

Review Article

***Echinococcus multilocularis* and Its Intermediate Host: A Model of Parasite-Host Interplay**

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Host-parasite interactions in the *E. multilocularis*-intermediate host model depend on a subtle balance between cellular immunity, which is responsible for host's resistance towards the metacestode, the larval stage of the parasite, and tolerance induction and maintenance. The pathological features of alveolar echinococcosis, the disease caused by *E. multilocularis*, are related both to parasitic growth and to host's immune response, leading to fibrosis and necrosis. The disease spectrum is clearly dependent on the genetic background of the host as well as on acquired disturbances of Th1-related immunity. The laminated layer of the metacestode, and especially its carbohydrate components, plays a major role in tolerance induction. Th2-type and anti-inflammatory cytokines, IL-10 and TGF- β , as well as nitric oxide, are involved in the maintenance of tolerance and partial inhibition of cytotoxic mechanisms. Results of studies in the experimental mouse model and in patients suggest that immune modulation with cytokines, such as interferon- α , or with specific antigens could be used in the future to treat patients with alveolar echinococcosis and/or to prevent this very severe parasitic disease.

1. Introduction

The infection of the intermediate hosts by the metacestode stage ("larval stage") of *Echinococcus multilocularis*, a cestode of the genus *Echinococcus*, is a good example to demonstrate the pivotal role the host immune response plays, favoring on one hand a partial control of infection, but yielding also a partial impairment of its own responsiveness [1–3]. Both tolerance and immunopathology may be observed in Alveolar Echinococcosis (AE), the disease due to *E. multilocularis* [4], and most of the mechanisms occurring seem to be triggered by a sophisticated modulation born out of a stage-specific parasite metabolism and respective immunomodulating strategy.

Tolerance is essential to ensure growth and development of the larval stage of the parasite in its host. In fact, parasite survival relies upon a mutual tolerance of the parasite and the host, and like all parasites, *E. multilocularis* modulates and even "uses" the host's immune system to ensure its

survival. Early killing of the parasite by the host would, of course, not allow the larva to grow and proliferate to reach the fertile stage (i.e., the production of protoscoleces) which allows its passage to the definitive host and thus its "adult" stage. Conversely, early killing of the host by the *Echinococcus* sp. larvae would lead to premature death of the host, and thus would also not allow the parasite to develop until the production of the fertile stage of the larva. *E. multilocularis* has evolved suitable strategies, at the recognition and effector stages of the immune response, to achieve its goal: to avoid both its premature death and the death of its host.

The immune response which develops against the larval stages of *E. multilocularis* accounts for a controlled parasite tissue development, but also for immunopathological events. In AE, hypersensitivity and immunopathology encompass several mechanisms: (1) mostly cell-related immune reactions leading locally, in the liver and other involved organs, to necrosis and fibrosis, (2) a rather Th2-dominated hypersensitivity reaction, although IgE-dependent clinical

manifestation are rare despite a constant IgE production by the host. Combination of epidemiological data on AE in endemic areas with immunological findings in humans and in experimentally infected murine intermediate hosts, has allowed us to design a rather comprehensive picture of the way how parasite and host survive, an understanding that may contribute to develop new treatment strategies comprising immune modulatory and stimulatory tools.

2. Susceptibility and Resistance of Animal Intermediate Hosts to *E. multilocularis*

E. multilocularis exhibits different growth rates and maturation characteristics in various species of hosts, that is, species of rodents or lagomorphs for *E. multilocularis*, but also of multiple other animal species such as swine and primates. These differences were first assigned to differences in strains/subspecies of the parasites; however, there is very little variation in *E. multilocularis* species [5, 6]. As rodents are the natural intermediate hosts of *E. multilocularis* in the conventional parasitic cycle, differences in host immune responses have been extensively studied in *E. multilocularis* experimental infection, and actually, differences in susceptibility/resistance, putatively related to respective immune responses, do occur in different murine models [7–11]. Impairment of cellular immunity (immune suppression) is followed by an increase in susceptibility to *E. multilocularis* in experimental animals. This was shown more than 30 years ago in immunosuppressed mice by Baron and Tanner [12] and was further demonstrated later on using SCID mice, which were shown to be highly susceptible compared to the wild strain and to reconstituted mice [13], and in nude mice [14]. A similar increase of susceptibility of experimental mice, associated with a decrease of delayed type hypersensitivity, was also observed in mice infected with *E. multilocularis* and treated with an immunosuppressive drug, cyclosporine, which interferes with IL-2 production in T-cells [15].

Conversely, cellular immune response against parasitic antigens is stronger in infected resistant mice, tested either using specific delayed-type hypersensitivity reactions *in vivo* [8] or specific proliferation of lymphocytes *in vitro* [11]. Resistance is increased by stimulation of the cellular immune response, as was shown with Bacille Calmette Guérin administration [16]. It was also shown that the antiparasitic effect of Isoprinosine treatment in mice infected by *E. multilocularis* [17] was at least partially due to immune stimulation by this immunomodulating agent [1].

3. Susceptibility and Resistance of Human Hosts to *E. multilocularis*

Liver transplantation has been performed in patients with very severe cases of AE since 1986. Observations in transplanted patients, who received immunosuppressive agents to prevent liver rejection, confirmed the increased susceptibility to *E. multilocularis* growth in humans upon impaired immune responsiveness. Increased susceptibility

was evidenced by a rapid increase in size of lung metastases, the development of brain metastases, late re-invasion of the transplanted liver by parasitic cell remnants, and even early re-invasion of the transplanted liver from a spleen metastasis [18, 19]. Similarly, a case of co-infection by *E. multilocularis* and Human Immunodeficiency Virus (HIV), leading to AIDS, has been reported, with a rapid and irreversible growth of *E. multilocularis* larvae in a young patient [20], leading to fatality. Associated to AIDS, restoration of immunity by appropriate antiretroviral therapy has led to reinstallation of the control of metacestode development [21]. Other cases have been observed in several European countries since then, as well as cases of AE associated with administration of other immunosuppressive drugs for autoimmune diseases (see [22], and personal communications through the EurEchinoReg network). AE thus appears to be another example of “opportunistic infection”.

In humans, a variety of clinical presentations of AE may be seen; however, pathological features and the frequent absence of protoscoleces suggest that, generally speaking, humans are relatively resistant to *E. multilocularis*. In fact, the implementation of mass screenings in endemic areas has revealed that the number of established infections in humans was far lower than the number of exposures to parasitic eggs [23, 24]. It may be assumed that a minority of individuals among humans (estimated to 1 out of 10 subjects) allows the development of the *E. multilocularis* metacestode after a contact with *E. multilocularis* oncospheres, the infectious component produced by the adult worm in the intestine of carnivores, which are definitive hosts [25].

The conceptual consequences of these findings in humans, added to the observations made in experimental rodents, cover two complementary, albeit non-mutually exclusive, assessments: (1) natural (immunological) mechanisms of defence (innate or acquired) are at work in the majority of human hosts, which are able to stop the larval growth at the very first stages or after the beginning of its development in the liver, (2) strategies are operating at the parasite’s level, which may counteract the immune system of the host and even take advantage of it for its own growth and survival. Studies performed in experimental animals as well as in humans with AE currently offer a rather comprehensive picture of the main events which operate at the very beginning of infection, when *E. multilocularis* settles within the host’s liver, and at the effector stage of the immune response, when *E. multilocularis* develops and progressively invades the liver and other organs of the hosts. These two stages will be presented successively although they are actually linked and interdependent.

4. Role of Parasite-Derived Molecules in the Modulation of the Host Response in View to Induce Immune Tolerance to the Parasite in Its Intermediate Host

In the host-parasite interplay, metacestode surface molecules as well as excretory/secretory (E/S) metabolic products are considered to function as important key players (reviewed

in [3]). The intraperitoneal murine infection model of AE offers the opportunity to study the direct effect of metabolic metacystode molecules on periparasitic peritoneal cells, including especially DCs, but also other immunologically relevant populations such as macrophages ($M\phi$), lymphocytes and other (inflammatory) cells that play a significant role in the putative control of (or respective failure to control) metacystode proliferation, and thus triggering of disease development. The *E. multilocularis* metacystode actively secretes or expresses molecules that putatively have potent effects on the immune system of the murine host. The production of these molecules and their chemical compositions might depend on the stage of the parasite (oncosphere, early vesicle or fully mature metacystode). However, little is known about biological effector molecules arising metabolically or somatically from the intrahepatic stages of the metacystode, although various *E. multilocularis* antigens, their epitopes and respective genes have been characterized. Every parasite molecule reaching the host environment has to pass the metacystode laminated layer. This laminated layer is an acellular carbohydrate-rich surface structure that protects the parasite from immunological and physiological reactions on part of the host. Among the main antigens described, a major carbohydrate named Em2 (G11) localizes on the surface of the laminated layer of the metacystode [26]. Em2 is a T-cell independent antigen, and the corresponding antibody response lacks antibody maturation [27]. Another polysaccharide-containing antigen, antigen C has been isolated and characterized from crude metacystode extract [28]. Similar investigations have yielded the finding of EmP2 [29, 30] and Em492 [31]. Em492 antigen is continuously released into the exterior medium and is also abundantly present in the parasite vesicle fluid. A suppressive effect on Con-A- or crude parasite extract-induced splenocyte proliferation in infected experimental mice was observed upon addition of Em492 antigen [31]. Protease treatment of Em492 antigen does not change its splenocyte proliferation inhibitory potential, while periodate treatment severely alters the functional properties of Em492 antigen, indicating that the effects of Em492 antigen is mediated through the carbohydrate part. Em492 antigen stimulates peritoneal macrophages from *E. multilocularis*-infected and -uninfected mice to produce increased levels of nitric oxide, leading to inhibition of splenocyte proliferation. On the other hand, Em492 antigen increases the levels of anti-CD3-induced apoptosis, which may contribute to a decrease of immune effector mechanisms. Another neutral glycosphingolipid has been identified as suppressor of human PBMCs proliferation following stimulation by phytohemagglutinin [32]. Hülsmeyer and co-workers [33] have then isolated novel mucin-type glycoforms from the metacystode of *E. multilocularis*, and these glycoforms contained mucin-type core-I type and core-II type structures that were further diversified by addition of GlcNAc or Gal residues. Koizumi et al. [34] reported on the synthesis of the glycan portions of a glycoprotein antigen of *E. multilocularis* in order to elucidate the interactions between oligosaccharides and sera of *E. multilocularis*-infected hosts. Stereocontrolled synthesis of branched tri-, tetra-, and pentasaccharides displaying

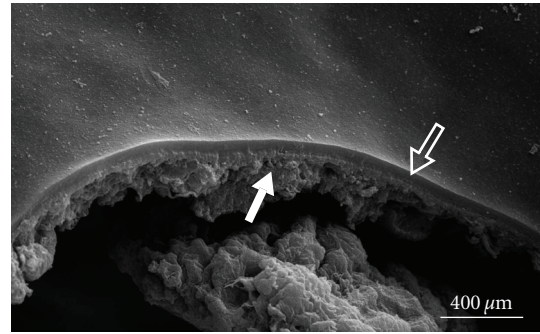


FIGURE 1: The laminated layer of *Echinococcus multilocularis*. Scanning electron microscopic visualization of an *Echinococcus multilocularis* metacystode vesicle trans-section: hollow arrow indicates outer laminated layer, white arrow indicates the inner germinal layer (Photo: A. Hemphill, IPB, Bern, Switzerland).

a Gal β 1- \rightarrow 3GalNAc core in the glycan portion of the glycoprotein antigen was achieved, which may become an interesting tool for further studies on their putative biological function. With regard to metabolized proteins, an *E. multilocularis*-protoscolex associated antigen of 62 kDa [35], two 70 and 90 kDa proteins [36], and several recombinant *E. multilocularis*-proteins (such as antigen II/3 [37] and its subfragments II/3-10 [38] and Em18 [39], or EM10 [40]), have all been published and discussed in view of a potential biological role. However, these antigens were mainly used to investigate respective immune responses with emphasis on immunodiagnosis of AE, and their biological functions have not been appropriately studied. EmAP (alkaline phosphatase) [41], an antigen which was shown to induce the production of antibodies associated with disease severity and resistance to treatment in AE patients [42], was also shown to induce only Th2-type cytokine secretion [43]. Aumüller et al. [44] used extracts from metacystodes of *E. multilocularis* to induce basophil degranulation, as well as the secretion of histamine, IL-4 and IL-13, in a dose-dependent manner. They concluded that *E. multilocularis* induces a Th2 response upon IL-4 release from basophils. Siles-Lucas and coworkers [45] identified and cloned a 14-3-3-gene of *E. multilocularis*, which appeared to play a key role in basic cellular events related to cellular proliferation, including signal transduction, cell-cycle control, cell differentiation, and cell survival [45, 46]. In a similar context, Kouguchi et al. [47] identified a cDNA clone, designated EMY162, that encoded a putatively secreted protein. EMY162 shares structural features with the EM95 antigen, for example, 31% amino acid sequence identity to EM95.

5. The Cross-Talk between *E. multilocularis* Larvae and Their Host through the Laminated Layer

As outlined above, the laminated layer is considered as a barrier between the parasite and the host (Figure 1). And, in fact, it may protect the growing larva from a direct

contact with the immune cells of the host, and especially the macrophages known as “epithelioid cells” that line the border of the germinal layer at the very early stage of parasitic development, and then the border of the laminated layer (Figure 2(a)); in addition, it may also protect the parasite against the attack by cytotoxic compounds such as activated complement proteins and NO. However, as described above, its main role in the protection of the parasite against the immune attack from the host seems to be mostly related to its immunomodulatory properties which inhibit immune cell activation directly or through the induction of immunoregulatory cytokines. Electron microscopic examination of the laminated layer suggests that, rather than a barrier, the laminated layer could well rather function as a “gate” between the parasitic germinal layer and vesicle and its host. The tegument of the germinal layer builds up a syncytium with numerous microtriches that protrude into the laminated layer; in addition, the release of membranous and vesiculated structures into the matrix of the laminated layer may be observed [3]. This “gate” could prove to be essential to ensure a regulated traffic of various substances between *Echinococcus* sp. and their hosts.

In fact, evidence of a “cross-talk” between the parasitic larva and its host is provided by a number of observations, the first one being the presence of high molecular weight host proteins within the “hydatid fluid” which was demonstrated at least 30 years ago both for *E. granulosus* [48, 49] and *E. multilocularis* [50]. Host immunoglobulins and albumin [48, 49, 51, 52], activators and inhibitors of the complement cascade [50, 53] and, recently, host-derived active matrix metalloproteinase 9, which was assumed to play a role in the periparasitic granulomatous reactions [54], were found in *Echinococcus* sp. hydatid fluid or bound to the cyst wall.

In addition, several lines of evidence now suggest that the larval development of *E. multilocularis* is triggered by cell signaling originating from the intermediate host [55, 56]. The phosphorylation of EmMPK1, a parasitic orthologue of the Extracellular signal Regulated Kinase (ERK) MAPK, is specifically induced in in vitro-cultured *E. multilocularis* metacestode vesicles, in response to exogenous host serum, hepatic cells and/or human epidermal growth factor (EGF). The *E. multilocularis* metacestode is thus able to “sense” host factors which results in an activation of the parasite MAPK cascade [57]. Cross-functioning between parasite-derived molecules and host liver was also described for parasite-derived enzymes: for instance, *E. multilocularis*-derived transglutaminase was shown to efficiently catalyze human liver-derived osteonectin cross-linking [58]. The fact that tissue-dwelling *E. multilocularis* expresses signaling systems with significant homologies to those of the host raises the highly interesting question whether cross-communication between cytokines and corresponding receptors of host and parasite can occur during an infection, that is, whether the parasite may also influence signaling mechanisms of host cells through the secretion of various molecules which might bind to host cell surface receptors. Such interactions could contribute to immunomodulatory activities of *E. multilocularis* or be involved in mechanisms of organotropism

and/or in host tissue destruction or regeneration during parasitic development. In a recent preliminary study, a significant influence of *E. multilocularis* metacestode on the activation of MAPKs signalling pathways was found in the liver cells both in vivo in infected patients and in vitro in cultured rat hepatocytes [59]. Significant changes in JNK phosphorylation was observed in hepatic cells in vivo, using hydatid fluid which contains a significant amount of host proteins. They were also observed using supernatant from axenic cultures of *E. multilocularis*, which is totally free of host components. This observation suggests that parasitic components, and not only factors from host origin, were actually acting on hepatocyte metabolic pathways.

It is possible that vesicle fluid from *E. multilocularis*, in addition to the already recognized proteins listed above, may also contain cytokines and growth factors from host origin and serve as storage for continuous release of factors both to the parasite and to the host through the laminated layer which appears critical at the host-parasite interface [3]. Dual interactions could thus ensure growth and survival of the parasite while interfering with host liver cells. As some of the cytokines which were proven to be important immunoregulatory factors leading to sustained tolerance, such as TGF- β , are quite important in the balance between cell proliferation and death/apoptosis, it may be suggested that the laminated layer could play a role similar to that of the placenta at the materno-foetal interface [60]: ensuring parasite growth and hepatic cell homeostasis while ensuring proper immune tolerance. Indeed, the *E. multilocularis* metacestode is sensitive to TGF- β signaling [61, 62] and the metacestode ERK-like kinase, EmMPK1, phosphorylates EmSmadD, a metacestode analogue of the Co-Smads of the TGF- β signaling pathway [63]. This is a novel and important field of research to understand better the subtle *Echinococcus*/host cross-talk and the complex triggering of the tolerance process.

6. Cells and Mechanisms Involved at the Early Stage of *E. multilocularis* Infection

At the time of initial encounter with its murine host, the metacestode might modulate the immune response. The changes that it induces are dynamic and depend on the stage of development, for example, ranging from oncosphere, to early stage vesicles up to a fully matured and fertile metacestode. Dendritic cells (DCs) and macrophages (M ϕ s) are among the first cells encountered by the parasite, which, by secreting and expressing certain molecules, has evolved mechanisms to suppress the major inflammatory and thus immunopathological pathway. Interaction of parasite metabolites with Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) that are expressed largely, but not exclusively, on DCs and M ϕ s is assumed to result in phenotypic changes and modification of the cytokine profiles produced by these cell types, but this has not yet been experimentally shown at the early post-oncospherical stage of infection of murine AE. Several experiments have suggested that *E. multilocularis*, either by cell-cell contact or

by its products, could inhibit and/or modulate the immune response at the very early stage of antigen presentation to T-cells. *E. multilocularis* was shown to modify the accessory cell function and interfere with antigen presentation through a parasite-derived macrophage modifying factor [64]. It was also shown that immature dendritic cells of human origin did not mature and had a reduced capacity to take up dextran via mannose receptors in the presence of crude non-fractionated *E. multilocularis* antigen; however, further maturation could be induced by proinflammatory cytokines; these mature dendritic cells, pulsed with *E. multilocularis* antigen, were slightly better inducers of T-cell proliferation than non-pulsed dendritic cells [65].

E. multilocularis-infected mouse macrophages as antigen-presenting cells exhibit a reduced ability to present a conventional antigen to specific responder lymph node T-cells when compared to normal macrophages [66]. Co-stimulatory signals are crucial for T-cell activation and it is well known that failure in the expression of one of the components of the receptor-ligand pairs may severely impair T-cell activation and induce tolerance by a mechanism generally called “anergy”. In the above mentioned experiment, using macrophages from *E. multilocularis*-infected mice, B7-1 (CD80) and B7-2 (CD86) remained unchanged, whereas CD40 was down-regulated and CD54 (=ICAM-1) slightly up-regulated. FACS analysis of peritoneal cells revealed a decrease in the percentage of CD4+ and CD8+ T-cells in *E. multilocularis*-infected mice. Taken together, the obstructed presenting activity of *E. multilocularis*-infected host macrophages appear to trigger an unresponsiveness of T-cells leading to the suppression of their clonal expansion during the chronic phase of AE infection [66].

By inducing functional changes in DCs and M ϕ s, the metacestode can achieve important shifts in T-cell subsets. An initial acute inflammatory Th1 response is subverted gradually to a Th2 response during the chronic phase of AE. Cytokines, such as IL-4, IL-5, IL-9 and IL-13, secreted largely by immune-cell types in response to parasite antigens, not only down-modulate the Th1 response but can also promote parasite expulsion and tissue renewal and repair [67]. The metacestode most likely achieves the Th2 expansion through the induction of regulatory cytokines, such as IL-10 and TGF- β [68]. As mentioned above, in murine AE, the cell-mediated immune response of the host plays an important role in controlling metacestode proliferation.

7. Cytokines Involved in the T-Cell Activation Stage of Immunity after *E. multilocularis* Infection

7.1. Th2 Cytokines. Cytokine profiles, due to the secretion of characteristic cytokines by (mostly but not only) T “helper” (Th) cells give an insight into immune mechanisms involved in host-infectious organism relationship and in the types of immune responses that are developed after the early stage of antigen and “pattern” recognition. Th1-cytokines [Interleukin- (IL)-2, Interferon- (IFN- γ)] are induced by IL-12 and mostly involved in T-cell-mediated cytotoxicity;

Th2-cytokines (IL-4, IL-5, IL-13) are induced by IL-4, and mostly involved in antibody-mediated non-cytotoxic immune responses; TH17-cytokine (IL-17) is induced by IL-23, modulated by IL-21 and IL-22 and TGF- β , and mostly involved in T-cell-mediated activation of innate immunity/inflammation, but also in tolerance [69].

High levels of Th2 cytokines are observed, including IL-4, IL-13 and IL-5, in addition to a relatively low level of secretion of IFN- γ , in patients with AE [70, 71], and in *E. multilocularis* infected mice [72]. Production of IL-5 by PBMCs, and among those very specifically by CD4 T lymphocytes, is induced by *Echinococcus* antigens not only in patients with AE but also in normal subjects [71]. Secretion of IgE and IgG4 antibodies is associated with the Th2 profile. Total IgE and specific IgE and IgG4 against *E. multilocularis* antigens are highly elevated in those patients with the most aggressive disease [73]. Disappearance of IgE and decline of IgG4 specific antibodies are significantly associated with regression of the lesions in patients treated with antiparasitic drugs, and both Th2-related antibody isotypes are the first to disappear after surgical cure [74, 75]; this represents indirect evidence of the prominent stimulation of Th2 cytokines by the viable parasite at a chronic stage.

In the experimental mouse model, a Th2 profile is also, globally, the hallmark of *Echinococcus* sp. infections at the chronic stage. In mice experimentally infected with *E. multilocularis*, three stages of cytokine secretion can be identified: (1) a first stage of Th1 cytokine secretion including IL-2 and IFN- γ , associated with a slow parasite growth; (2) a second stage characterized by a mixed secretion of Th1 and Th2 cytokine secretion, especially IL-5 and IL-10, associated with rapid parasite growth; and finally (3) a last stage of immune suppression with a nearly complete inhibition of lymphocyte proliferation and of cytokine secretion following specific or non-specific stimulation [76].

7.2. Cytokines Leading to Tolerance. The main cytokines involved in immune tolerance are IL-10 and TGF- β . Most of the studies in AE as well as in the experimental models have first focussed onto IL-10. The anti-inflammatory properties of IL-10 are well known, especially through the inhibition of macrophage activation and cytotoxic functions [77].

Spontaneous secretion of IL-10 by the PBMCs is the immunological hallmark of patients with progressing lesions of AE [71]. Conversely, IL-10 is significantly lower in patients with abortive lesions [78]. IL-10 is measurable in the serum of the patients with AE at higher concentrations than in control subjects [79]. A variety of cell types are involved in the secretion of IL-10 by resting and stimulated PBMC in patients with AE, especially CD4 and CD8 T-cells, but also non-T non-B cells [71]. “Suppressor” CD8 T-cells, induced by parasite products, were reported to be involved in tolerance to *E. multilocularis* [80, 81]. However, the relationship between the capacity of these cells to secrete IL-10 and their “suppressor” activity is unknown. A preliminary report has confirmed that locally, in the periparasitic granuloma, T-cells secreted IL-10 and the data suggest that IL-10 production is highest closer to the parasitic vesicles [82]. After experimental infection with *E. multilocularis*, IL-10 secretion by spleen

cells is slightly delayed and is part of the cytokine profile observed in the second phase of *E. multilocularis* growth [76]. Similar changes were also observed when measuring IL-10 levels in the serum of infected mice: they remained low before 80 days post-infection and then increased sharply at 100 days post-infection when they reached a peak [83].

The presence of TGF- β secreting cells in the periparasitic granuloma surrounding *E. multilocularis* vesicles in the liver of patients with AE has been recognised only very recently [68] and exploring TGF- β in its multiple functions in *E. multilocularis* infection is still an open field of research. To our knowledge, no information is available on the production of TGF- β by PBMCs in AE. Evidence of TGF- β production in experimental *E. multilocularis* infection is given by a recent study on the effect of combined pentoxifylline and albendazole on parasite growth: with this combination the inhibition rate of cyst growth was 88% and was associated with a marked decrease of IL-10 and TGF- β which were elevated in control mice [84].

7.3. Pro-Inflammatory Cytokines and Chemokines. All studies on the cytokine profile in AE, in humans and in the experimental models, have stressed that it was never a “pure” Th2 profile, but always a rather mixed profile, including the so-called “Th0” profile, even in late stages of the disease. Significant amounts of Th1 cytokines such as IL-2, IL-12, IFN- γ , as well as pro-inflammatory cytokines (IL-1, TNF- α , and IL-6) have been found to be secreted by peripheral blood mononuclear cells in most studies in humans infected by *E. multilocularis*. However, the relative ratios of these cytokines as well as of the chemokines produced when PBMCs from patients are cultivated with parasitic antigens give a rather complex picture. A recent study showed that production of the proinflammatory cytokines IL-1 α and IL-18 by *E. multilocularis* vesicle antigen-stimulated PBMC was reduced in AE patients, regulatory IL-10 was similar, but parasite vesicle-induced IL-8 was dominant and clearly elevated in patients [85]. Such selective and opposite dynamics of inflammatory cytokines and chemokine release may prevent pathogenic inflammation, and constitute an appropriate response for attraction of effector cells into the periparasitic tissues with the capacity to limit *E. multilocularis* metacyst proliferation and dissemination.

In the experimental model of secondary infection in mice, the levels of Th1 cytokines as well as pro-inflammatory cytokines was initially elevated, and then progressively decreased while Th2 cytokines and IL-10 increased [76]. Unfortunately, nothing is known on the involvement of Th17, IL-17 secreting T-cells, and of IL-21, -22 and -23 in the development of immune cell infiltration around the parasitic vesicles and their relationship with immunoregulatory cells in echinococcosis. This newly recognised subset of T helper cells appears to be essential for the development of T-cell-derived inflammatory reactions (delayed-type hypersensitivity reactions and granuloma-type pathological pictures) [69]. No doubt that this discovery opens a new and exciting field of research to better understand the tolerance/cytotoxicity balance and time-course in AE. The recruitment and presence of all potential actors of Th17-

driven immune reaction in the lesions highly suggests that the IL-23/IL-21/IL-22/IL-17 pathway is actually operating in echinococcosis; however, it has to be formally demonstrated and its modulation/inhibition by the regulatory cytokines should be carefully studied.

Attempts at enhancing Th1-related immune responses have resulted in increased resistance to *E. multilocularis* infection in experimental mice. Treatment with IFN- γ either before or after experimental infection has been shown to be only partially effective in reducing larval growth, although it was able to moderately increase the periparasitic fibrotic process [86]. Conversely, pretreatment of mice with IL-12 is extremely efficient in preventing the development of lesions and leads to abortive parasitic vesicles surrounded by fully efficient periparasitic immune cell infiltration and fibrosis [87]. IFN- α is also able to prevent larval growth in experimental mice [72]. Associated with this preventive effect of IFN- α , a significant modulation of cytokine secretion, with a significant decrease in IL-13 and increase in IFN- γ by peritoneal macrophages and spleen cells was observed in the treated mice [72]. Isolated attempts of treatment with IFN- γ in patients at a late stage of AE were no more successful than those performed in experimental mice and they could not modify host's cytokine profile significantly [88]. Conversely, IFN- α was shown to favour the regression of AE lesions while reversing the abnormal Th2-skewed cytokine profile in a patient with AE [89].

8. Effector and Regulatory Cells and Mechanisms in *E. multilocularis* Infection

Anti-inflammatory cytokines do interfere in T helper/regulatory/cytotoxic lymphocyte differentiation. In patients with AE, long after the initiation of the disease, the generation of memory Th1 CD4⁺ T-cells was shown to be impaired [90]. Furthermore, in such patients a biased CD4/CD8 T-cell ratio is observed: a marked increase of the CD4/CD8 ratio, mainly due to a decreased number of CD8 T-cells among peripheral T lymphocytes, and the predominance of CD8 T lymphocytes within the periparasitic granuloma characterize “susceptible” patients with a severe and progressing disease [91] (Figure 2(b)). In patients with progressing/severe AE, CD8-T-cells have been shown to produce Th2 cytokines as well as IL-10 and TGF- β [68, 71, 92]. In infected mice, the first two stages of cytokine production are also characterized by differential infiltration of the periparasitic liver with CD4, and then CD8 T lymphocytes, following different kinetics in resistant versus susceptible mice [9].

In addition, cytokines and other mechanisms may play a role in specifically inhibiting effector immune mechanisms, and particularly cytotoxicity mediated by cells of the innate immune response (NK cells, macrophages) and by cells of the adaptive immune response (cytotoxic T-cells). The crucial role of macrophages in the effector phase of the immune response towards *E. multilocularis* has been reported, and their stimulation results in enhanced parasite killing and protection of the host [25, 93]. On the other hand, an impairment of chemotactic and phagocytic properties of cells of

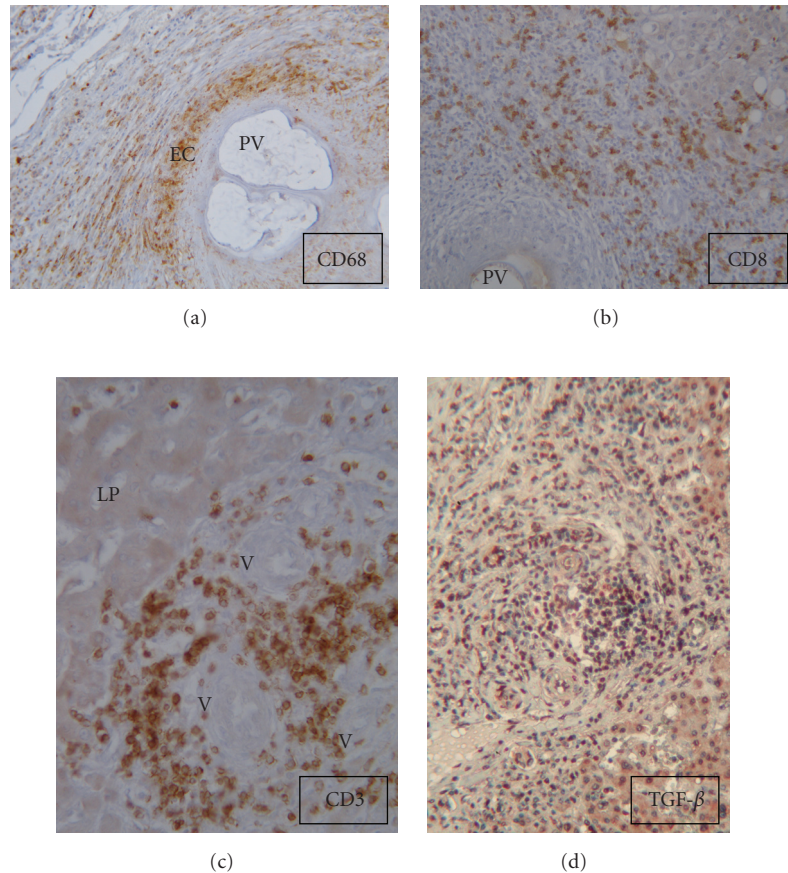


FIGURE 2: Cells of the periparasitic granuloma in *E. multilocularis* infection in humans; immunostaining of liver sections of patients with alveolar echinococcosis. (a) the “epithelioid cells” (EC) are CD68+ macrophages with an epithelium-like arrangement, located at close contact to the laminated layer of the parasitic vesicle (PV); (b) CD8+ T-cells represent the major cell population of the periparasitic infiltrate; (c) infiltration by CD3+ T-cells is especially prominent around the new vessels (V) developed at the periphery of the granuloma, at the border of the invaded liver parenchyma (LP); (d) most of the infiltrating T-cells express TGF- β . (Photo: B. Kantelip and DA Vuitton, WHO-Collaborating Centre; Université de Franche-Comté, Besançon, France).

the phagocytic system has been shown in *E. multilocularis* hosts [72, 94, 95]. This suggests that anti-inflammatory effects, mediated by cytokines, are actually operative at the systemic level. A crucial- and ambiguous- role of macrophages regarding NO secretion has been disclosed in murine *E. multilocularis* infection. In fact, NO may be both cytotoxic against parasites and immuno-modulatory by inhibiting cell activation. Both were found to be operating and it was especially demonstrated that the high periparasitic Nitric Oxide (NO) production by peritoneal exudate cells contributed to periparasitic immunosuppression [96, 97]; this explains why, paradoxically, iNOS deficient mice exhibit a significantly lower susceptibility towards experimental infection [98].

Contact inhibition of effector macrophages could be induced either by anti-inflammatory cytokine secretion or, more directly, by germinal layer-derived molecules through the laminated layer. Analysis of the cell surface markers of the epithelioid macrophages lining the parasitic vesicles in humans (Figure 2(a)) has shown a very unusual expression of these markers, particularly a high expression of CD 25,

the inducible chain of the IL-2 receptor, a chain usually only expressed by activated T-cells and especially CD4 T-regulatory cells [99]. Such an abnormal expression of CD 25 on macrophages has been shown in other granulomatous diseases and could be related to a particular functional state of the macrophages, either involved in tolerance induction or inhibited in its cytotoxic properties, or both.

NK- and/or T-cell-dependent cytotoxic mechanisms may be impaired by the cytokines secreted abundantly in the periparasitic immune cell infiltrate and/or through cell-cell interaction mechanisms. The quasi-absence of NK cells and the inhibition of the expression of the co-stimulatory receptor NKG2D at the surface of CD8-T-cells in the periparasitic granuloma has been shown in patients with AE. Despite the presence of its ligand, MICA/B, at the surface of hepatic cells, epithelioid cells and of the parasite germinal layer itself, cytotoxicity of CD8 T-cells might thus be severely impaired [68]. The lack of expression of NKG2D on CD8 T-cells was not related to the presence of the soluble form of MICA/B, since this soluble form could not be detected in these patients. A role for TGF- β is highly likely since it

was massively expressed by the lymphocytes surrounding the parasitic vesicles (Figure 2(d)).

Characterisation of regulatory cells is just at its beginning in *E. multilocularis* infection. An important role of CD4+ CD25+ T-regulatory cells has been suggested by a recent study in patients with AE [85]. Such a phenotype is quite characteristic of T-regulatory cells and they are likely responsible, at least partially, for the secretion of the anti-inflammatory/tolerogenic cytokines, such as IL-10 which is constantly elevated in AE patients. In this study, an increased number of CD4+ CD25+ T-cells was observed in the patients, compared to healthy controls. In the same study, after 48 h of co-culture, *E. multilocularis* metacystode culture supernatant and *E. multilocularis* vesicles depressed the release of the pro-inflammatory cytokine interleukin (IL)-12 by PBMC. This effect was dose-dependent and a suppression of tumour necrosis factor (TNF)- α and IL-12 was observed even when PBMC were activated with lipopolysaccharide (LPS). Comparing proinflammatory cytokine release by AE patients and controls showed that the release of IL-12 and TNF- α was reduced in AE patients, and was accompanied by a reduced release of the Th2-type chemokine CCL17 (*thymus and activation regulated chemokine*, TARC), suggesting an anti-inflammatory response to *E. multilocularis* metacystode in AE patients.

9. Relationship between the “Delayed Type Hypersensitivity” Responses and Local Pathological Events/Clinical Presentation, Signs and Symptoms in AE

Despite the tolerance that is exerted to protect the parasite against host's immune defences, effector mechanisms are nevertheless present. They are responsible for the attraction of various types of immune cells to the site of larval growth, and especially to the infected liver. “Delayed-type hypersensitivity reactions”, the immune effector mechanisms also known as “type IV” hypersensitivity, are characterised by immune cell recruitment, tissue infiltration, and neo-angiogenesis, followed by necrosis and fibrosis. Involvement of such reactions is particularly striking in AE, where chronically established larval development is characterized by a sustained infiltration of the host's immune cells which becomes organised as a “granuloma” and associated with necrosis and fibrosis [9, 91]. Granulomatous reactions are typical of delayed-type hypersensitivity reactions that are not fully efficient. Their association with histopathological characteristics such as “giant cells” (macrophages with multiple nuclei) and calcifications (which are the ultimate outcome of macrophage activation) is unique. Such granulomatous reactions contribute to the immunopathological events responsible for some of the complications of the underlying diseases, as observed in tuberculosis, leprosy, leishmaniasis or schistosomiasis.

Necrosis of the lesions is one of the hallmarks of AE in humans. In the liver, expression of IL-1, IL-6 and TNF- α mRNAs were observed in macrophages at the periphery of the granuloma in all studies patients, and close to the para-

sitic vesicles in patients with severe diseases and pathological pictures of lesion necrosis [99]. TNF- α could thus contribute to the necrotic process which gives the typical ultrasound and CT-scan pictures of AE, and leads to several complications in patients with AE. However, TNFs represent also a major factor to prevent metacystode growth, as is exemplified by the exacerbation of *E. multilocularis* metacystode growth in transgenic mice deficient for Lymphotoxin and TNF- α [100].

Fibrosis is also a hallmark of AE, leading to a complete disappearance of the liver parenchyma and to the death of the metacystode, with vesicles embedded in an acellular tissue composed nearly entirely of cross-linked collagens [101]. The diffusion of the fibrotic process even far from the parasitic lesions strongly suggests a major role for cytokines in collagen synthesis. They may also be involved in cross-linking the collagen bundles in humans [58, 101–104], as well as in the experimental models [10]. TGF- β , present in the cell infiltrate surrounding the parasitic lesions, in addition to its role in maintaining tolerance, is likely involved in the development of fibrosis in AE; however, this has never been studied until now. The parasite itself could also be involved in the collagen cross-linking process, since a transglutaminase of parasitic origin has been shown to be strongly expressed in and at the border of the parasitic vesicles and is able to efficiently cross-link collagens of human origin in vitro [58]. Development of fibrosis has been shown to be either quantitatively or qualitatively correlated to protection in experimental animals [10, 86], and is thus, usually, considered to be beneficial to the host. Fibrosis, in addition to the laminated layer, could be responsible for the protection of the parasite against any contact with both cytotoxic and antibody-secreting cells of the host and vice-versa. It may explain the low rate of anaphylactic symptoms in patients with AE [104, 105]: the extremely fibrotic lesions of AE cannot rupture, and the echinococcal fluid may well be never in contact with mast cell-bound IgE, despite their constant presence, which could be demonstrated in vitro [104]. In fact allergic symptoms rarely occur in patients with AE, only while parasitic cells are migrating to other organs than the liver and are eventually leading to metastases, especially through pulmonary embolism [105]. However, fibrosis is also the main cause for bile duct and vessel obstructions and thus, the pathophysiological background of chronic cholestasis, angiocholitis, portal hypertension, Budd Chiari syndrome and/or vena cava obstruction [105]. It may also be one of the reasons for the poor transport of antiparasitic drugs to the lesion.

One of the striking features observed in experimental murine AE (and also in naturally acquired AE of humans [104, 105]) is the absence of hypereosinophilia, a common feature of helminth-related diseases. The mobilization of eosinophils is known to be a crucial immunological event that plays an important role in the host defense against helminths. Eotaxin, a CC-proinflammatory chemokine, is one of several described chemoattractants for eosinophils. In addition, also IL-5 may mobilize these cells [106] but its role remains controversial. Eosinophils possess granules containing a variety of toxic molecules [major basic protein

(MBP), peroxidase, neurotoxin, histaminase and others] which are active against many multicellular parasites, in particular helminths [107]. In many examples of nematode infections, eosinophilia is a marked characteristic, and eosinophils directly cause profound damage to the tegument of the worms, in which a marked reduction of fertility and longevity is observed [108]. On the other hand, IL-5 and eosinophils have no detectable effects on the infection with selected cestodes or trematodes [109]. An extravasation of eosinophils causing eosinophilia in the peritoneal cavity has been demonstrated to be beneficial for the host by causing damage to the immigrant immature worm of *Fasciola hepatica*, resulting in the erosion of the tegumental syncytium [110]. Recently it could be shown that metacystode antigens (VF and E/S) exhibit proteolytic activity on eotaxin in vitro [111]. Inhibition of eotaxin activity may suppress the mobilization of eosinophils in *E. multilocularis*-infected hosts. Eotaxin is considered one of the main activators and chemo-attractants of resident eosinophils [112]. In experimental murine AE, the detected eotaxin inactivation by VF and E/S products may contribute to explain the absence of eosinophils within the peritoneal cavity of AE-secondary infected mice. Absent eosinophils thus may be a part of a series of events that maintain a low level of inflammation in *E. multilocularis*-infected hosts.

Finally, angiogenesis is one of the aspects of the periparasitic cell-mediated immune reactions which have been most neglected in the immuno-pathological studies of echinococcosis. Immunostaining of extracellular matrix proteins in the periparasitic areas of *E. multilocularis* infections have shown impressive pictures of neo-angiogenesis in the periparasitic granuloma [10, 101]. Angiogenesis is likely involved in the traffic of immune cells to and from the lesions; T-cells are especially numerous around the neo-vessels at the periphery of the periparasitic granuloma (Figure 2(c)). It may also explain some aspects of AE imaging, with a delayed reinforcement of the periphery of the lesions at CT-scan after injection [113] and an uptake of fluoro-deoxyglucose on PET-scan images [114]. Last but not least, as observed in malignant tumours, it may also be involved in the metastatic process observed in AE, a major component of the severity of this disease. Preliminary unpublished observations suggest that angiogenesis could be promoted by cytokine-like substances secreted by the metacystode itself.

10. Role of Host Immunogenetics in the Balance between Tolerance and Effector/Hypersensitivity Mechanisms

The role of some specific parasite-derived substances/antigens for the final orientation of the immune response and the relative importance of cell-mediated effector immune response *versus* tolerance in *Echinococcus* sp. infection is ambiguous. Until the beginning of the 1980s, every exposure to *E. multilocularis* oncospheres was believed to be followed by larval development and occurrence of the disease. A more careful evaluation of epidemiological data

in humans and animals in the same endemic areas first questioned this assumption [115, 116]. Then the implementation of mass screening in endemic areas revealed that the number of serologically “positive” cases were far higher than that of patent cases, disclosed by liver ultrasonography [23, 24, 117]. Positive serological results in individuals living in endemic areas (i.e., subjects with specific antibodies against *E. multilocularis* and who share the same risk factors) may account for at least 4 different situations: (1) “patent”, overt disease with symptoms, (2) “latent”, non-apparent disease, (3) calcified dead lesions in the liver and (4) no apparent lesions [2]. The clustering of subjects with specific antibodies in those areas where the number of “patent” cases is highest strongly suggests that these subjects actually had contacts with the parasite in the previous years. Some genetic factors have been found to associate with these various outcomes in humans. There is a significant association between HLA DR 11 and protection, HLA DPB1*0401 and susceptibility, and HLA DR3 and DQ2 and severe clinical evolution of the disease [118]. The HLA B8, DR3, DQ2 haplotype is observed with an unusual frequency in patients with autoimmune diseases characterised by an increased and inappropriate humoral immunity and a relatively inefficient cellular immune response. In fact, studies on cytokine secretion by PBMC from HLA DR3+, DQ2+ patients with AE, compared with patients without this HLA haplotype, have shown that the spontaneous secretion of IL-10 was much higher in HLA DR3+, DQ2+ patients [43]. A limited number of family cases have been observed in endemic areas that suggest an inherited pattern in populations which share the same risk factors [119]. Preliminary data indicate that relatives with progressive forms of *E. multilocularis* infection shared both HLA haplotypes and the associated clinical presentation of the disease [120]. Other genes within the MHC that are known to be related to the initiation and/or the effector phase of the immune response could also be involved. No significant correlation was observed between occurrence and/or severity of the disease and polymorphism in TNF promoter gene. However, 63% of 94 patients with AE from the same European endemic area as mentioned above had the homozygote Thr-Thr form of the TAP2 665 codon site, versus 45% of controls [121].

11. Conclusion

Results of observations in humans and experimental studies in animals suggest that, in the absence of fully effective anti-parasitic chemotherapy for AE, modulation of the host's immune response could be envisaged to fight against the parasite and to prevent the disease and/or its complications. As shown above, recombinant IFN- α 2a would be the best “immunological drug” candidate. Other approaches are based on the use of specific antigenic components of *Echinococcus* sp. as preventive or therapeutic vaccines [122–124]. The Eg95 vaccine has already reached field validation for its use in *E. granulosus* infection of sheep [125], and similar or novel antigens could be adapted to *E. multilocularis* infection of intermediate hosts. In murine AE, preliminary experimental reports suggest that several

antigenic compounds may provide good protection against primary infection, for example, Em14-3-3 [122], Em95 [123], EMY162 [126] and EmTetraspanin [124]. Dependent of the economical and ethical feasibility of such an approach, a preventive vaccine for *E. multilocularis* infection in humans may theoretically be envisaged, while a therapeutic approach still deserves further detailed investigation.

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