Review

Cell PRESS

Understanding the laminated layer of larval *Echinococcus* II: immunology

Alvaro Díaz[1], Cecilia Casaravilla[1], Judith E. Allen[2], Robert B. Sim[3,4] and Ana M. Ferreira[1]

[1] Cátedra de Inmunología. Departamento de Biociencias, Facultad de Química/IQB, Facultad de Ciencias. Universidad de la República, Montevideo CP 11600, Uruguay
[2] Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh, UK, EH9 3JT
[3] MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford. Oxford, UK, OX1 3QU
[4] Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, UK, OX1 3QT

The laminated layer (LL) is the massive carbohydrate-rich structure that protects *Echinococcus* larvae, which cause cystic echinococcosis (hydatid disease) and alveolar echinococcosis. Increased understanding of the biochemistry of the LL is allowing a more informed analysis of its immunology. The LL not only protects the parasite against host attack but also shapes the overall immune response against it. Because of its dense glycosylation, it probably contains few T-cell epitopes, being important instead in T-cell independent antibody responses. Crucially, it is decoded in non-inflammatory fashion by innate immunity, surely contributing to the strong immune-regulation observed in *Echinococcus* infections. Defining the active LL molecular motifs and corresponding host innate receptors is a feasible and promising goal in the field of helminth-derived immune-regulatory molecules.

Echinococcus metacestodes and the laminated layer

The larval stages (metacestodes) of the cestodes belonging to the genus *Echinococcus* cause larval echinococcoses (traditionally referred to as hydatid disease) in mammals, including humans [1]. They have a basic bladder-like morphology and establish in the parenchymas of internal organs, especially liver and lungs. They are protected by a layer of extracellular, carbohydrate-rich material, termed the laminated layer (LL), which is synthesized by the underlying, cellular, germinal layer (GL). The molecular structure of the LL is described in a separate review [2]. In brief, the LL is essentially a meshwork formed by highly *O*-glycosylated glycoproteins of the mucin type (Figure 1). The structure of the glycans decorating these mucins, largely based on galactose, is now partly known, although the sequences of the corresponding peptide backbones are not. The mucin meshwork apparently accounts for the LL in *Echinococcus multilocularis,* agent of alveolar echinococcosis, and probably the LL of a minor species, *Echinococcus vogeli*. By contrast, the LL of *Echinococcus granulosus*, agent of cystic echinococcosis (hydatid disease *sensu strictu*), additionally contains nanodeposits of calcium *myo*-inositol hexakisphosphate.

The LL is said to be the crucial element of the host–parasite interfaces in larval echinococcoses. Being the

Corresponding author: Díaz, A. (adiaz@fq.edu.uy).

Glossary

Complement system: this is a proteolytic cascade present in plasma and extracellular fluids that results in the generation of proinflammatory mediators and the opsonization and in some cases lysis of target cells. Complement can be activated through the classical, lectin and alternative pathways (see Figure (2a)).
Regulatory T cells (T_{reg}): are T cells, usually of the $CD4^+$ type, specialized in controlling the proliferation and actions of effector T cells. They include natural T_{reg}, generated in the thymus, and adaptive T_{reg} induced in the periphery with the help of the suppressive cytokine TGF-β; both these sets express the marker FoxP3. They also include $FoxP3^-$ peripheral regulatory cells such as Tr1 cells, induced by IL-10. Generally speaking T_{reg} display both antigen-specific and non-specific suppressive mechanisms. Part of their action on effector T cells is mediated by their conditioning of antigen-presenting cells in a non-inflammatory direction.
Tegument: is the outermost cellular layer of platyhelminths. It has a syncytial organization, that is, it comprises many nuclei that share a cytoplasm bounded by a single plasma membrane.
Th1: is the subset of effector $CD4^+$ T cells characterized by the production of interferon-γ as well other cytokines. They are specialized in controlling intracellular bacterial and viral pathogens. By extension, the terms Th1 responses or type 1 responses refers to the whole set of responses orchestrated by Th1 cells, usually encompassing classical macrophage activation and specific isotypes of IgG antibodies. Th1 responses, participate together with Th17 responses, in autoimmune pathology.
Th2: is the subset of effector T $CD4^+$ cells characterized by the production of IL-4, IL-5, IL-13 as well other cytokines. They are thought to be specialized in controlling helminth as well as arthropod ectoparasite infections. By extension, the terms Th2 responses or type 2 responses refers to the whole set of responses orchestrated by Th2 cells, usually encompassing alternative activation of macrophages, expansion/recruitment of eosinophils, specific isotypes of IgG antibodies, and IgE antibodies. Pathological Th2 responses cause allergy and fibrosis.
Th17: is a recently recognized subset of effector $CD4^+$ T cells, characterized by the production of the cytokine IL-17. Th17 cells are highly inflammatory, are thought to be specialized in the control of extracellular unicellular pathogens, and are prominent in autoimmune pathology. They are generated in the presence of TGF-β plus inflammatory cytokines.
T-independent type-2 antigens: are antigens, such as microbial polysaccharides, that feature multiple repetitive epitopes and are thus capable of extensively cross-linking B cell receptors. They are distinguished from T-independent type-1 antigens such as LPS by their limited ability to induce polyclonal B cell responses. The generation of antibodies against these antigens can occur in the absence of T cell help, but can be influenced by T-cell cytokines.

1471-4922/$ – see front matter doi:10.1016/j.pt.2011.01.008 Trends in Parasitology, June 2011, Vol. 27, No. 6

Tolerogenic: this term refers to those molecules and cells within the immune system that promote tolerance, that is, the absence of effector response against a particular set of antigens. It applies in particular to the promotion of active tolerance, based on the instruction and expansion of regulatory T cells. For example, dendritic cells that instruct regulatory T cells are called tolerogenic dendritic cells.

parasite structure exposed to the host, it engages in multiple interactions with the immune system, and shapes to a large extent the immunology of larval echinococcal infections. The LL is an insoluble and unusual biological structure. It is extraordinarily difficult to perform immunology research on a material that we do not understand, both in terms of theoretical reasoning and in practice. The present article attempts to extract information useful for immunological studies from the structural understanding of the LL gained over the past few years; this includes practical aspects, summarized in Boxes 1–3. The article also integrates the available immunological data on the LL.

Immunology of larval *Echinococcus* infections

The immunology of the larval echinococcoses has been reviewed [3,4]. As expected of a helminth infection, responses are biased towards the Th2 arm. However, the Th2 shift is not as marked as in other helminth infections, and a Th1-type component is detectable. As *Echinococcus* metacestodes often live as long as their (immunocompetent) hosts, they must express efficient immune-evasion mechanisms. Although this capacity is shared by all pathogens capable of chronic infection, it is particularly striking in *Echinococcus* larvae, probably the largest pathogens to dwell in mammalian internal organs.

Most of the literature ascribes immune evasion by larval *Echinococcus* to the induction of permissive Th2 responses, as opposed to parasite-damaging Th1 responses [3,5]. This is somewhat at odds with current thinking in most of helminth immunology, which proposes that evasion results from parasite stimulation of host regulatory responses [6,7]. In this view, regulatory responses blunt Th2 responses (specialized in controlling helminth infections), giving rise to 'modified type 2 responses,' which allow parasite survival. Certainly, interventions that boost Th1 responses (Th1/inflammatory cytokines, TLR agonists) result in enhanced control of the larval echinococcoses [4]. This should not necessarily be taken as evidence that *Echinococcus* larvae evade immunity by inducing Th2 responses. A variety of effector mechanisms (Th1, Th2 and others), when boosted away from the control exerted by regulatory responses, can probably damage the parasites. These parasites are also probably evolutionarily adapted to downregulating a Th2-dominated profile of response, and when this profile is artificially shifted [8,9], the regulatory mechanisms might not operate as efficiently. This view is compatible with Th1-associated effector mechanisms being more important against *Echinococcus*, and more generally against larval cestodes [10], than against most other helminths.

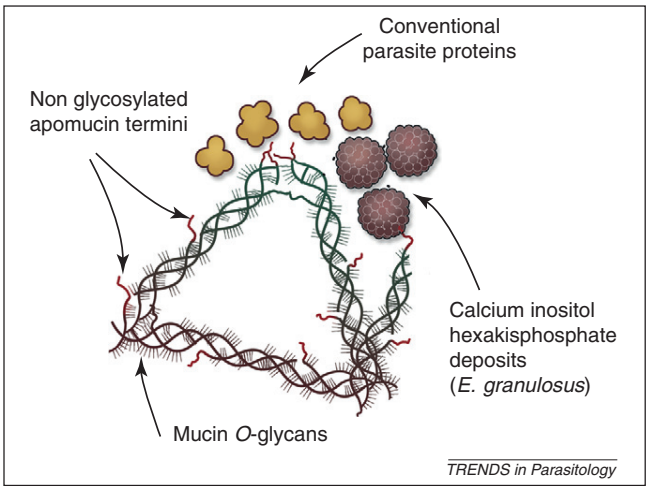

Figure 1. Diagrammatic summary of the molecular architecture of the LL. The LL is essentially a meshwork of mucins, that is, highly *O*-glycosylated glycoproteins. In addition to the glycans, mucins can expose non-glycosylated peptides at the termini of their protein backbones. The *E. granulosus* LL, but apparently not the *E. multilocularis* or *E. vogeli* LL, additionally contains abundant deposits of calcium inositol hexakisphosphate. Non-LL-intrinsic secreted parasite proteins (conventional parasite proteins) can adsorb on the LL, as do abundant host-derived proteins, which are not depicted. The elements shown are not to scale: calcium InsP_6 deposits are 40 nm in diameter, and mucin-based fibrils are approximately 10 nm thick (whereas 'typical' globular proteins measure around 5 nm in diameter).

Box 1. Processing the LL for immunological research: host components and GL

The *E. granulosus* LL generally is not attached to the surrounding host tissue. In cysts from natural infections, release of the internal pressure by puncture causes the hydatid wall (HW; LL plus GL) to detach from host tissue. In the situations in which local inflammation persists, a significant minority of the epithelioid macrophages remain attached to the LL. In mouse intraperitoneal infections, larvae are surrounded by a layer of inflammatory cell remnants and collagen. This layer (slightly more yellow in colour than the HW) can sometimes be removed by rubbing the intact cysts [12] (or more easily, the HW after cyst puncture) against filter paper.

The LL contains abundant (mostly host-derived) adsorbed proteins [33,46,49,70,71]. Remains of inflammatory cells attached to the LL (mentioned above) can also contribute host proteins to HW starting materials. Washing with 2 M NaCl extracts most of the LL-associated non-structural proteins, although much of the immunoglobulin remains. The newly developed techniques for *in vitro*, including axenic, cultivation of *E. multilocularis* [77], will be of much help for obtaining host-free materials.

Separating the LL from the GL mechanically is not straightforward. For *E. granulosus* cysts from mouse i.p. infections, overnight incubation in 2 M NaCl at 4 °C results in collapse of the GL, presumably as a consequence of parasite death. The hydatid cyst fluid and GL remains can then be aspired cleanly with a syringe, leaving behind the 'cyst ghost.' GL detachment can also be obtained quickly by incubating cysts in 0.1% Triton X-100 [47]. In all cyst types, (most of) the GL can be dissolved away from the LL using detergents, reagents for RNA extraction (containing phenol and guanidine isothiocyanate), or 6 M urea. Urea causes some alteration in the LL ultrastructure, and extracts some presumably intrinsic molecules away from the *E. multilocularis* LL [37,78].

The cytosolic proteins P-29 and cyclophillin P have been used as markers for the GL content of extracts as opposed to the LL [79]. LL markers completely absent from the GL might not exist, but galactose to mannose ratios in total hydrolysates (very high in LL, lower in GL [2]) would give indication of relative LL *versus* GL contents. As in large (several cm in diameter) *E. granulosus* cysts, the LL is much thicker than the GL, crude HW samples of this type are quantitatively almost entirely LL.

Box 2. Processing the LL for immunological research: *E. granulosus* InsP_6 deposits

Keeping hydatid wall samples in buffers without calcium causes some degree of solubilization of InsP_6. This is more marked with decreasing pH, and can be prevented by including $CaCl_2$ in the buffer [80]. InsP_6 can be extracted away from the LL, even from intact larvae, by using calcium-chelating agents [79]. The amounts of calcium associated with InsP_6 are surprisingly high, up to 1.65 mmol of Ca^{2+} per g LL dry mass [80]. Therefore in excess of 2 mmol of EDTA per g LL dry mass must be used (buffer concentration has to be in excess of twice the EDTA concentration to avoid acidification). InsP_6 can also be eliminated by enzymatic hydrolysis with phytase in carefully chosen buffer (Ca^{2+} concentration and pH) conditions [80]. In all cases, InsP_6 removal is accompanied by a readily observed increase in the translucence of the LL. The native InsP_6 deposits can be purified by prolonged alkaline treatment, which dissolves away the mucins [81]. Mixing solutions of commercial sodium InsP_6 with a 5-fold or higher molar excess of Ca^{2+} (as $CaCl_2$) at alkaline pH yields a precipitate with the same stoichiometry as the *E. granulosus* deposits. Soluble InsP_6 is neither compatible with physiological conditions (it essentially requires the absence of free calcium), nor in the least representative of the solid deposits found in the *E. granulosus* LL.

Much evidence exists for immune-suppressive mechanisms in hydatid infections [4,11,12]. Innate immune cells conditioned by infection or by secreted metacestode products *in vitro* adopt non-inflammatory and/or suppressive phenotypes [13–15]. The regulatory cytokines IL-10 and TGF-β are abundantly expressed by leukocytes in infected hosts, especially in the immediate vicinity of the parasite [12,16–19]. T cell responses become progressively depressed during experimental *E. multilocularis* infection [20]. In human *E. multilocularis* infections, $CD4^+$ T cell responses are weak [21,22]. In *E. granulosus*, direct evidence for dominant suppression exists, from adoptive transfer experiments in which lymph node cells from infected mice suppress antibody responses to unrelated antigens [23]. The central component of regulatory responses, regulatory T cells (T_{reg}), has been recently found to be strongly expanded in mouse *E. multilocularis* infection [15]. These T_{reg} include canonical $CD4^+$ $FoxP3^+$ cells (see 'regulatory T cells' in the Glossary) but also $CD8^+$ $FoxP3^+$ cells. The surprisingly strong expansion (for an extracellular pathogen) of $CD8^+$ T cells was a previously known feature of human alveolar echinococcosis [22,24]. Several lines of evidence suggest that $CD8^+$ T cells are important in suppression in larval echinococcal infections: their expansion correlates with weaker effector responses, they accumulate selectively around the parasite, and can suppress T cell proliferation [19,20,25–28]. The regulatory bias might also be reflected in humoral responses, as suggested by the prominence of the (human) isotype IgG4 [29], associated with conditioning of B cells by T_{reg} [30].

Immune regulation is also evident at the histopathological level in *E. granulosus*. Whereas the infecting stages elicit local inflammation, striking inflammatory resolution accompanies metacestode establishment, resulting in a poorly- or non-infiltrated collagen capsule, called the adventitious layer [1]. Resolution does not always take place, and local inflammatory status can vary among different cysts in an individual, or even regions of the same cyst. Resolution requires a correct parasite–host match, which is absent in infection of cattle by the G1 (sheep) strain of *E. granulosus*, in which inflammation persists [27]. *E. vogeli* infection in natural intermediate hosts also results in inflammatory resolution [31]. By contrast, *E. multilocularis* infection, whether in rodents or humans, is accompanied by chronic inflammation [32]. Whether in non-resolutive *E. granulosus* or *E. multilocularis* infection, inflammation is typically granulomatous. Usually, a rim

Box 3. Processing the LL for immunological research: mucins and their glycans

The LL mucin meshwork is insoluble. It can be solubilized by treatments that destroy the integrity of the mucins, such as β-elimination, which releases the glycans and fragments the peptide backbones extensively [50]. Solubilization can also be achieved by boiling in SDS–PAGE sample buffer containing 6 M urea; however, usefulness of this material is limited by the difficulty of removing SDS. In our experience, the most representative soluble preparations of *E. granulosus* LL mucins are obtained by reducing disulphides (dithiotreitol), alkylation of thiols (iodoacetamide), elimination of excess reagents from the insoluble residue by washing/centrifugation, and finally sonication [64]. Disulphide reduction loosens the mucin meshwork, allowing the use of only mild sonication (desirable, as sonication entails uncontrolled breakage of covalent bonds). Adsorbed proteins and calcium InsP_6 can be eliminated before the procedure as in Boxes 1 and 2, respectively.

Particulate preparations are prepared by grinding the previously dehydrated HW or LL using mortar and pestle and/or between polished glass plates. The particles must be re-hydrated carefully (avoiding formation of clumps). Thereafter particles can be handled as a suspension, including filtering through fine mesh. InsP_6 can be removed before or after the dehydration/pulverization/rehydration cycle.

The *E. granulosus* or *E. vogeli* LL structural mucins cannot be visualized by conventional SDS–PAGE [64,82], but agarose electrophoresis allows visualization of the *E. granulosus* mucins, which appear highly aggregated and heterogeneous [64]. For *E. multilocularis* the mucins are visualized as 'smears' at the top of conventional SDS–PAGE gels [78].

Monoclonal antibodies and lectins are useful reagents by virtue of their specificity. However, except for free glycans, LL-derived fractions affinity-purified with such reagents contain additional glycans (not targeted by the antibody or lectin used) that are bound to the same mucin backbone. For example, materials purified from *E. multilocularis* by the Em2(G11) antibody (fine specificity unknown) contain diverse glycans, including targets of the lectin PNA (non-decorated core 1), and of the E492 antibody, which probably recognizes Galα1-4 Gal termini [20,37,68,83]. Similarly, material purified using E492 also contains non-decorated core 1 [37]. Moreover, because the same terminal carbohydrate decoration can be present in unrelated molecules (other mucins, *N*-glycoproteins, glycolipids), material purified from different parasite tissues/stages by a particular antibody cannot be taken to constitute a single 'antigen.'

The free mucin glycans can be obtained by reductive β-elimination, followed by desalting, for example by addition of methanol and rotavaporation, use of cation exchange resin and finally gel filtration [50,83]. Some LL glycans have been synthesized in the laboratory [84]. It is unlikely that free, monovalent, glycans are immunologically active except as competitive inhibitors of their multivalent versions. Biotin-tagged glycans, which could be immobilized on streptavidin-coated surfaces, have recently been synthesized [85].

The LL is refractory to immunohistochemical staining. Oxidation of carbohydrates by periodate, or deglycosylation by trifluoromethanesulfonic acid, could allow immunostaining of LL-associated proteins that are otherwise not revealed [86].

of palisading epithelioid and giant multinucleated macrophages is in direct contact with the LL, a mononuclear cell infiltrate being present more distally intermixed with layers of collagen and fibroblasts [25,27,32,33]. Eosinophils usually are present but not dominant. The granulomatous reaction is $CD4^+$ cell-dependent [34], and as pointed out previously [4] it would not be surprising if it has a Th17 component. The species differences in inflammatory resolution must be related to the fact that although *E. granulosus* grows only by concentric enlargement (and *E. vogeli* has limited capacity for outward budding), *E. multilocularis* aggressively invades host tissues through outward budding. Not only will the invasive species generate more danger signals through tissue disruption, but it can also more readily afford the cost of host inflammation. Indeed, necrosis of part of the *E. multilocularis* larval mass, which is usually seen, is compatible with completion of the life-cycle of this parasite, as new parasite tissue is permanently generated.

Overall, larval *Echinococcus* infections probably induce strong regulatory responses, which control effector (Th2, Th1, and possibly Th17) responses. Although the degree of inflammatory control attained differs among species, the immune-regulatory mechanisms could be qualitatively similar, but be weaker and/or offset by stronger pro-inflammatory signals in invasive alveolar echinococcosis.

The laminated layer and host immunity: general remarks

The main evolutionary pressures giving rise to the LL might have been the needs to: (i) provide mechanical support for metacestode turgidity, probably instrumental to parasite growth, and (ii) protect GL cells from host immunity. These functions must be fulfilled while allowing the passage of nutrients and waste products, and permitting parasite growth. The evolutionary answer to these requirements has been a physically coherent, elastic, hydrophilic meshwork, which allows the diffusion of macromolecules up to at least 150 kDa [35], but shields the GL from host leukocytes. As attested by death of established metacestodes when inflammatory resolution fails, the LL does not make the parasite completely insensitive to host inflammation. Rather, it confers a partial protection against host effectors, but additionally, and crucially, it appears to downregulate inflammation, as discussed below.

The massive LL must be the major source of *Echinococcus* molecules that the immune system of the infected host encounters. This encompasses the adhesion of leukocytes to the external LL surface and the interaction between soluble host recognition molecules and the large solvent-exposed area represented by the entire thickness of the LL. In addition, parasite growth requires shedding of material from the LL outer strata [2]. Molecules recognized by LL-reactive monoclonal antibodies are released from *E. multilocularis* vesicles *in vitro* [36,37]. In experimental infections, host macrophages adhere to the LL outer surface and phagocytose LL-derived particles [32,38].

The large exposure of the host immune system to the LL, and the overall regulatory response profile in larval echinococcoses, together suggest that LL constituents induce regulation. This widely held view [3,4,12] is supported by observations that inflammatory resolution in *E. granulosus* and *E. vogeli* infections coincides in time with LL deployment [39,40], which takes place two to six weeks post-infection depending on the model [2]. Also in broad agreement, generation of suppressor cells and IL-10 responses do not require host exposure to the pre-cystic stages, because they occur after experimental metacestode transplant that bypasses exposure to the early stages [12,23].

Current immunological reasoning dictates that if the LL does induce regulation, it must be interpreted by the innate immune system as a non-dangerous material, thus triggering pathways that induce adaptive regulatory responses [6]. Indeed, immune evasion by chronic pathogens is now believed to pivot on expansion and/or local recruitment of natural and/or adaptive T_{reg} [41], as well as induction of IL-10 expression by effector T cells [42]. It is thought that the presentation, by conditioned myeloid cells to naïve T cells, of pathogen antigens together with tolerogenic signals allows pathogen antigenic specificities to become associated with regulatory responses (as normally happens for the specificities of the host). Because these responses involve certain non-antigen-specific mechanisms, some degree of non-specific suppression also results. Importantly, because innate immune cells encounter pathogen molecules mostly as large assemblies, tolerogenic (or other polarization) signals present in one molecule will spill over to other molecules in the same pathogen. We therefore reason that any molecular information for tolerogenic (and any other) response profiles present in the LL constituents must impact on the response against most other *Echinococcus* antigens. Thus innate recognition of the LL is a central aspect of hydatid immunology. Although this might seem paradoxical, because the LL is not present during early infection when innate immunity is sometimes thought to be most important, adaptive responses are determined by signals from innate immunity all along chronic processes [43].

Innate recognition of the laminated layer: complement

Studies on complement (Figure 2(a)) constituted the first evidence that the LL is deciphered in an essentially non-inflammatory way by innate immunity [44]. Indeed, the *E. granulosus* LL activates complement only poorly, by contrast to hydatid cyst fluid and protoscoleces [45]. This is largely the consequence of the activity of the host complement inhibitor factor H, which is present in host extracellular fluids, and becomes concentrated on the LL [46]. The LL component that recruits factor H is distinct from the $InsP_6$ deposits, and is thus deduced to correspond to the mucins [47]. The classical factor H ligands on complement-non-activating surfaces are anionic carbohydrates, including sialylated oligosaccharides and heparin-like glycosaminoglycans [48]. Host proteins other than factor H that associate with the LL are also classed as heparin-binding [33,49]. By contrast, all the known LL carbohydrates are neutral [50]. Certain mammalian extracellular matrix proteins bind factor H and other heparin-binding proteins independently of carbohydrates [51,52]. It is therefore possible that the sites with affinity for factor H in the

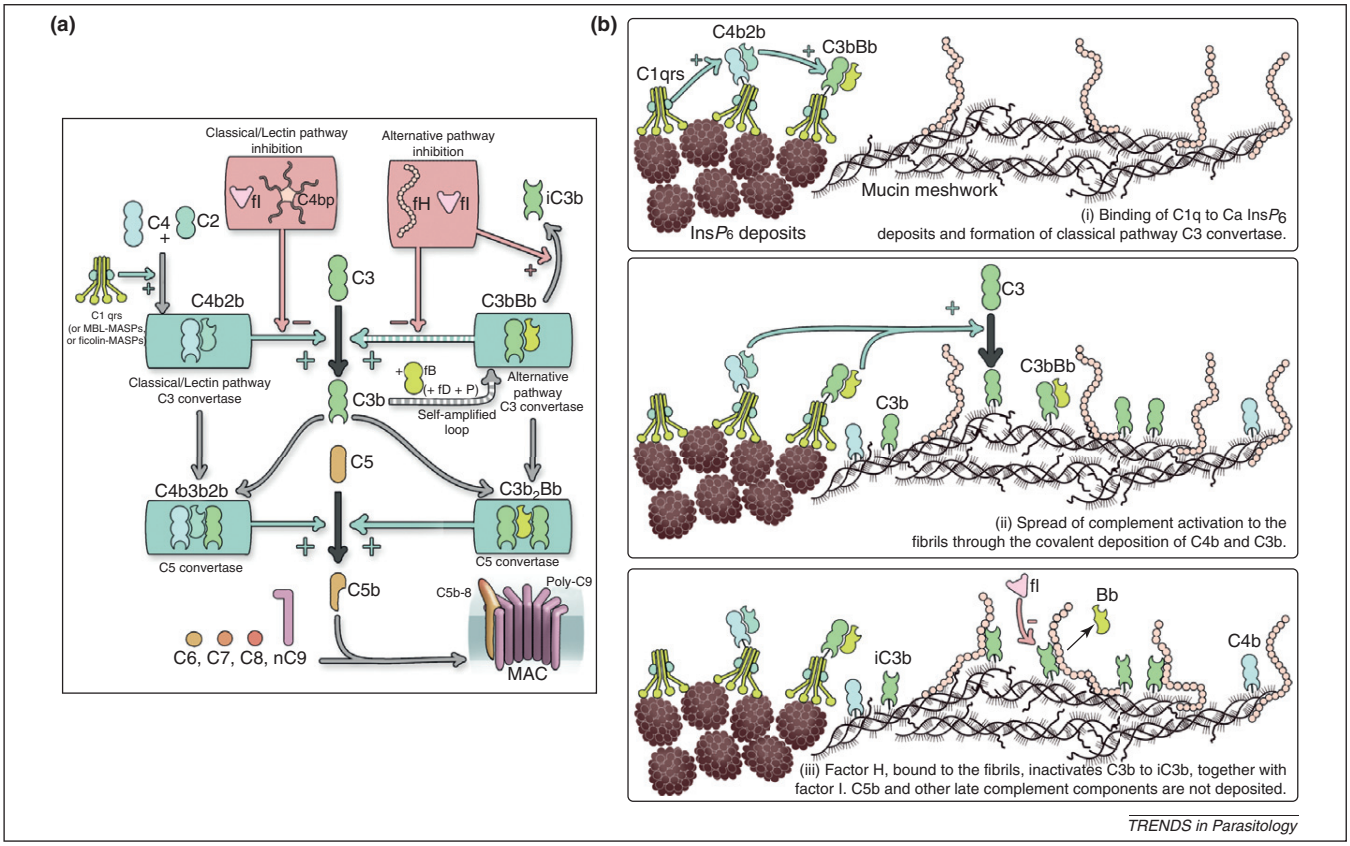

Figure 2. The *E. granulosus* LL and host complement. The complement cascade **(a)** can be activated by the classical, lectin, and alternative pathways. The classical pathway starts with the binding of the C1q subcomponent of the C1 (or C1qrs) complex to antigen-bound antibodies and also to certain molecular arrays exposed on cells or particles of pathogen or self origin. The lectin pathway is initiated by the recognition of specific carbohydrates by mannan-binding lectin (MBL) and consequent activation of MBL-associated serine proteinases (MASPs). The C1qrs and MBL-MASP complexes are analogous. The alternative pathway is initiated essentially by a default mechanism, explained below. All three pathways give rise to activated, transient enzyme complexes termed C3 convertases, which cleave and activate the central complement component, C3. Activated C3 binds covalently and essentially non-specifically to nearby biological surfaces. Because activated C3 (C3b) is the precursor of the alternative pathway C3 convertase, this pathway amplifies activation initiated through the other two pathways. This is also the basis of the default mechanism of activation: the alternative pathway self-amplifies on those surfaces that lack specific inhibitors. Self cells and extracellular matrix are protected by a range of inhibitors including factor H (fH), a soluble protein that is recruited to surfaces bearing sialic acid and/or other anionic sugars. fH dissociates the Bb component from C3b, and further allows the specific proteinase factor I (fI) to inactivate C3b to iC3b, then further degraded to C3d/C3dg (not shown). An analogous control mechanism, also involving fI, exists for the classical/lectin pathways. C3 convertases are precursors of so-called C5 convertases, which activate C5. Deposition of the terminal complement component C5 through to C9 leads to the formation of the MAC, which can form microscopic holes in cell membranes. The proposed forms of interaction between complement and the LL are given in **(b)** C1q binds to InsP_6 deposits and initiates classical pathway activation **(i)**. Activation spreads to the mucins **(ii)**, which contrary to calcium InsP_6, have acceptor sites for the covalent deposition of C3b and C4b (the activated form of the C4 component of the classical/lectin pathways). Effective alternative pathway-mediated amplification of the initial activation is curtailed by the action of fH, for which binding sites exist on the mucins **(iii)**. Initiation of complement activation by the InsP_6 deposits has been inferred but not proven to occur through the classical pathway. The association of factor H with nonglycosylated apomucin stretches is speculative. Abbreviations: fD, factor D; P, properdin; MAC, membrane-attack complex.

LL are located in non-glycosylated stretches of the apomucins [2].

Curiously, the InsP_6 deposits cause initiation of complement deposition, which then extends to the mucins [47]. This initiation probably occurs through adsorption of the C1q component of C1 [47] (Figure 2b). Compatible with this observation, no inhibitors for the enzymatic activity of the C1 s component of C1 are present in the LL [53]. Additional classical pathway initiation could be brought about by LL-bound antibodies. As for the complement lectin pathway, mannan-binding lectin (MBL) does not associate with the LL *in vivo* [53] (the ficolins have not been analyzed). Crucially, any complement activation initiated on the LL via the classical pathway is efficiently controlled further down the complement cascade. A major factor in this control is the inhibition, via factor H recruitment, of the amplification loop represented by the alternative pathway [47]. Initiation of classical pathway activation through direct C1q binding, coupled with restriction further down the cascade via binding of factor H (and/or the related classical pathway inhibitor C4 bp), constitutes the hallmark of the interaction with complement of mammalian extracellular matrix proteins and apoptotic cell surfaces [51]. These 'self' molecules display considerable deposition of C4 and C3, but poor activation of terminal components. By contrast, standard microbial surfaces trigger the whole cascade, with strong release of pro-inflammatory C5a and terminal complement complex formation. Similar to this dichotomy, the LL is slightly poorer than other *E. granulosus* materials at activating C3, but much poorer at activating the late components [45]. As summarized in Figure 2b, the *E. granulosus* LL is adapted to avoiding full activation of complement, while allowing low-level deposition of complement early components. Interestingly, this mode of activation causes the LL to become covalently opsonized with the C3 inactivation fragment iC3b, shown

in other systems to condition dendritic cells towards tolerogenic phenotypes [54].

Innate recognition of the laminated layer: cell-surface receptors

The decoding of metazoan pathogens including the platyhelminths is carried out centrally by cell-surface receptors, expressed in dendritic cells, macrophages and other innate cells. Recognition of pathogen surface carbohydrates by lectin receptors is central in this context. The recognition of materials derived from *Schistosoma* is thought to involve the interaction between carbohydrate motifs termed Lewis antigens, abundant in the surface of this parasite and secreted glycoproteins, and the receptor Dendritic Cell-Specific ICAM-3-Grabbing Nonintegrin (DC-SIGN) [55]. Lewis antigens, or other fucose-contaning motifs present in *Schistosoma* that elicit responses from innate immune cells, are altogether absent from the *Echinococcus* LL [50]. In the more closely related (taenid cestode) *Taenia crassiceps* system, metacestode surface carbohydrates are central in the shaping of host responses, through their interaction with as yet unknown receptors in dendritic cells [56]. However, profound differences exist between the major carbohydrates exposed by *Taenia* and *Echinococcus* metacestodes (Box 4). Hence data from other platyhelminth systems should not be extrapolated to the idea that the LL carbohydrates are necessarily active with respect to host dendritic cells. In fact, the LL carbohydrate structures [50] do not suggest candidate innate cell-surface receptors, with the exception of the liver-specific Kupffer cell receptor (KCR) and hepatocyte asialoglycoprotein receptor (ASGR). These receptors recognize galactose-terminated glycans including the non-decorated core 1 [57], which are very abundant in the LL. The liver, which is globally the prime infection site for *Echinococcus* larvae, is being increasingly recognized as an immune organ specialized in inducing T_{reg} responses [58]. The liver galactose-specific receptors in particular have been implicated in tolerance induction to allogeneic de-sialylated cells [59]. Therefore the LL carbohydrates might constitute an adaptation to engage liver-specific receptors for the tolerogenic presentation of parasite antigens. Evidently, *Echinococcus* larvae develop correctly in many other anatomical locations. However, the liver, through the ASGR and the KCR in particular, is responsible for clearing terminal galactose-bearing (non-sialylated or de-sialylated) glycoproteins and cells from systemic circulation [60], and tolerance induced in the liver can impact decisively on distant organs [58]. It is thus conceivable that even when metacestodes are lodged in non-liver sites, LL-derived mucin aggregates target *Echinococcus* antigens to the liver, inducing tolerance that influences the infection site. On the KCR specifically, the complication exists that this rodent receptor is not conserved throughout mammals. Rodents are natural hosts for *E. multilocularis* but not for *E. granulosus*, which, however, develops surprisingly well in mice.

Another intriguing finding from the structural data is that a particular carbohydrate motif (terminal N-acetyllactosamine) is systemically avoided in *E. granulosus*, but present in an *E. multilocularis* LL fraction [2]. This motif is known to induce granuloma formation in a model system [55]. Thus interspecies comparisons with regards to exposed *versus* cryptic LL carbohydrate determinants and their immunological correlates would be worthwhile.

Functional assays suggest that the LL probably contains no agonists for Toll-like receptors. An *E. multilocularis* metacestode extract (containing LL plus GL components) did not cause dendritic cell maturation [61]. *E. multilocularis* or *E. granulosus* LL extracts do not elicit nitric oxide from macrophages [62]. Rather, LL materials inhibit the nitric oxide response of macrophages stimulated with LPS or interferon-γ, those from *E. granulosus* more strongly than those from *E. multilocularis* [62,63].

In sum, the liver-specific lectin receptors must be taken into account when considering the decoding of the LL carbohydrates by the host. LL extracts are immunologically active (in a regulatory direction) towards non-liver innate immune cells. In addition to mucin glycans, non-glycosylated apomucin stretches and as mentioned above opsonizing complement components, should be taken into account as candidate active components of such extracts.

The laminated layer and adaptive immunity: T cell responses

Because of their very high degree of glycosylation and (consequent) resistance to proteolysis [64], the LL structural mucins probably yield few T-cell epitopes. In possible agreement, a purified *E. multilocularis* LL mucin does not induce proliferation of T-cells from infected mice [65]. As indicated above, we envisage LL components as impacting on T-cell responses mostly through polarizing and/or suppressive signals from innate immunity. In addition,

Box 4. Surface carbohydrates of *Taenia* and *Echinococcus* metacestodes: a tricky comparison

Taenia and *Echinococcus* metacestodes are both essentially bladder-like and bounded by a syncytial tegument, the surface of which is rich in carbohydrates. In the *Taenia crassiceps* model system, the metacestode surface glycoproteins (retrieved as *in vitro* excretion-secretion extracts) condition dendritic cells so as to polarize T cell responses towards the Th2 arm, and can also induce myeloid suppressor cells [56,87]. Both types of activities are clearly dependent on the carbohydrate part of these glycoproteins. However, *Taenia* metacestodes lack a structure homologous to the LL. Whereas the LL is made up from *O*-glycoproteins [2], lectin-binding studies in *T. crassiceps*, *Taenia solium* and *Taenia taeniaeformis* suggest strongly that the *Taenia* metacestode surface and secretions are dominated by *N*-glycoproteins [56,88–90]. Host receptors usually recognize pathogen carbohydrates principally through the motifs (encompassing two or three monosaccharide units) present at their non-reducing termini, that is, farthest away from the protein backbone in the case of glycoproteins. No such terminal motifs in common seem to exist between the *T. crassiceps* *N*-glycans [91] and the *E. granulosus* LL *O*-glycans [50]. The *T. crassiceps* *N*-glycans are structurally similar to known *E. granulosus* *N*-glycans [92], but with important differences in their non-reducing terminal motifs. *T. crassiceps* features an unusual Fucα1-3GlcNAc as dominant terminal motif, and this is absent from *E. granulosus*. In turn, some *E. granulosus* *N*-glycans feature the P_1-like motif Galα1-4Galβ1-4GlcNAc (in common with LL *O*-glycans), apparently absent from *T. crassiceps*. By contrast, certain *T. crassiceps* and *E. granulosus* *N*-glycans share the presence of N-acetyl lactosamine (Galβ1-4GlcNAc) at their termini. A role for *N*-glycans in the decoding of larval *Echinococcus* by innate immunity should not be discounted; for example, the major immunogenic protein (and hence diagnostic antigen) called antigen 5 is an *N*-glycoprotein [93].

LL components might influence the processing of conventional protein antigens internalized together with them. In addition to protease-resistance of the mucins, calcium InsP_6 found in the *E. granulosus* LL is a strong proton sink that might antagonize phagosome acidification. In agreement with the general picture of immune-regulation, a fraction from the *E. multilocularis* LL causes strong inhibition of antigen-specific and polyclonal splenocyte proliferation [37].

The laminated layer and adaptive immunity: antibody responses

Humoral responses in *Echinococcus* infections prominently feature low avidity, anti- carbohydrate antibodies [66]. A proportion of these, as well as antibodies elicited in T cell-deficient mice, react with LL fractions [20,37,65,67]. It remains to be proven that the LL is sufficient as a stimulus to induce these responses, as priming in early infection could be important, and protoscoleces (often used for experimental infection) induce anti-carbohydrate T-independent antibodies that cross-react with the LL [37,68]. Conversely, persistence of LL remains from aborted infections appears to be necessary and sufficient for maintenance of antibodies against LL mucins [32]. T-independent type-2 responses require second signals in addition to surface immunoglobulin cross-linking, one of which might be given through the CR2 receptor for C3 inactivation fragments (iC3b, C3dg) [69]. The self-limiting complement activation described above (Figure 2b) could therefore contribute to T-independent antibody generation.

The specificity of the antibodies against LL carbohydrates is unknown, although terminal α-Gal residues constitute an important target [67] (which probably explains cross-reactivity with protoscoleces [68]). The LL has strongly bound immunoglobulins, particularly IgG [70,71]. Although part of these antibodies are surely infection-elicited, natural antibodies (for example against non-decorated core 1), and antibodies bound by their F_c portions [72], cannot be ruled out.

The laminated layer and immune effectors

Echinococcus immunology has been conceptually divided into an 'establishment' phase during which the parasite is most susceptible to host effectors, and an 'established metacestode' phase [73]. Thus, although oncospheres are killed in immunized hosts, probably by complement-fixing antibodies, established metacestodes are spared (concomitant immunity). The difference between the two phases is thought to be made by the LL. In agreement, single vesicles of *E. multilocularis* metacestode can establish successful infection, as long as their LL is intact [74].

Parasite protection by the LL must largely derive from its barrier function for leukocytes. The LL is probably not easily degraded by host enzymes, as suggested by its persistence after parasite death [32]. An intact LL, even in its mm-thick *E. granulosus* version, does not confer complete protection to the parasite. *In vitro*, elicited nitric oxide can damage the larvae [63]. Further, the granulomatous response is certainly detrimental for both *E. multilocularis* and *E. granulosus* [27,34]. The host mediators

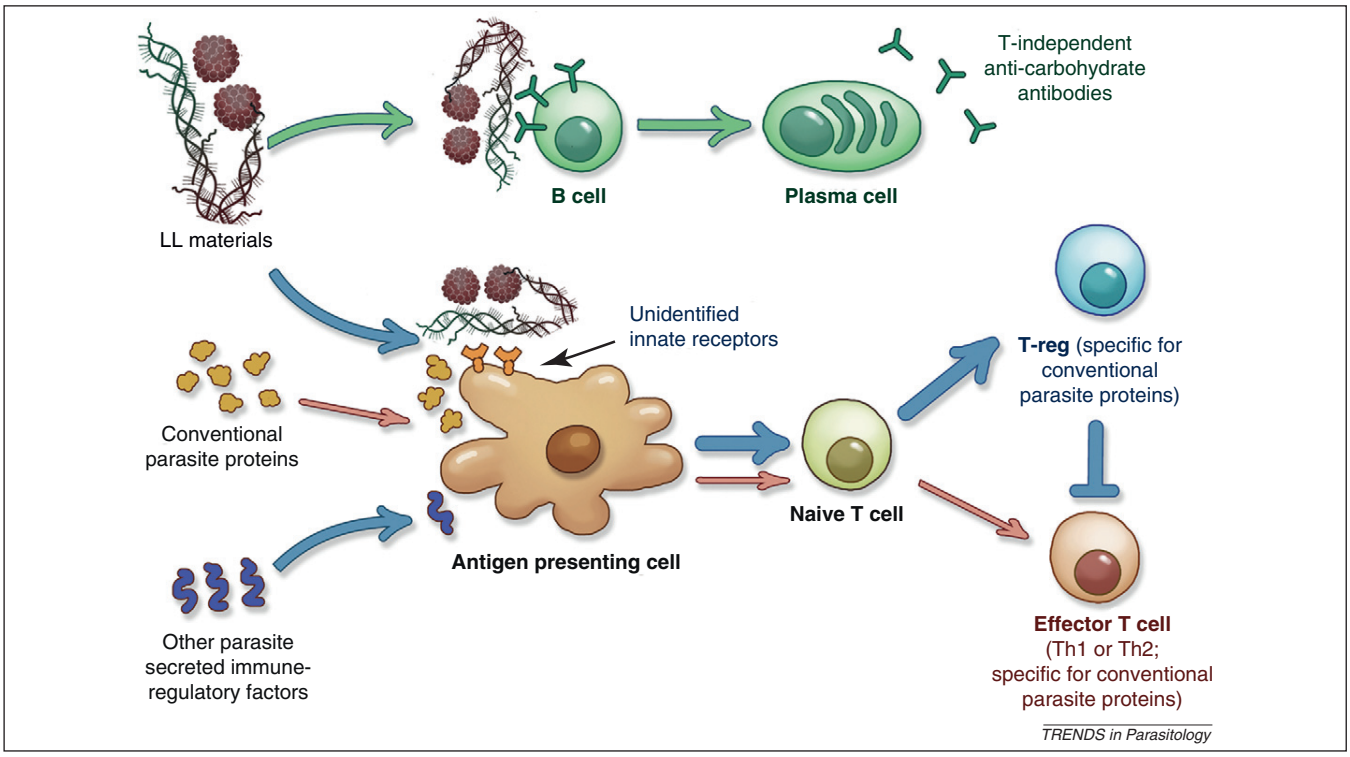

Figure 3. The LL and immune cell responses: a possible scenario. The interactions depicted are inferred, largely on the basis of: **(i)** non-inflammatory decoding of LL materials by myeloid cells; **(ii)** the scarcity of T-cell epitopes in the LL mucins predicted by the structural information available; **(iii)** evidence of LL-reactive T-independent antibodies. The existence of non-LL parasite secreted immune-regulatory factors is hypothesized from the fact that reactivation of inflammation usually accompanies parasite (*E. granulosus*) death. The category 'conventional parasite proteins' includes well-known immunodiagnostic antigens such as antigen B and antigen 5, which are the targets of antibody and T-cell proliferation responses to the infection [94]. These proteins are deduced to provide T-cell dependent epitopes for effector responses and also for adaptive T_{reg} that control those effector responses.

involved are unknown, but tissue-resorbing digestive proteases are secreted in large amounts by the epithelioid macrophages [33].

Eosinophils, a cell type probably specialized in combating helminth parasites, appear to have important effector capabilities against *Echinococcus* metacestodes. In mouse secondary *E. granulosus* infection, eosinophil recruitment can restrict metacestode growth [75]. In human hydatid infection, eosinophil-derived protein effectors are found within the LL [76]. Eosinophils might be able to erode the LL, as observed in certain tissue samples of bovine hydatid disease [27]. However, recruitment of this cell type is under most circumstances kept to a minimum, probably through the control of Th2 responses by T_{reg}.

It is unknown how the LL protects the metacestode from antibodies. Antibodies binding the acellular LL itself would be damaging to the parasite only via complement-mediated inflammation, essentially ruled out by the data discussed above. Whereas IgM appears not to diffuse freely through the LL [39], IgG does [35], and could therefore bind to the GL tegument. Whereas antibody-dependent cellular cytotoxicity mechanisms are excluded, complement-activation on the tegument could conceivably be controlled through factor H-binding mucins common with the LL [2]. Also, part of the infection-induced antibodies (e.g. human IgG4 [29]) do not fix complement. Complementarily, a major proportion of the antibodies potentially binding the GL could be diverted onto the LL. Interestingly, lack of B cells does not exacerbate susceptibility of mice to *E. multilocularis* [34].

Concluding remarks

The major forms of interaction between the LL and host immunity are summarized in Figure 3. Of these, the most interesting is its non-inflammatory decoding by innate immunity and probable role in the induction of regulation. It must be stressed that there are at present more questions than certainties about this hypothesis (Box 5).

Regulation must also be induced through LL-unrelated parasite molecules, as suggested by the increased inflammation and diminished regulatory cytokines associated with metacestode death, upon which the LL persists [12]. An immune-modulatory candidate that has been the subject of considerable study is 'antigen B' [5], a member of a cestode-specific family of hydrophobic ligand binding proteins. Although the conditioning of dendritic cells by antigen B shifts their T cell-polarizing activity away from Th1 and towards Th2, the induction of tolerogenic capacity has not been analyzed.

Given their probable immune-regulatory properties and exceptional abundance (a single *E. granulosus* cyst can supply some ten grams dry mass) the LL is an interesting starting material in the area of helminth-derived immunosuppressive molecules. This is more so now that most of obstacles discouraging helminth immunologists to work with it have been surmounted.

Box 5. Outstanding questions and future directions

(i) What is the molecular motif responsible for host factor H recruitment to the LL?
(ii) Does iC3b deposited on the LL have a role in generation of T-cell independent antibodies and/or in tolerogenesis?
(iii) What are the innate immune receptors engaged by LL mucins, especially on dendritic cells and macrophages?
(iv) Can antigen-presenting cells process LL-derived materials effectively? Do these materials interfere with processing of other parasite antigens?
(v) Can LL-derived materials effectively induce antigen-presenting cells to instruct adaptive T_{reg} in the periphery?
(vi) Does engagement of liver-specific lectins by LL carbohydrates play a role in tolerance induction, through tolerogenic presentation by liver myeloid cells and/or hepatocytes? Can this function when the parasite is lodged in non-hepatic sites, through liver capture of circulating LL molecules and liver-induced systemic tolerance?
(vii) Are the *E. granulosus* - *E. multilocularis* differences in inflammatory potential explained in part by differences in LL biochemistry?

Acknowledgements

The authors are thankful to Gerardo Lin (from AD's group) for skillfully preparing the figures. They also acknowledge Cecilia Fernández, Florencia Irigoín, and Gustavo Salinas (Universidad de la República, Uruguay) for critical reading of manuscript drafts and helpful discussions. They also thank Andrew S. MacDonald (University of Edimburgh) for expert advice on dendritic cells. The collaboration between AD and JEA was funded by a Royal Society joint grant (2008–2009). In addition, CC was supported by a British Council scholarship. We apologize to the many authors whose valuable results contributed to this article but could not be cited directly.

References

1 Thompson, R.C.A. (1995) Biology and systematics of *Echinococcus*. In *Echinococcus and Hydatid Disease* (Thompson, R.C.A. and Lymbery, A.J., eds), pp. 1–50, CAB International
2 Díaz, A. *et al.* (2011) Understanding the laminated layer of larval *Echinococcus* I: structure. *Trends Parasitol.* DOI: 10.1016/j.pt.2010.12.012 (http://www.cell.com/trends/parasitology/home)
3 Conchedda, M. *et al.* (2004) Immunobiology of cystic echinococcosis. *Parassitologia* 46, 375–380
4 Vuitton, D.A. and Gottstein, B. (2010) *Echinococcus multilocularis* and its intermediate host: a model of parasite-host interplay. *J. Biomed. Biotechnol.* DOI: 10.1155/2010/923193
5 Siracusano, A. *et al.* (2008) Molecular cross-talk in host-parasite relationships: the intriguing immunomodulatory role of *Echinococcus* antigen B in cystic echinococcosis. *Int. J. Parasitol.* 38, 1371–1376
6 Díaz, A. and Allen, J.E. (2007) Mapping immune response profiles: the emerging scenario from helminth immunology. *Eur. J. Immunol.* 37, 3319–3326
7 Maizels, R.M. *et al.* (2004) Helminth parasites–masters of regulation. *Immunol. Rev.* 201, 89–116
8 Emery, I. *et al.* (1998) *In vivo* treatment with recombinant IL-12 protects C57BL/6J mice against secondary alveolar echinococcosis. *Parasite Immunol.* 20, 81–91
9 Reuben, J.M. *et al.* (1978) Immunoprophylaxis with BCG of experimental *Echinococcus multilocularis* infections. *Infect. Immun.* 21, 135–139
10 Rodríguez-Sosa, M. *et al.* (2002) Cutting edge: susceptibility to the larval stage of the helminth parasite *Taenia crassiceps* is mediated by Th2 response induced via STAT6 signaling. *J. Immunol.* 168, 3135–3139
11 Riley, E.M. *et al.* (1986) *Echinococcus granulosus* infection in mice: host responses during primary and secondary infection. *Parasitology* 92 (Pt 2), 391–403
12 Rogan, M.T. (1998) T-cell activity associated with secondary infections and implanted cysts of *Echinococcus granulosus* in BALB/c mice. *Parasite Immunol.* 20, 527–533
13 Hubner, M.P. *et al.* (2006) *Echinococcus multilocularis* metacestodes modulate cellular cytokine and chemokine release by peripheral blood mononuclear cells in alveolar echinococcosis patients. *Clin. Exp. Immunol.* 145, 243–251

14 Mejri, N. and Gottstein, B. (2006) Intraperitoneal *Echinococcus multilocularis* infection in C57BL/6 mice affects CD40 and B7 costimulator expression on peritoneal macrophages and impairs peritoneal T cell activation. *Parasite Immunol.* 28, 373–385
15 Mejri, N. *et al.* (2011) Intraperitoneal *Echinococcus multilocularis* infection in mice modulates peritoneal $CD4^+$ and $CD8^+$ regulatory T cell development. *Parasitol. Int.* 60, 45–53
16 Godot, V. *et al.* (2000) Resistance/susceptibility to *Echinococcus multilocularis* infection and cytokine profile in humans. I. Comparison of patients with progressive and abortive lesions. *Clin. Exp. Immunol.* 121, 484–490
17 Godot, V. *et al.* (1997) Increased basal production of interleukin-10 by peripheral blood mononuclear cells in human alveolar echinococcosis. *Eur. Cytokine Netw.* 8, 401–408
18 Mondragón-de-la-Peña, C. *et al.* (2002) *Echinococcus granulosus* down regulates the hepatic expression of inflammatory cytokines IL-6 and TNF alpha in BALB/c mice. *Parasite* 9, 351–356
19 Zhang, S. *et al.* (2008) Expression of major histocompatibility complex class I chain-related molecule A, NKG2D, and transforming growth factor-beta in the liver of humans with alveolar echinococcosis: new actors in the tolerance to parasites? *J. Infect. Dis.* 197, 1341–1349
20 Gottstein, B. *et al.* (1994) *Echinococcus multilocularis*: parasite-specific humoral and cellular immune response subsets in mouse strains susceptible (AKR, C57B1/6J) or 'resistant' (C57B1/10) to secondary alveolar echinococcosis. *Clin. Exp. Immunol.* 96, 245–252
21 Manfras, B.J. *et al.* (2004) Impeded Th1 CD4 memory T cell generation in chronic-persisting liver infection with *Echinococcus multilocularis*. *Int. Immunol.* 16, 43–50
22 Manfras, B.J. *et al.* (2002) Increased activation and oligoclonality of peripheral $CD8^+$ T cells in the chronic human helminth infection alveolar echinococcosis. *Infect. Immun.* 70, 1168–1174
23 Allan, D. *et al.* (1981) A study of immunoregulation of BALB/c mice by *Echinococcus granulosus equinus* during prolonged infection. *Parasite Immunol.* 3, 137–142
24 Kilwinski, J. *et al.* (1999) T lymphocyte cytokine profile at a single cell level in alveolar Echinococcosis. *Cytokine* 11, 373–381
25 Bresson-Hadni, S. *et al.* (1990) Cellular immunity in experimental *Echinococcus multilocularis* infection. II. Sequential and comparative phenotypic study of the periparasitic mononuclear cells in resistant and sensitive mice. *Clin. Exp. Immunol.* 82, 378–383
26 Kizaki, T. *et al.* (1991) Immune suppression induced by protoscoleces of *Echinococcus multilocularis* in mice. Evidence for the presence of CD8dull suppressor cells in spleens of mice intraperitoneally infected with *E. multilocularis*. *J. Immunol.* 147, 1659–1666
27 Sakamoto, T. and Cabrera, P.A. (2003) Immunohistochemical observations on cellular response in unilocular hydatid lesions and lymph nodes of cattle. *Acta Trop.* 85, 271–279
28 Vuitton, D.A. *et al.* (1989) Cellular immune response in *Echinococcus multilocularis* infection in humans. II. Natural killer cell activity and cell subpopulations in the blood and in the periparasitic granuloma of patients with alveolar echinococcosis. *Clin. Exp. Immunol.* 78, 67–74
29 Dreweck, C.M. *et al.* (1997) Subclass-specific serological reactivity and IgG4-specific antigen recognition in human echinococcosis. *Trop. Med. Int. Health* 2, 779–787
30 Adjobimey, T. and Hoerauf, A. (2010) Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation. *Ann. Trop. Med. Parasitol.* 104, 455–464
31 Rausch, R.L. *et al.* (1981) Characteristics of the larval *Echinococcus vogeli* Rausch and Bernstein, 1972 in the natural intermediate host, the paca, *Cuniculus paca* L. (*Rodentia: Dasyproctidae*). *Am. J. Trop. Med. Hyg.* 30, 1043–1052
32 Gottstein, B. and Hemphill, A. (1997) Immunopathology of *Echinococcosis*. *Chem. Immunol.* 66, 177–208
33 Díaz, A. *et al.* (2000) Expression of the proteinase specialized in bone resorption, cathepsin K, in granulomatous inflammation. *Mol. Med.* 6, 648–659
34 Dai, W.J. *et al.* (2004) *Echinococcus multilocularis* proliferation in mice and respective parasite 14-3-3 gene expression is mainly controlled by an alphabeta CD4 T-cell-mediated immune response. *Immunology* 112, 481–488
35 Coltorti, E.A. and Varela-Díaz, V.M. (1974) *Echinococcus granulosus*: penetration of macromolecules and their localization in the parasite membranes of cysts. *Exp. Parasitol.* 35, 225–231
36 Gottstein, B. *et al.* (1992) *Echinococcus multilocularis*: immunological study on the "Em2-positive" laminated layer during *in vitro* and *in vivo* post-oncospheral and larval development. *Parasitol. Res.* 78, 291–297
37 Walker, M. *et al.* (2004) Isolation and characterization of a secretory component of *Echinococcus multilocularis* metacestodes potentially involved in modulating the host-parasite interface. *Infect. Immun.* 72, 527–536
38 Richards, K.S. *et al.* (1983) *Echinococcus granulosus equinus*: an ultrastructural study of murine tissue response to hydatid cysts. *Parasitology* 86, 407–417
39 Breijo, M. *et al.* (2008) *Echinococcus granulosus*: the establishment of the metacestode is associated with control of complement-mediated early inflammation. *Exp. Parasitol.* 118, 188–196
40 Rausch, R. (1954) Studies on the helminth fauna of Alaska. XX. The histogenesis of the alveolar larva of *Echinococcus* species. *J. Infect. Dis.* 94, 178–186
41 Grainger, J.R. *et al.* (2010) Microbe-dendritic cell dialog controls regulatory T-cell fate. *Immunol. Rev.* 234, 305–316
42 Jankovic, D. *et al.* (2010) IL-10 production by $CD4^+$ effector T cells: a mechanism for self-regulation. *Mucosal Immunol.* 3, 239–246
43 Veldhoen, M. *et al.* (2006) Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. *Nat. Immunol.* 7, 1151–1156
44 Ferreira, A.M. *et al.* (2000) How *Echinococcus granulosus* deals with complement. *Parasitol. Today* 16, 168–172
45 Irigoín, F. *et al.* (1996) Comparison of complement activation *in vitro* by different *Echinococcus granulosus* extracts. *Parasite Immunol.* 18, 371–375
46 Díaz, A. *et al.* (1997) Complement evasion by *Echinococcus granulosus*: sequestration of host factor H in the hydatid cyst wall. *J. Immunol.* 158, 3779–3786
47 Irigoín, F. *et al.* (2008) Resistance of the *Echinococcus granulosus* cyst wall to complement activation: analysis of the role of $InsP_6$ deposits. *Parasite Immunol.* 30, 354–364
48 Meri, S. and Pangburn, M.K. (1990) Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. *Proc. Natl. Acad. Sci. U.S.A.* 87, 3982–3986
49 Díaz, A. *et al.* (2000) Host-derived annexin II at the host-parasite interface of the *Echinococcus granulosus* hydatid cyst. *Mol. Biochem. Parasitol.* 110, 171–176
50 Díaz, A. *et al.* (2009) The major surface carbohydrates of the *Echinococcus granulosus* cyst: mucin-type *O*-glycans decorated by novel galactose-based structures. *Biochemistry* 48, 11678–11691
51 Sjoberg, A.P. *et al.* (2009) Complement activation and inhibition: a delicate balance. *Trends Immunol.* 30, 83–90
52 Tillgren, V. *et al.* (2009) The tyrosine sulfate-rich domains of the LRR proteins fibromodulin and osteoadherin bind motifs of basic clusters in a variety of heparin-binding proteins, including bioactive factors. *J. Biol. Chem.* 284, 28543–28553
53 Díaz, A. *et al.* (1999) Control of host complement activation by the *Echinococcus granulosus* hydatid cyst. *Immunopharmacology* 42, 91–98
54 Verbovetski, I. *et al.* (2002) Opsonization of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. *J. Exp. Med.* 196, 1553–1561
55 Hokke, C.H. *et al.* (2007) Glycomics-driven discoveries in schistosome research. *Exp. Parasitol.* 117, 275–283
56 Terrazas, C.A. *et al.* (2010) Impaired pro-inflammatory cytokine production and increased Th2-biasing ability of dendritic cells exposed to *Taenia* excreted/secreted antigens: a critical role for carbohydrates but not for STAT6 signaling. *Int. J. Parasitol.* 40, 1051–1062
57 Coombs, P.J. *et al.* (2006) Two categories of mammalian galactose-binding receptors distinguished by glycan array profiling. *Glycobiology* 16, 1C–7C
58 Carambia, A. and Herkel, J. (2010) CD4 T cells in hepatic immune tolerance. *J. Autoimmun.* 34, 23–28
59 Suda, T. *et al.* (1988) Prevention of suppression of alloreactive capacity following intravenous injection of neuraminidase-treated allogeneic cells by co-injection of agents competing for asialoglycoprotein receptor. *Reg. Immunol.* 1, 24–31
60 Grewal, P.K. (2010) The Ashwell-Morell receptor. *Methods Enzymol.* 479, 223–241

61 Jenne, L. *et al.* (2001) Dendritic cells pulsed with unfractionated helminthic proteins to generate antiparasitic cytotoxic T lymphocyte. *Parasite Immunol.* 23, 195–201

62 Andrade, M.A. *et al.* (2004) *Echinococcus multilocularis* laminated-layer components and the E14t 14-3-3 recombinant protein decrease NO production by activated rat macrophages in vitro. *Nitric Oxide* 10, 150–155

63 Steers, N.J. *et al.* (2001) In-vitro susceptibility of hydatid cysts of *Echinococcus granulosus* to nitric oxide and the effect of the laminated layer on nitric oxide production. *Parasite Immunol.* 23, 411–417

64 Casaravilla, C. and Díaz, A. (2010) Studies on the structural mucins of the *Echinococcus granulosus* laminated layer. *Mol. Biochem. Parasitol.* 174, 132–136

65 Dai, W.J. *et al.* (2001) Major carbohydrate antigen of *Echinococcus multilocularis* induces an immunoglobulin G response independent of alphabeta$^+$ CD4$^+$ T cells. *Infect. Immun.* 69, 6074–6083

66 Sterla, S. *et al.* (1999) *Echinococcus granulosus* human infection stimulates low avidity anticarbohydrate IgG2 and high avidity antipeptide IgG4 antibodies. *Parasite Immunol.* 21, 27–34

67 Russi, S. *et al.* (1974) Isolation and characterization of a blood P1 active carbohydrate antigen of *Echinococcus granulosus* cyst membrane. *J. Immunol.* 112, 1061–1069

68 Baz, A. *et al.* (1999) Antibody response in CD4-depleted mice after immunization or during early infection with *Echinococcus granulosus*. *Parasite Immunol.* 21, 141–150

69 Vos, Q. *et al.* (2000) B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. *Immunol. Rev.* 176, 154–170

70 Varela-Díaz, V.M. and Coltorti, E.A. (1973) The presence of host immunoglobulins in hydatid cyst membranes. *J. Parasitol.* 59, 484–488

71 Taherkhani, H. *et al.* (2007) Antibody responses in human cystic hydatid disease to the laminated layer of *Echinococcus granulosus*. *Parasitol. Res.* 101, 647–652

72 Alkarmi, T.O. *et al.* (1988) *Echinococcus multilocularis*: the non-specific binding of different species of immunoglobulins to alveolar hydatid cysts grown in vivo and in vitro. *Parasite Immunol.* 10, 443–457

73 Rogan, M.T. and Craig, P.S. (1997) Immunology of *Echinococcus granulosus* infections. *Acta Trop.* 67, 7–17

74 Gottstein, B. *et al.* (2002) An intact laminated layer is important for the establishment of secondary *Echinococcus multilocularis* infection. *Parasitol. Res.* 88, 822–828

75 Ferreira, A.M. *et al.* (2000) Contribution of C5-mediated mechanisms to host defence against *Echinococcus granulosus* hydatid infection. *Parasite Immunol.* 22, 445–453

76 Ramos, A.L. *et al.* (2006) Eosinophil cationic protein damages protoscoleces *in vitro* and is present in the hydatid cyst. *Parasite Immunol.* 28, 347–355

77 Brehm, K. (2010) *Echinococcus multilocularis* as an experimental model in stem cell research and molecular host-parasite interaction. *Parasitology* 137, 537–555

78 Ingold, K. *et al.* (2000) High molecular mass glycans are major structural elements associated with the laminated layer of *in vitro* cultivated *Echinococcus multilocularis* metacestodes. *Int. J. Parasitol.* 30, 207–214

79 Irigoín, F. *et al.* (2002) *myo*-Inositol hexakisphosphate is a major component of an extracellular structure in the parasitic cestode *Echinococcus granulosus*. *Biochem. J.* 362, 297–304

80 Irigoín, F. *et al.* (2004) Unique precipitation and exocytosis of a calcium salt of *myo*-inositol hexakisphosphate in larval *Echinococcus granulosus*. *J. Cell Biochem.* 93, 1272–1281

81 Casaravilla, C. *et al.* (2006) Characterization of *myo*-inositol hexakisphosphate deposits from larval *Echinococcus granulosus*. *FEBS J.* 273, 3192–3203

82 Ingold, K. *et al.* (2001) Characterization of the laminated layer of *in vitro* cultivated *Echinococcus vogeli* metacestodes. *J. Parasitol.* 87, 55–64

83 Hulsmeier, A.J. *et al.* (2002) A major *Echinococcus multilocularis* antigen is a mucin-type glycoprotein. *J. Biol. Chem.* 277, 5742–5748

84 Koizumi, A. *et al.* (2009) Synthetic studies on the carbohydrate moiety of the antigen from the parasite *Echinococcus multilocularis*. *Carbohydr. Res.* 344, 856–868

85 Koizumi, A. *et al.* (2011) Synthesis of the carbohydrate moiety from the parasite *Echinococcus multilocularis* and their antigenicity against human sera. *Eur. J. Med. Chem.*, in press

86 Stadelmann, B. *et al.* (2010) *Echinococcus multilocularis* phosphoglucose isomerase (EmPGI): a glycolytic enzyme involved in metacestode growth and parasite-host cell interactions. *Int. J. Parasitol.* 40, 1563–1574

87 Gómez-García, L. *et al.* (2005) Intact glycans from cestode antigens are involved in innate activation of myeloid suppressor cells. *Parasite Immunol.* 27, 395–405

88 Obregon-Henao, A. *et al.* (2003) *In situ* detection of antigenic glycoproteins in *Taenia solium* metacestodes. *J. Parasitol.* 89, 726–732

89 Restrepo, B.I. *et al.* (2000) Characterisation of the carbohydrate components of *Taenia solium* metacestode glycoprotein antigens. *Int. J. Parasitol.* 30, 689–696

90 Sandeman, R.M. and Williams, J.F. (1984) Lectin binding to cystic stages of *Taenia taeniaeformis*. *J. Parasitol.* 70, 661–667

91 Lee, J.J. *et al.* (2005) Mass spectrometric characterisation of *Taenia crassiceps* metacestode N-glycans. *Mol. Biochem. Parasitol.* 143, 245–249

92 Khoo, K.H. *et al.* (1997) Structural characterization of the N-glycans from *Echinococcus granulosus* hydatid cyst membrane and protoscoleces. *Mol. Biochem. Parasitol.* 86, 237–248

93 March, F. *et al.* (1991) *Echinococcus granulosus*: antigen characterization by chemical treatment and enzymatic deglycosylation. *Exp. Parasitol.* 73, 433–439

94 Profumo, E. *et al.* (1994) Cellular and humoral responses to antigenic subunits of *Echinococcus granulosus* cyst fluid in hydatid patients. *Parasite Immunol.* 16, 393–398