC-based regulatory T-cell conversion assay *in vitro*. This indicates a major role for E/S products of the parasite metacestodes in host immunomodulation. Furthermore this finding implies that the regulatory T-cell expansion in *E. multilocularis* -infected hosts is a parasite-driven process, rather than just a mere homeostatic mechanism of any chronically infected host in order to limit the disease pathology, as previously reported for other diseases [295,296]. *In vivo* analyses carried out in this work further supported the idea of parasite-driven Treg expansion in AE. Indeed, the recruitment of Foxp3+ Treg took place at a time point where the experimentally infected hosts were not chronically infected (day 7). This is in contrast with a past study where Foxp3+ Treg expansion was first reported in heavily-infected hosts (> day 42) [235]. The latter study, although informative, was equivocal with respect to the basis of Treg expansion. It was not possible to differentiate between a parasite-driven process and a homeostatic response of the chronically infected host as the cause of the observed Treg expansion. My present observation of an early recruitment of Treg in *E. multilocularis*-infected mice therefore ascertains the ability of the parasite metacestodes to actively drive host Treg expansion *in vivo*. However, with respect to the early (day 7 post injection) expansion of Treg in mice following metacestode injection in this study, the data also suggest the possibility of a mitogenic effect of the parasite on natural/pre-existing Tregs. Future studies using differential markers for natural vs. induced Tregs like Neuropilin 1 [297] should be conducted to distinguish between the proliferation of natural Tregs (neuropilin 1^{high}) and/or the conversion of inducible Tregs (neuropilin1^{low}) by *E. multilocularis* metacestodes *in vivo*. All the same, expanding natural [298,299] and/or inducible Foxp3+ Tregs [300] has already been reported for parasitic helminths with large implication for disease establishment and/or progression [286,298,301]. Taking advantage of the recently developed bacterial artificial chromosome–transgenic mice termed “depletion of regulatory T-cell” or DEREG mice

[302,303], the question whether a similar role for Tregs (as a whole) in disease establishment and/or progression applies during AE can now be addressed. The induction of Treg by helminth E/S products has already been described. Grainger *et al*., [286] have elegantly demonstrated a similar potency of E/S products of the parasitic nematode *H. polygyrus* via the host TGF-β pathway [286]. Similarly, the blockade of the host TGF-β pathway abrogated the ability of metacestode E/S products to drive Foxp3 expansion in T-cells suggesting that this pathway is instrumental for the Treg-converting ability of metacestode E/S products. Furthermore, this metacestode Treg-converting potency was equally lost when TGF-ß was neutralized with antibodies pointing out that metacestode products require the host TGF-β to expand regulatory T-cells, as previously reported for a major egg antigen of the related parasitic flatworm *S. mansoni* [304].

As a candidate factor mediating host Treg expansion, a TGF-β/Activin subfamily member with highest homology to mammalian inhibin beta A subunit could be identified *in silico* within *E. multilocularis* genome. In mammals, heterodimers of inhibin α and inhibin beta A / beta B are denominated inhibin A and B, respectively, while homodimers of either inhibin beta A or inhibin beta B are called activin A and activin B [230]. Since further *in silico* searches within the *E. multilocularis* genome failed to identify any other inhibin homologue, I termed the inhibin beta A subunit homologue *E. multilocularis* activin or EmACT, given that most probably only homodimer chains of the inhibin beta A orthologue can be formed by the parasite. Interestingly, EmACT appeared to be secreted by the parasite metacestodes suggestive of a role at the host-parasite interface. Indeed, subsequent results indicated that recombinant EmACT (rEmACT) was able to promote TGF-β-driven Foxp3+ Treg conversion in a manner similar to what has been shown for mammalian activin A [228], thus suggesting a possible role of this factor in the Treg converting potency of *E. multilocularis* metacestode E/S products.

Of note, primary cells and protoscolex secretions failed to promote Foxp3+ Treg conversion but could also secrete EmACT *in vitro* (Suppl. Fig. 5). Strikingly, EmACT appeared to be differentially processed in these stages (Suppl. Fig. 5) illustrating the complexity of post-translational modifications in TGF-β superfamily members and the ensuing effect on their activities. It is therefore likely that posttranslational modifications, like N-glycosylations, that have commonly been reported to affect TGF-β superfamily members' activity [305–307], could

regulate the activity of EmACT in *E. multilocularis* E/S products. On the other hand, two follistatin homologues (Suppl. Fig. 6) could be identified within *E. multilocularis* genome with one flanked at its N-terminus by a signal sequence suggesting secretion. Since follistatins are specific inhibitors of activins [308,309], it is reasonable to hypothesize that the parasite, similarly to its mammalian host, is using this regulatory loop to control EmACT activity throughout development. Whether the post translational modifications of EmACT and/or the differential expression of parasite follistatins govern the dissimilarities observed with *E. multilocularis* larvae E/S products with respect to Foxp3+ Treg conversion clearly requires further investigations.

The data of this study also indicated that EmACT failed to induce DC maturation. Additionally, rEmACT also failed to impair DC responsiveness to subsequent LPS challenge, unlike what was previously observed with metacestode E/S products and reported for activin A [229,310] . Interestingly, rEmACT did not promote Foxp3+ Treg conversion in the absence of TGF-β, as was observed for metacestode E/S products. Although the latter observation is in agreement with previous studies where CD4+ T-cells failed to upregulate Foxp3 expression in response to activin A alone [229], unless co-stimulated with TGF-β [228], limitations in the present experimental settings can not be ruled out. Using supernatant of transiently transfected mammalian cell lines is a common approach for the preliminary assessment of novel factors [205,311] but still hold the drawback of yielding a limited and uncontrolled amount of the factor [205]. In fact, since in another study, purified activin A could singly elicit host Treg conversion in a dose-dependent manner in the absence of TGF-β [228], a reasonable step to take from here would therefore be the dose-dependent assessment of purified rEmACT alone in the DC stimulation assay and the Treg conversion assay. However, the TGF-ß-dependent Treg converting capacity of rEmACT corroborates with the mode of action of *E. multilocularis* metacestode E/S products. Indeed, the neutralization of TGF-β abrogated the rate of Foxp3+ Treg conversion with metacestode E/S products. This stands as indirect evidence for EmACT as the Treg promoting factor secreted by the parasite metacestodes. Other possible parasite factor (s) that could support TGF-β-driven Treg conversion through the TGF-β pathway are bone morphogenetic proteins (BMPs). Indeed, BMP-2 and BMP-4 have been shown to act in synergy with TGF-β to promote Foxp3+ Treg induction [312]. Two BMPs homologues (Suppl. Fig. 3) have been identified by *in silico* search within the *E. multilocularis* genome and although not

studied in this work, these factors are awaiting an in-depth characterization. I also can not dismiss the implication of T2 ribonucleases which, with respect to *Schistosoma mansoni* hepatotoxic T2 ribonuclease omega-1 [304], could be able to drive a TGF-β-dependent Foxp3+ Treg expansion and DC modulation. Of note, an *in silico* search lead to the identification of an omega-1 homologue (EmOMG) within the parasite genome which clearly warrants further analysis in this respect (Suppl. Fig. 4). However, the role of EmACT in the metacestode-driven host T-cell modulation gained more support with the observation that, similarly to metacestode E/S products and as reported for activin A [229], rEmACT triggered IL-10 secretion by CD4+ T-cells *in vitro*. Of interest, IL-10 as an anti-inflammatory cytokine [265], has been extensively reported to peak during murine and human infection with *E. multilocularis* larvae [130,142,143,145,151,163,164,170,313,314] and is therefore suggested to play a crucial role in the maintenance of tolerance and partial inhibition of cytotoxic mechanisms [152,153]. Interestingly, the mechanisms governing Foxp3 expression and IL-10 secretion by activin A-treated CD4+ T-cells involve both the TGF-β and p38 map kinase pathways [315]. Whether EmACT utilizes both the host TGF-β pathway and the p38 MAPK cascade to mediate the aforementioned effects would therefore be interesting to test.

Collectively, these findings demonstrate the long suspected but unproven harnessing of one evolutionary conserved TGF-β superfamily cytokines by larvae of the fox tapeworm *E. multilocularis* in particular and helminths in general, to subvert host immune cell functions. EmACT, an activin orthologue secreted by *E. multilocularis* metacestode E/S products, promoted regulatory functions within T-cell populations in a manner similar to metacestode secretions. Not only could these data prove to be valuable for novel anti-AE strategies but, beyond the level of parasitology, provide a lead factor for the control of detrimental immune responses and their associated pathologies in humans such as autoimmune diseases and allergies.

4.3 An IFN-gamma-inducing protein secreted by *Echinococcus multilocularis* larvae is important for parasite development.

IFN-γ and its associated effects dominate the very early post-oncospheral phases of infection with *E. multilocularis* [129,154,155] and have been shown to be potentially deleterious to the parasite larvae [133,136,137,170,237]. The resulting Th1 immune response is progressively suppressed as the metacestodes are formed and disseminate within the host [234]. The mechanisms mediating the early onset of host protective Th1 responses against *E. multilocularis* larvae are still not known. I herein characterized a factor homologous to human T-cell immunomodulatory protein (TIP). Since human TIP is known to trigger the release of IFN-γ, TNF and IL-10 by T-cells [205], I characterized and functionally assessed the parasite TIP homologue, renamed EmTIP, with regard to an eventual ability to trigger the release of these cytokines by CD4+ T-cells.

This gene renamed Em*tip* codes for an atypical FG-GAP repeat containing protein EmTIP (*E. multilocularis* T-cell immunomodulatory protein). I showed that EmTIP is expressed by all larval stages of the parasites and released in soluble forms by primary cells and protoscoleces but not metacestodes. I also showed that EmTIP localized to the inter-cellular space of the parasite larvae and that antibodies directed against EmTIP limited the proliferation of the parasite cells and restrained them in their ability to regenerate into metacestode vesicles *in vitro*. Finally, I 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 an antigen important for the early phases of *Echinococcus multilocularis* larvae development and at the same time capable of eliciting IFN-γ release by CD4+ T-cells.

Even though the precise function of the T-cell immunomodulatory protein family is largely unknown and has so far only been associated with a protective effect in a graft-versus-host model amid a complex cytokine profile (IFN-γ, IL-10 and TNF) [205], they are widely conserved throughout the animal kingdom. A structural feature of all TIP proteins is the presence of FG-GAP repeats, which are present in 2 atypical copies in EmTIP as opposed to one copy in human and mouse TIP. In mammals, FG-GAP repeat-containing proteins have principally been associated with cell-cell/ECM (extracellular matrix) interactions [316,317]. Alpha-Integrins

constitute a group of such 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 [318–321]. Similar to cell-cell/ECM interaction-mediating integrins alpha [316,317], a signal sequence and a transmembrane domain are found within EmTIP and thus suggest a membrane-bound/extracellular localization. Indeed, the observation that EmTIP could be detected in the parasite lysates and as a soluble form in the secretions of early and late parasite larvae corroborates this point. EmTIP secretion, in spite of its transmembrane domain, is congruent with the secretion of TIP by mammalian cells [205]. Moreover, EmTIP localization to the inter-cellular space within all the parasite larvae provide yet additional support to its role in the cell-cell/ECM interactome as described for FG-GAP repeat-conatining proteins [317].

Interestingly, antibodies directed against the putative interacting domain of the FG-GAP repeat-containing protein EmTIP i.e. the FG-GAP repeat-containing portion [236,321–323] could limit *E. multilocularis* primary/stem cell proliferation. Since cell-cell/ECM interactions are necessary for stem cell proliferation in various organisms [324–326], this observation suggests that EmTIP antibody-mediated binding could affect the parasite cell-cell/ECM interactions. Therefore EmTIP would probably be an important component of the parasite cell-cell/ECM interactome. Although the mechanism through which anti-EmTIP antibodies inhibit *E. multilocularis* cell proliferation remains unknown, it is likely that these antibodies sequester soluble EmTIP or cover membrane-associated EmTIP, and thus interfere with any eventual ligand binding and/or transduce an inhibitory signal. Assessing whether these proposed mechanisms would separately or altogether account for the impairment of *E. multilocularis* cell proliferation would clearly provide valuable information on the biology of *E. multilocularis*.

Furthermore, I showed that anti-EmTIP antibodies inhibited the formation of mature metacestode vesicles from primary cells *in vitro*. Given the suggested importance of this process as a model for the early transformation from oncosphere into metacestode [190,191] and the process of metastasis formation [190,191,272,327], this finding suggests that EmTIP might play a role during the onset of AE and most probably for the metastatic growth of the parasite larvae *in vivo*. Indeed, *E. multilocularis* primary cells have been suggested to mediate the early expansion of the parasite metacestode tissue and to drive the parasite dissemination within the host [14]. Therefore, the *in vitro* impairment of *E. multilocularis* primary cell culture transition into

metacestodes as a result of EmTIP blockade/binding suggests that this approach holds considerable promise for the control of AE onset and progression. First, assessing the role of the antibody-mediated EmTIP blockade *in vivo* during *Echinococcus* infection is needed. Given the prospect of additional parasiticidal mechanisms like ADCC (antibody-dependent cell cytotoxicity), likely to follow EmTIP recognition by host antibodies *in vivo*, it would also be desirable to see the potency of EmTIP antigen as a candidate vaccine.

Rather encouraging for this potential host-protective role of EmTIP is the observation that EmTIP triggered the release of IFN-γ by CD4+ T-cells *in vitro*, a cytokine shown to promote anti-parasitic events in *E. multilocularis*-infected hosts [133,136,137,170,237]. It therefore appears that EmTIP homologies with TIP enabled the parasite factor to instruct a similar pro-IFN-γ response in T-cells. Which receptor(s) on these cells is/are recruited by T-cell immunomodulatory proteins and which signaling pathway(s) is/are of importance are yet to be determined. But again, this effect of EmTIP is unprecedented and provides the first possible explanation for the early Th1 response reported during AE [129,152–154]. Clearly, if released *in vivo* as observed *in vitro* during the immunoprecipitation assays, EmTIP would interact with the host CD4+ T-cell. This interaction might lead to IFN-γ release, which would support T-cell polarization towards Th1.

The present *in vitro* stimulation system, although failing to show IL-10 release in response to EmTIP-containing supernatants, does not establish that the purified protein would similarly fail in this regard. Indeed, the supernatant of TIP-transfected cells used in a previous study [205] could uniquely trigger IFN-γ release by T-cells. When purified, TIP could in addition to IFN-γ secretion prompt the release of IL-10 and TNF by the stimulated T-cells [205]. An affinity purification of EmTIP is therefore needed to pursue the T-cell stimulation assays. In this respect, I already generated an EmTIP-expressing cell line which would facilitate the obtention of large amounts of EmTIP-containing supernatants.

TIP has up to 12 N-glycosylation sites and is secreted as highly glycosylated form [205], EmTIP only has 2 potential N-glycosylation sites and only appears secreted at a higher molecular weight by protoscoleces. Interstingly, neither primary cells nor Em*tip*-transfected mammalian cells could release this higher molecular weight form. This could explain the differences observed with regards to the induction of IFN-γ release by CD4+ T-cells following exposure to these

supernatants. Whereas primary cell E/S products and recombinant EmTIP could clearly trigger IFN-γ release on the one side, protoscolex E/S products failed to show this ability. Thus, the presence of this additional band of higher molecular weight in protoscolex E/S products could be explained here as a posttranslational variant associated with feedback regulation/inhibition, whereas the lower molecular weight (58kDa) EmTIP variant, as secreted by primary cells and transfected HEK cells, would be associated with the induction of IFN-γ release by T-cells. Fractionation studies on protoscolex E/S products in order to separately assess the IFN-γ inducing potency of these 2 variants in T-cells could provide more insights towards this issue. Additionally, the differential secretome of *E. multilocularis* larval stage as previously suggested in this work could clearly account for an anti-IFN-γ potency of protoscolex released metabolites which would be absent from primary cell E/S products. In any case, the present data indicate that EmTIP as expressed by the parasite primary cells strongly evokes IFN-γ production by CD4+ T-cells. Whether EmTIP is secreted (or cleaved off) by the primary cell to affect the host T-cells is rather unlikely since the metacestode failed to release EmTIP, probably as a result of their morphology (i.e. fluid-filled cyst, surrounded by a laminated layer). Indeed at this stage EmTIP is most probably retained completely within the intracystic compartment as observed in the immunohistochemical experiments. Therefore, EmTIP would rather appear to be released in an attempt to fulfill developmental functions, accumulating within the cellular matrices and defining a suitable niche for the parasite cell proliferation and differentiation.

In conclusion, given the implication of EmTIP in *Echinococcus multilocularis* stem cell proliferation and cyst formation and the essential role of this phenomenon in disease establishment and metastasis formation and in keeping with the ability of this factor to promote IFN-γ release by CD4+ T-cells, I strongly recommend the assessment of EmTIP as a candidate vaccine. Moreover, I propose the investigation of the latter with regard to the identification of lead target for anti-echinococcal prophylaxis on asymptomatic seropositive patients as well as in post-operative instances as an attempt to limit relapses.

4.4 A model for the immunomodulation of hosts by *Echinococcus multilocularis*.

Understanding the events and associated molecular bases of the host immunomodulation by parasitic helminths in the course of an infection is of clear benefits for the development of more efficient deworming strategies. Additionally, beyond the level of parasitology, unraveling and utilizing the molecular stratagems used by long-lasting parasitic helminths like *E. multilocularis* to persist within immunocompetent hosts could clearly provide new tools to counterpose detrimental immune responses and the ensuing human pathologies like autoimmune diseases, allergies or acute graft rejection during transplantation. In the light of such multiple applications of the understanding of host immune response to parasitic helminths in general and to our model the fox-tapeworm *E. multilocularis* in particular, I will herein draw a possible picture of the immunological events governing hosts colonization by our model the fox-tapeworm *E. multilocularis* (Fig. 37) using previous investigations and evidences provided by the present work.

In susceptible murine hosts, upon uptake, *E. multilocularis* eggs / oncospheres fail to trigger an augmentation of total IgG, IgA or IgM within the intestine [128]. However, the local amount of anti-echinococcocal immunoglobulins is enhanced by the parasite eggs entry. The eggs / oncospheres trigger a strong local production of anti-echinococcocal IgG and IgA [128]. Additionally, lymphocytes then gradually infiltrate the lamina propria of the intestine. The intestinal mucosa permeability is significantly increased allowing the transit of the parasite oncospheres [128]. At the systemic level, the humoral response appears non-specifically suppressed with a reduced amount of total IgG, IgA and IgM [128]. Slowly, the intestinal response vanishes with the oncospheres now extraintestinal. The intestinal mucosa permeability also returns to normal [128]. At this level, little is known on the profile of host-protective responses. It would be interesting to assess the role of the local and systemic anti-echinococcal immunoglobulins on the containment of the parasite oncospheres within the gut and short upon gaining access to the host interior milieu. Given the widely approved central role of IgA in mucosal immunity [328], recent lines of research are underway to identify IgA-inducing antigens from the parasites as vaccination approaches [329,330].

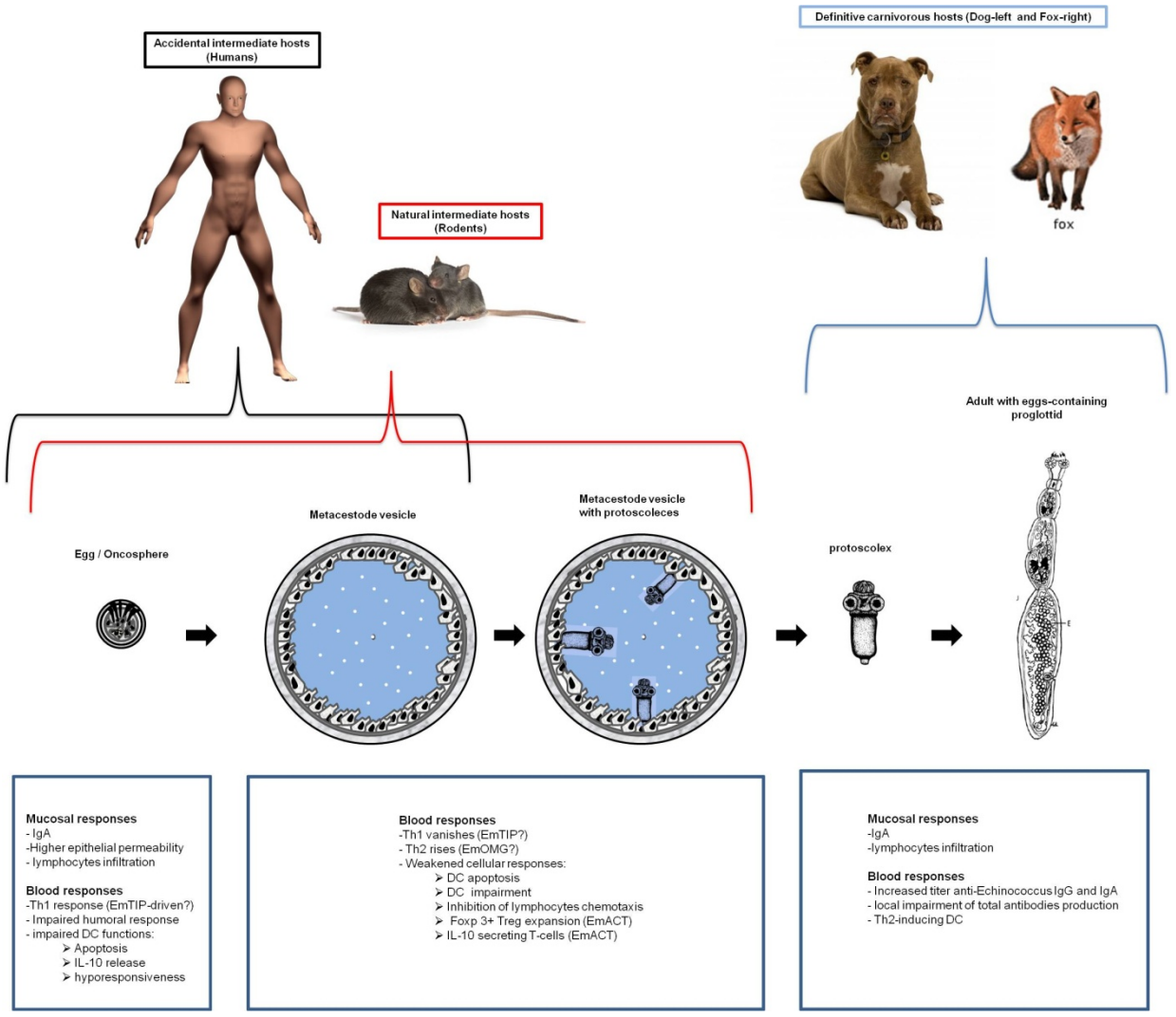


Figure 37: Alterations of the immune system in response to *E. multilocularis* larvae: a model.

An interesting point here is the early and non-specific impairment of the systemic host humoral response by *E. multilocularis* oncospheres [128]. This reconciles with the present consensus of a dominating cellular Th1 response following passage of the oncospheres through the intestinal barrier [129,152–155]. It is enviseageable that following the oncosphere drainage and in the process of metamorphosis, its population of undifferentiated cells release factors like EmTIP that are important for cellular proliferation and cyst formation. In the process, however, EmTIP triggers the release of IFN-γ by host CD4+ T-cells. Given, the deleterious effect of IFN-γ-driven

immune cell activation on the parasite larvae [136,137], the completion of the oncosphere metarmophosis and the formation of metacestode are likely to be severely compromised. This is consistent with the frequent observation of abortive cases of the disease in patients confirmed seropositive for *E. multilocularis* antigens [331–334]. However, the parasite cells can eventually reprogram the host immune sentinels like DC that are encountered during their migration from the intestinal entry site to the liver. Aided by their released products, the oncospheres induce apoptosis and evoke IL-10 production in DC. Moreover, to presumably counter the inflammation resulting from the intestinal epithelia damage as a result of the oncosphere passage, the oncosphere secretions render these DC hyporesponsive to bystander inflammatory stimuli. This immunosuppressive environment most probably helps alleviate the deleterious effects of IFN-γ-activated macrophages and cytotoxic cells that result from EmTIP release. This altogether most probably enables a certain degree of survival for the parasite cells in the face of a dominated Th1 immune response and, with the help of factors like EmTIP, drives the metamorphosis of the early oncospheres into metacestodes

The development of *E. multilocularis* metacestode coincides with the decline of parasiticidal Th1 responses [170,237]. As the potential Th1 driving factor, EmTIP is no longer secreted by the parasite at this stage and given the ability of the parasite somatic and secretory factors to drive T-independent responses [175], a dominating Th2 response gradually arises [134,142,163]. Shedded carbohydrate rich components of the laminated layer [174] might also instruct the recruited phagocytes to preferentially direct Th2 polarization. Moreover, Th2-driving factors like the parasite omega-1 homologue EmOMG could account for a Th1- to TH2 switch. A role has, however, still to be demonstrated for this factor in the course of an infection. Furthermore, the metacestode might support the commitment of T-cell to a Th2 profile by promoting IL-4 release by basophils [176]. The metacestode also targets the host DC. In keeping with the pivotal role of DC in T-cell polarization [54,181,335], metacestodes alter DC away from a classical Th1-inducing profile. To continue minimizing the host protective responses, the metacestodes sustain host DC killing by apoptosis via its released products. Interestingly, presumably due to protection by a thick accelular membrane, the metacestode stops inducing IL-10 production in DC, as opposed to the early oncospheres. This could additionally occur as a result of the fact that the infection has now progressed over the initial immune recognition phase where innate immune functions were predominantly actives. Now that the host might have recruited cells from

the adaptive immune response, metacestodes would accordingly redirect some of their immunomodulatory tools against the host adaptive immune response, probably as a result of a long evolutionary process of adaptation to parasitism. Again, the metacestode efficiently limits the chemotaxis of inflammatory cells [150,171] to possibly achieve a more permissive environment and thus quietly thrive. To further counter the host immunity in general, the metacestodes now release factors like EmACT which induce regulatory properties in the host T-cells. As a result, the gradually establishing Th2 response is also negatively affected, therefore allowing a silent and lengthy dwelling of the metacestode until a chronic stage is attained. A particularly critical aspect of this model is the presumption that regulator T-cells are important for the parasite persistence. Further investigation is urgently needed in order to attest or refute such a role for these cells. Whatever the case, the switch from a larvicidal Th1 to a Th2 response that is supposedly supported by the induction of an immunoregulatory network both at the level of the host innate and adaptive immune responses, is clearly a result of the metacestode driven fine-tuning of the host immune system. In this setting, the metacestode proliferates, metastasizes and, in the case of highly susceptible rodents, produces protoscoleces. The heavily infected rodent hosts are now easy preys for the definitive carnivorous host as a result of their slowness of gate.

In the gut of the definitive host, *E. multilocularis* protoscoleces excyst and closely adhere to the definitive intestinal submucosa [165]. The protoscoleces and its secretions evoke mucosal immune responses triggering the chemotaxis and activation of IgA-producing cells at the local level [165]. Supposedly, mucosal DC are also altered by the parasite secretions and mature into Th2-inducing DC that would most probably foster the production of anti-echinococcocal antibodies. The higher the anti-echinococcal titer of these hosts the more protected they are thus the more impaired is the protoscoleces development of gravid proglottids [165,336]. The protoscoleces also trigger a systemic response in their carnivorous hosts given their tight anchorage to the intestinal sub-mucosa [165]. The systemic response appears finely downregulated in the vicinity of the scolex, limited in both cytotoxic and and effector cell activity [165]. A central role for the protosoclex E/S products is highly likely mediating these effects. Neverthless, serum IgG and IgA titers become elevated in response to the protoscoleces and tightly correlates with the protection against the partasite [165].

In conclusion, the present model proposes novel, appealing clues for the modulation of the hosts immune responses by *E. multilocularis* larvae. An important role is demonstrated for the parasite secretions of which two evolutionarily conserved parasite factors EmTIP and EmACT shed more light to the mechanisms of the early host Th1 response and the mitigation of potentially host-protective immune responses, respectively. An in-depth investigation of the scope of the present factors at the molecular level with regards to the control of AE and inflammatory diseases should therefore be performed.

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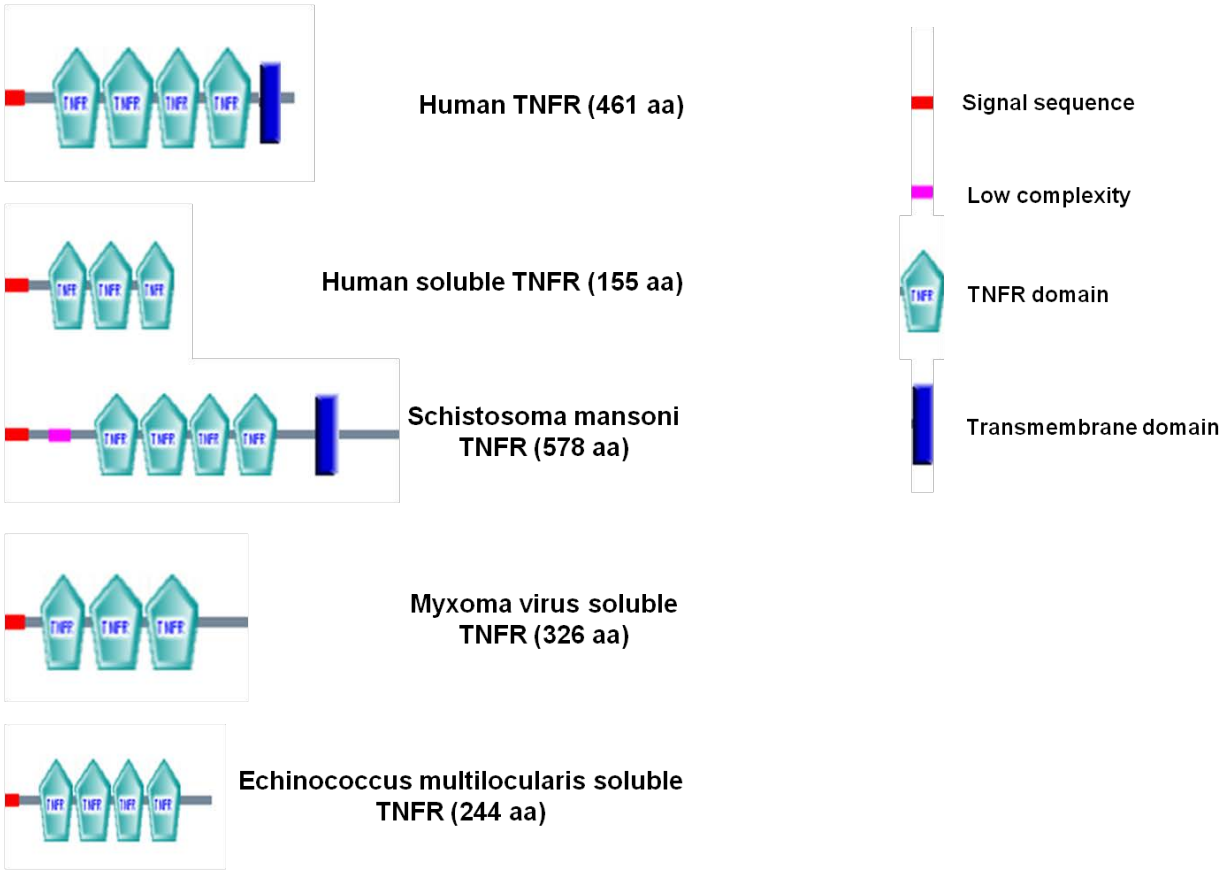
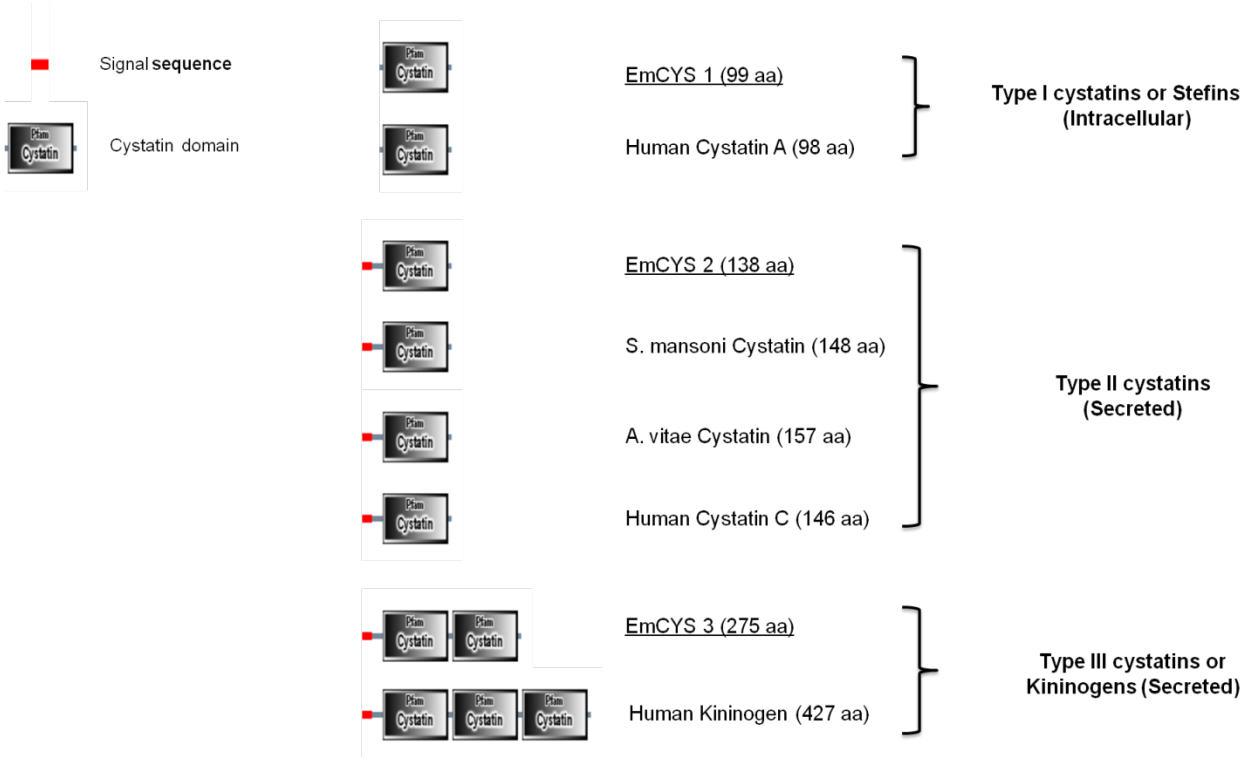
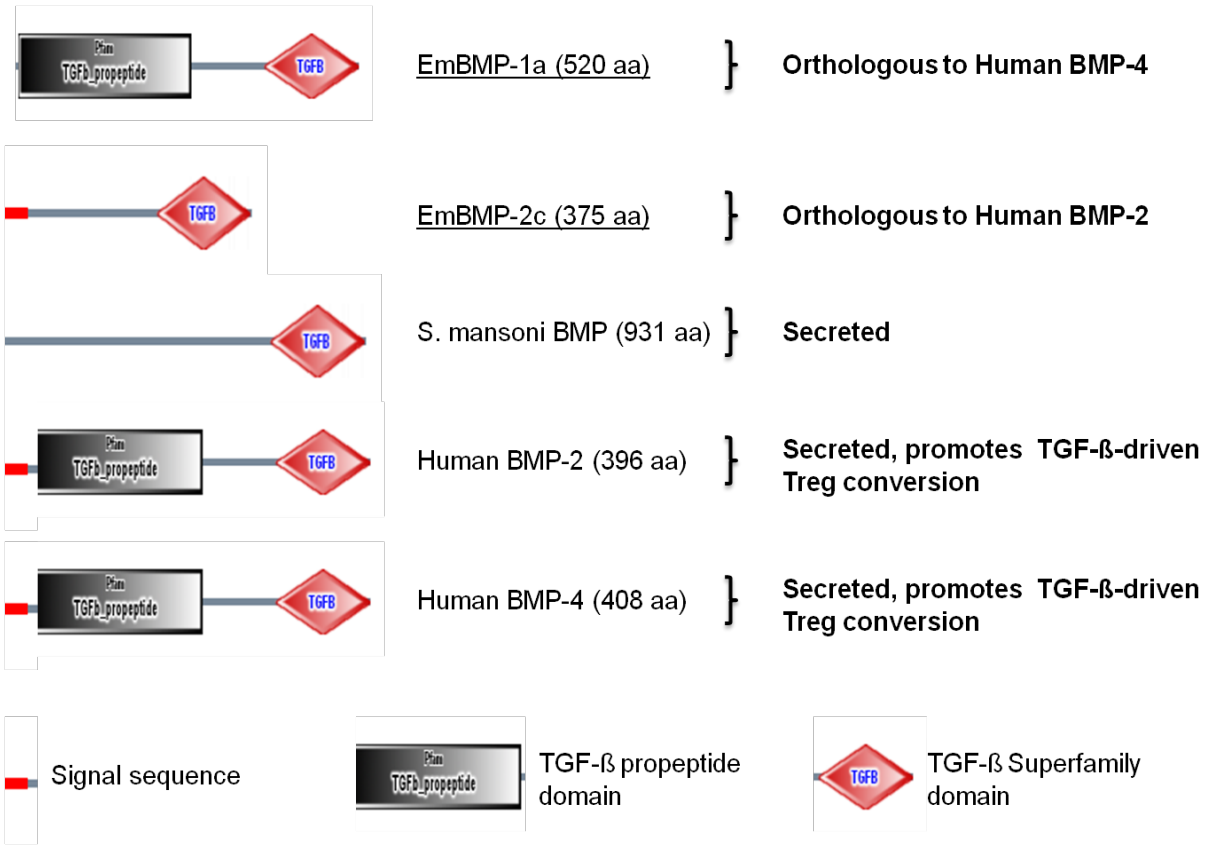
6. SUPPLEMENTARY FIGURES



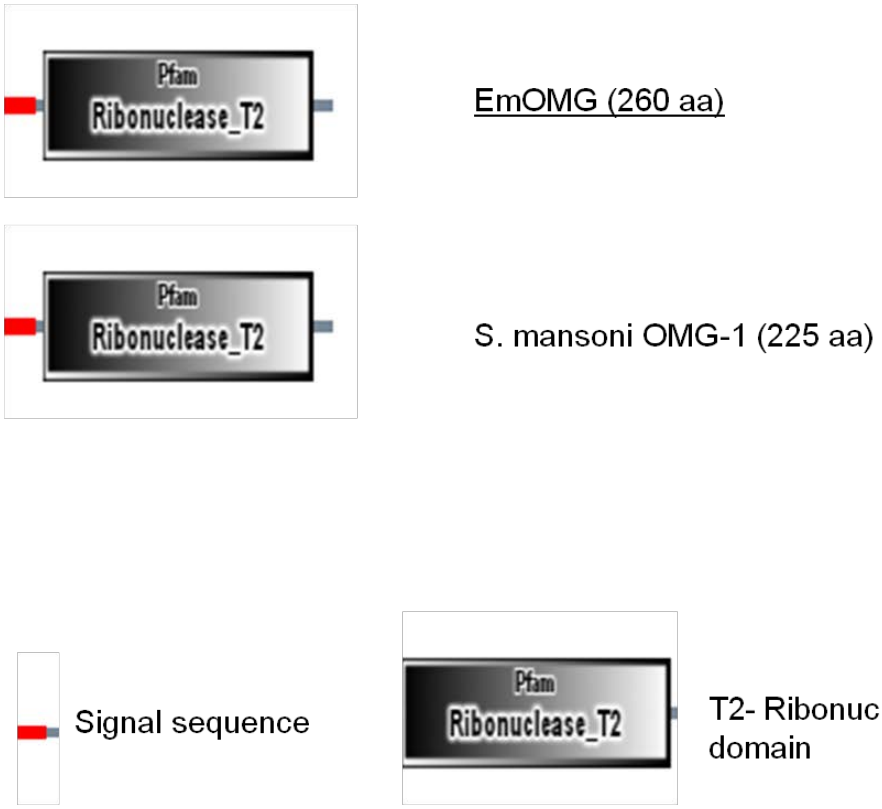
Supplementary Figure 1: Domains of the *Echinococcus multilocularis* putative Tumour Necrosis Factor Receptor in comparison to other functionally characterized TNFRs. Shown is a putative secreted TNFR retrieved from the genome sequencing project of *Echinococcus multilocularis* (EmuJ_000990500) in comparison to other membrane bound and soluble TNFRs functionally characterized. The domains were identified *in sillico* using SMART (http://smart.embl-heidelberg.de/).



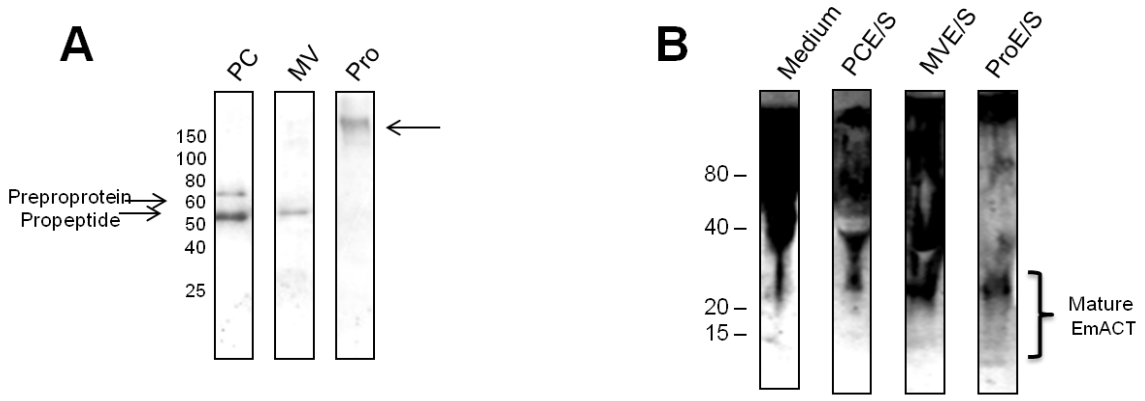
Supplementary Figure 2: *Echinococcus multilocularis* Cystatins in comparison to other functionally characterized cystatins (cysteine protease inhibitors). Shown are three experimentally identified cystatins of *Echinococcus multilocularis* structurally clustered with functionally characterized cystatins. EmCYS1 (EmuJ_000159200) is shown as a stefin or intracellular cystatin. EmCYS2 appears a type 2 secreted cystatin whereas EmCYS3 (EmuJ_000849600) is an atypical type 3 multidomain cystatin. The domains were identified *in sillico* using SMART (http://smart.embl-heidelberg.de/).



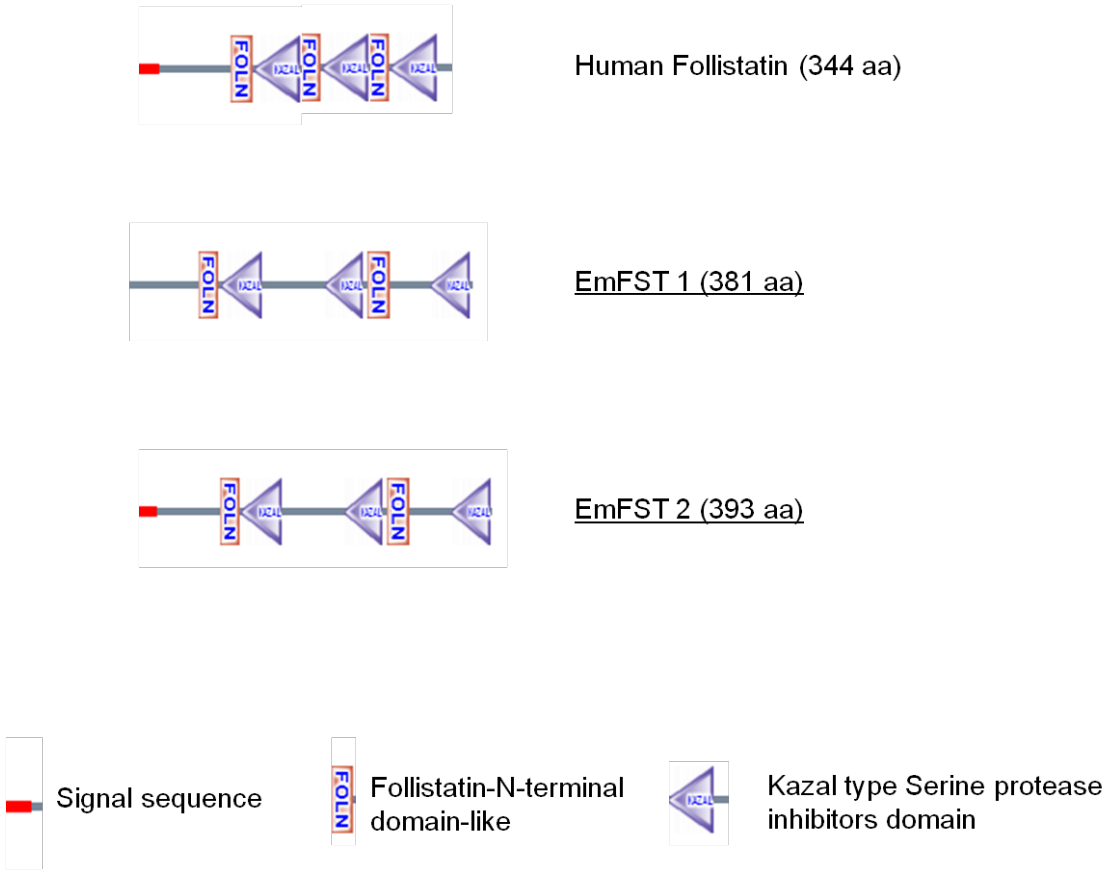
Supplementary Figure 3: *Echinococcus multilocularis* bone morphogenetic proteins. Shown are two experimentally identified BMPs of *Echinococcus multilocularis* structurally related to the functionally characterized human BMP-2 and BMP-4. EmBMP-1a (EmuJ_000181200) is shown as primarily related to Human BMP-4 whereas EmBMP-2c (EmuJ_000460200) relates to Human BMP-2 as per reciprocal BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). S. mansoni BMP-like protein is also depicted illustrating the possibility of secretion despite the absence of a N terminal signal sequence. The domains were identified *in sillico* by SMART analysis (http://smart.embl-heidelberg.de/).



Supplementary Figure 4: *Echinococcus multilocularis* T2- Ribonuclease or Omega-1 orthologue. The domains were identified *in sillico* by SMART analysis (http://smart.embl-heidelberg.de/).



Supplementary Figure 5: Expression and secretion of EmACT by *in vitro* cultivated larvae of *E. multilocularis*. Shown in **A** is the level of EmACT expression within the parasite larvae lysates suggesting a complex post translational modification in a stage-specific manner. In **B** is depicted the detection of EmACT in the parasite secretions (PC: primary cells; MV: metacestode vesicles; Pro: protoscoleces) revealing EmACT secretion by all larvae in differently processed forms. PS: single experiment.



Supplementary Figure 6: *Echinococcus multilocularis* putative Follistatins. Shown are *E. multilocularis* Follistatins i.e EmFST1 (EmuJ_000278400) and EmFST2 (Emu-J000189700) as referenced to Human follistatin or activin A inhibitor. The domains were identified *in sillico* by SMART analysis (http://smart.embl-heidelberg.de/).

7. LIST OF ABBREVIATIONS

7-AAD	7-Amino-actinomycin D
aa	amino acid
Ab	Antibodies
ABZ	Albendazole
AE	Alveolar Echinococcosis
Ag	Antigen
ALK	Activin receptor-like kinase
APC	Antigen-presenting Cell
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMDC	Bone marrow-derived DC
BrdU	Bromodeoxyuridine
BZs	Benzimidazoles
CCL	Chemotactic Cytokines Ligand
CCR	Chemotactic Cytokines Receptor
CD	Cluster of Differentiation
cDNA	complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
Cov.	Coverage
DAMPs	Damage Associated Molecular Patterns
DC	Dendritic Cell (s)
DMEM	Dulbecco's Modified Eagle Medium
E/S	Excretory/Secretory or Excretions/Secretions
ELISA	Enzyme-linked immunosorbent assay
EmACT	*Echinococcus multilocularis* activin
EmAP	*Echinococcus multilocularis* Alkaline Phosphatase
EmTIP	*Echinococcus multilocularis* T-cell Immunomodulatory Protein
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FG-GAP	Phenyl-alanyl-glycyl - glycyl-alanyl-proline
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency virus
Id.	Identity

Ig	Immunoglobulin
LPS	Lipopolysaccharide
MBZ	Mebendazole
MHC	Major Histocompatibility Complex
Mult	*Meriones unguiculatus*
MV	Metacestode Vesicle (s)
OD	Optical density
OVA	Ovalbumin
p.i	Post infection
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffer Saline
PC	Primary Cell (s)
PCR	Polymerase Chain Reaction
Pro	Protoscolex (ces)
RH-	Rat reuber Hepatoma
RPMI	Roswell Park Memorial Institute Medium
SCID	Cevere Combined Immunodeficiency
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
Sim.	Similarity
TCR	T Cell Receptor
Teff	Effector T cells
TGF-β	Transforming Growth Factor
Th	T helper
Tregs	Regulatory T cells
UV	Ultraviolet
Vs.	Versus
WHO	World Health Organization

8. PUBLICATIONS

Parts of this work have been / will be published in peer reviewed journals and have been presented on international conferences as talks or poster presentations.

8.1 Original publications

Nono JK, Pletinckx K, Lutz MB, Brehm K.

Excretory/secretory-products of *Echinococcus multilocularis* larvae induce apoptosis and tolerogenic properties in dendritic cells *in vitro*.

PLoS Negl Trop Dis. **2012**;6(2):e1516.

Nono JK, Lutz MB, Brehm K.

Expansion of Foxp3+ regulatory T cells in mice infected with the Tapeworm *Echinococcus multilocularis.*

Manuscript in preparation

Nono JK, Epping K, Lutz MB, Brehm K.

An Activin Homologue Secreted by the *Echinococcus multilocularis* Metacestode Promotes Regulatory T-cell Conversion *in vitro*.

Manuscript in preparation

Nono JK, Lutz MB, Brehm K.

EmTIP, a T-cell immunomodulatory protein secreted by the tapeworm *Echinococcus multilocularis* is important for early larval development.

Manuscript in preparation

8.2. Conference contributions

Nono JK, Pletinckx K, Lutz MB, Brehm K. (Talk)

"E/S products of *E. multilocularis* larvae induce apoptosis and tolerogenic properties in Dendritic cells *in vitro*."

Joint Meeting of the German Societies for Tropical Medicine and International Health and German Society for Parasitology; Heidelberg; Germany. 15 - 17 Mar 2012.

Nono JK, Pletinckx K, Lutz MB, Brehm K. (Talk)

"E/S products of *E. multilocularis* larvae impede Dendritic cell responsiveness *in vitro*."

International Meeting "HIV and Infectious Diseases" ; International Research Training Group 1522; Wuerzburg; Germany. 19 - 20 Jan 2012.

Nono JK, Pletinckx K, Lutz MB, Brehm K. (Poster)

"Primary Cells of *E. multilocularis* trigger murine Dendritic cells to release Interleukin-10."

Joint Meeting of the German Societies for Parasitology and Protozoology; Düsseldorf; Germany. 16 - 20 Mar 2010;

9. CURRICULUM VITAE

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University of Würzburg, Germany
Institute for Hygiene and Microbiology

Jan 2009- Dec 2012

PhD Studies
University of Würzburg, Germany
Institute for Hygiene and Microbiology
Thesis: "Immunomodulation through Excretory/Secretory Products of the parasitic Helminth *Echinococcus multilocularis*"

Supervision: Prof. Dr. Klaus Brehm (Würzburg, Germany)
Prof. Dr. Manfred Lutz (Würzburg, Germany)
Prof. Dr. Frank Brombacher (Cape Town, South Africa)
Prof. Dr. Joachim Morschhäuser (Würzburg, Germany)

Oct 2005- Jul 2008

Graduate Studies (Master of Science)
University of Yaounde I, Cameroon
Biochemistry Department
Thesis: "Establishment of a PCR based technique for the detection of Constant Spring mutants among malaria treated patients in Cameroon"

Supervision: PD. Dr. Wilfred Mbacham (LAPHEB)
Dr. Sarah Riwom (Biochemistry Dept)

Oct 2002- Sept 2005

Undergraduate Studies (Bachelor of Science)
University of Yaounde I, Cameroon
Biochemistry Department

Würzburg

Date Signature