Experimental Parasitology 158 (2015) 23–30

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Minireview

# The laminated layer: Recent advances and insights into *Echinococcus* biology and evolution

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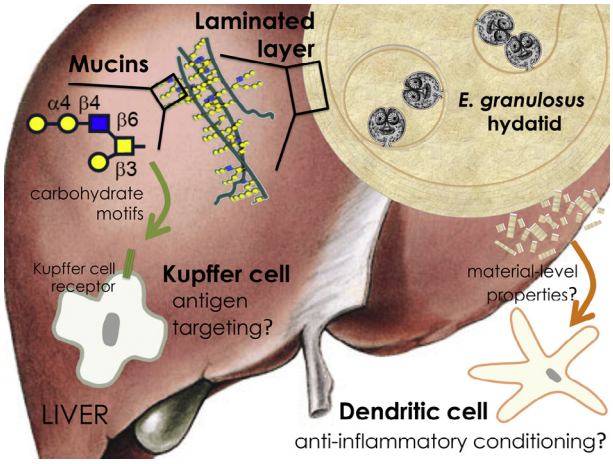
## HIGHLIGHTS

- *Echinococcus* larvae are protected by the acellular, mucin-based laminated layer (LL).
- *E. granulosus* probably uses more mucin protein backbones than *E. multilocularis*.
- In addition, the mucin glycans in *E. granulosus* undergo more elongation.
- The LL glycans may be optimized to interact with liver-specific host receptors.
- The LL also has carbohydrate-independent modulatory effects on innate immune cells.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 23 December 2014
Received in revised form 9 March 2015
Accepted 20 March 2015
Available online 25 March 2015

*Keywords:*
*Echinococcus*
Laminated layer
Mucin
Carbohydrate
Liver
Lectin

## ABSTRACT

The laminated layer is the unique mucin-based extracellular matrix that protects *Echinococcus* larvae, and thus to an important extent, shapes host–parasite relationships in the larval echinococcoses. In 2011, we published twin reviews summarizing what was known about this structure. Since then, important advances have been made. Complete genomes and some RNAseq data are now available for *E. multilocularis* and *E. granulosus*, leading to the inference that the *E. multilocularis* LL is probably formed by a single type of mucin backbone, while a second apomucin subfamily additionally contributes to the *E. granulosus* LL. Previously suspected differences between *E. granulosus* and *E. multilocularis* in mucin glycan size have been confirmed and pinned down to the virtual absence of Galβ1–3 chains in *E. multilocularis*. The LL carbohydrates from both species have been found to interact selectively with the Kupffer cell receptor expressed in rodent liver macrophages, highlighting the ancestral adaptations to rodents as intermediate hosts and to the liver as infection site. Finally, LL particles have been shown to possess carbohydrate-independent mechanisms profoundly conditioning non-liver-specific dendritic cells and macrophages.

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http://dx.doi.org/10.1016/j.exppara.2015.03.019

These advances are discussed in an integrated way, and in the context of the newly determined phylogeny of *Echinococcus* and its taenid relatives.



**Contents**



## 1. Introduction

The taenid cestodes belonging to the genus *Echinococcus* (Thompson and Jenkins, 2014) have two-host life cycles that in all cases include a sexual stage in the intestine of a carnivore and a larval stage in the tissues of non-carnivorous/omnivorous mammals. These life-cycles are based on a predator–prey relationship between the definitive host (harboring the parasite adult stage) and the intermediate hosts (harboring the larval stage). Infection by the larval stages (larval echinococcoses) can affect humans (Brunetti and White, 2012), which are thus accidental intermediate hosts. Intermediate host infection comes about after ingestion of eggs (passed out with the definitive host feces), which hatch releasing oncospheres that penetrate the intestinal wall, and are carried by blood or lymph to internal organs. Oncospheres develop into larvae known as metacestodes, which have a basic bladder-like morphology, and give rise within them to protoscoleces, infective for the definitive hosts.

Ten or eleven *Echinococcus* species are currently recognized (Lymbery et al., 2014; Thompson and Jenkins, 2014). These include six or seven species split apart from what was historically *E. granulosus*. This (paraphyletic) group of species (Nakao et al., 2013a, 2013b), now called *E. granulosus sensu lato*, uses different ungulate species as intermediate hosts. The metacestode develops as a large, unilocular turgid "cyst" (more correctly termed a "hydatid"), which grows through concentric enlargement only. This group includes *E. granulosus* (*sensu strictu*), i.e. what was formerly known as the "sheep strain", the most studied species of the group. Outside this group, the genus includes the neotropical species *E. vogeli* and *E. oligarthra*, having rodent intermediate hosts and metacestode morphologies with small variations with respect to the hydatid (D'Alessandro and Rausch, 2008). Finally, the genus also includes the sister species *E. shiqicus* and *E. multilocularis*, with lagomorph and rodent intermediate hosts respectively (Vuitton and Gottstein, 2010; Xiao et al., 2006). Larval *E. shiqicus* develops in a hydatid-like morphology. In contrast, the *E. multilocularis* metacestode generates outward protrusions that invade the surrounding host tissue (Mehlhorn et al., 1983). Hence *E. multilocularis* larvae develop as a network of interconnected vesicles and tubules than can take over the whole organ (liver) and even invade other organs. Thus human infection by this species is the most lethal of helminthiasis (Brunetti and White, 2012; Thompson and Jenkins, 2014).

Throughout the genus, metacestodes are bounded by a thin layer of cells (germinal layer, GL), outwardly protected by an acellular structure known as the laminated (or laminar) layer (LL). The LL, unique and distinctive of the genus *Echinococcus*, is undoubtedly a major component of the adaptation of these parasites to dwell in internal organs of immunocompetent mammals over years or decades. In 2011, we published twin reviews summarizing what was known about the LL, and attempting to fill the gaps with informed speculations (Díaz et al., 2011a, 2011b). In short, the LL is a specialized extracellular matrix synthesized by the GL, and more specifically by its outermost syncytial layer, termed the tegument. As the tegument is only one cell-thick, tegumental cells must engage in intense biosynthetic activity in order to generate the building blocks for the massive LL. The LL is based on mucins, i.e. glycoproteins with many points of a particular type of glycosylation (mucin *O*-type glycosylation). Whereas mucins normally form the loose mucus barriers (Corfield, 2015), the LL is much more structured, allowing live hydatids to be turgid. Across the genus, LL thickness varies considerably, reaching 3 mm in *E. granulosus sensu strictu* (and being similarly thick in *E. granulosus sensu lato*), up to 400 μm in *E. vogeli*, 5–38 μm in *E. shiqicus*, and 10–12 μm in *E. multilocularis* (Bortoletti and Ferretti, 1978; Rausch, 1954; Rausch et al., 1981; Xiao et al., 2005). In addition to mucins, the LL of *E. granulosus* contains abundant nanodeposits of a calcium salt of inositol hexakisphosphate (Ins$P_6$) (Casaravilla et al., 2006; Irigoín et al., 2002, 2004). This is a curious adaptation, since Ins$P_6$ is an intracellular molecule in all other biological systems studied (Irvine and Schell, 2001). The adaptation is absent from *E. multilocularis* on the basis of biochemical evidence (Irigoín et al., 2002). From transmission electron microscopy data, a technique by which the *E. granulosus* Ins$P_6$ deposits appear as a conspicuous feature (Irigoín et al., 2004), the adaptation would be present in *E. equinus* (Richards et al., 1983) but absent in *E. vogeli* (Ingold et al., 2001). Thus presence of the calcium Ins$P_6$ deposits appears to correlate with adaptation to infect large mammals.

The LL is widely thought to be a crucial element of the host–parasite relationship in the larval echinococcoses. Its roles include shielding the parasite from direct attack by host immune cells, and probably down-regulating local inflammation (Díaz et al., 2011a, 2011b). The evidence for the second aspect is still indirect. It includes the observation that in experimental *E. granulosus* infections, the local inflammation subsides at the same time as the LL is deployed (Breijo et al., 2008). It also includes, for *E. multilocularis,* the observation that the portions of the invasive protrusions that are freshly formed, and thus still devoid of LL, are lined with inflammatory cells, while those nearby areas that are already covered by LL are lined with collagen and fibroblasts (Mehlhorn et al., 1983).

In the following two sections of this review, we focus on recent advances on the peptide (apomucin) and carbohydrate (mucin *O*-glycan) components of the mucins that make up the LL. We then summarize advances on how the LL is decoded by the immune system. We propose that this decoding includes, but is not restricted to, specific interactions between LL mucin carbohydrates and host receptors, and place it in the context of *Echinococcus* evolution.

## 2. Advances on the LL structure: apomucins

The sequences of apomucins making up the *E. granulosus* LL had been previously inferred from (pre-RNAseq) transcriptomic data, as sequences highly expressed in the GL and essentially absent from the other stages/tissues sampled (Díaz et al., 2011a, 2011b; Parkinson

et al., 2012). These sequences code for (mostly short) secreted apomucins, each comprising a 10–11 residue-long non-glycosylated N-terminal extension, a mucin domain in which a very high density of *O*-glycosylation is predicted, and a C-terminal signal for the incorporation of a GPI anchor. Both the leader peptide and the GPI anchor signal are common for all these apomucins, which are thus a protein family. An attempt to obtain proteomic data on these mucins failed, only host proteins being detected in LL samples treated in a number of ways (AD, C. Batthyány and R. Durán, unpublished). Nonetheless, additional useful information on the apomucin family has been recently contributed by the publication of the *E. granulosus* and *E. multilocularis* (plus two further taenid) genomes, as well as RNA seq data (Tsai et al., 2013; Zheng et al., 2013) (most of the information is summarized in fig. S12.1 in Tsai et al., 2013). Firstly, the family exists only in *Echinococcus*, suggesting that it appeared concomitantly with the LL (see evolutionary discussion later). For the most highly expressed apomucin, featuring an acidic non-glycosylated N-terminus and defining by itself a sub-family, there is a single gene in each of the *E. granulosus* and *E. multilocularis* genomes. These sequences had been named EGC0000317 and EMC0000019 respectively in Parkinson et al. (2012) and are named as EgrG_000742900.1 and EmuJ_000742900.1 respectively in Tsai et al. (2013). In addition, the *E. granulosus* ortholog was named EGR_08371 in Zheng et al. (2013). More conveniently, the apomucin was baptized as MUC-1 in Koziol et al. (2014). The divergence in sequence between *E. granulosus* and *E. multilocularis* MUC-1 is striking, suggesting that at least one of the orthologs has evolved under positive selective pressure. The main differences are in the glycosylated domain, in which the *E. multilocularis* ortholog has 3 charged residues, instead of 1 in *E. granulosus.* The new transcriptomic data (Tsai et al., 2013; Zheng et al., 2013) confirm that MUC-1 is expressed with selectivity in the GL with respect to other tissues/stages, in the two species. For *E. multilocularis*, this was also confirmed by quantitative PCR and *in situ* hybridization (Koziol et al., 2014). This latter technique showed that within the GL the protein is, as expected, expressed in the tegument. However, unexpectedly, expression was also observed in the lining of the brood capsules, the structures that give rise to protoscoleces, suggesting that MUC-1 can also contribute to conventional glycocalyces. It has to be taken into account that while assembly into the LL in all probability requires that the putative GPI anchor is cleaved off, the same mucins could, under conditions in which the anchor is retained, contribute to cellular glycocalyces.

With respect to the three other LL apomucins previously predicted (EGC0004254, EGC0002904, EGC0005092; Parkinson et al., 2012), the genomic data show that they belong to a sub-family with at least half a dozen members in each species, which for simplicity will be referred to here as “MUC-2 sub-family”. Here the existence of several similar genes, complicated by the repetitive nature of the glycosylated domains, makes gene prediction difficult. Thus two of the three *E. granulosus* sequences listed earlier, obtained by an approach that privileged full-length mRNAs (Parkinson et al., 2012), were not found in the genome of this species (neither putative orthologs in the higher quality one of *E. multilocularis*, species in which they may be genuinely absent) (Tsai et al., 2013; Zheng et al., 2013). Even with these uncertainties, it can be summarized that, in *E. granulosus,* MUC-2 sub-family members exist both with an unpaired cysteine in the N-terminal extension and without cysteine, while in *E. multilocularis* all MUC-2 members appear to have the unpaired cysteine (Tsai et al., 2013). Additionally, for two *E. granulosus* MUC-2 sub-family members on which there are RNAseq data, in one case (EG_05124 in the nomenclature of Zheng et al., 2013, lacking cysteine) there is selective expression in the GL, while in the other case (EG_09538 (Zheng et al., 2013), containing cysteine) there is expression in GL and in oncospheres (but not in protoscoleces or adults). In the case of *E. multilocularis*, only a single MUC-2 member (EmuJ_000408200 in the nomenclature in Tsai et al., 2013) is expressed at all in the GL, and then at levels similar to those measured for the other two stages sampled. Indeed, the quotient of expression between the sum of the MUC-2 family members and MUC-1 is approximately 1/4 for *E. granulosus*, but 1/255 for *E. multilocularis*. Although as mentioned gene predictions and hence RNAseq data are incomplete for the MUC-2 family, this affects *E. granulosus*, whose genome was sequenced to low coverage, but probably not *E. multilocularis*. Thus the actual difference in these quotients must be larger than suggested by the numbers discussed earlier.

In summary, the available data indicate that it is highly likely that MUC-1 is a major LL constituent in both *E. granulosus* and *E. multilocularis*, and thus the major glycans elucidated probably decorate this backbone. In addition, the data suggest that while the *E. multilocularis* LL could be made up from MUC-1 only, the *E. granulosus* LL would be made up from MUC-1 plus MUC-2 family members, both cysteine-containing and not. The effects of disulphide reducing agents on the *E. granulosus* LL (Casaravilla and Díaz, 2010; Casaravilla et al., 2014) are consistent with this conclusion. Although disulphide reduction has not been tried on the *E. multilocularis* LL, certainly proteolysis causes its solubilization in experiments in which the *E. granulosus* LL retains its structure (our unpublished observations), suggesting inter-species differences in mucin cross-linking. So both the differential contribution of MUC-2 members and sequence differences in MUC-1 probably underlie the physical differences between the LL of the two species. In turn, these physical differences must relate to the adaptation, in the case of *E. multilocularis*, to grow by outward budding of the GL (Mehlhorn et al., 1983). This implies that GL-derived cellular projections have to grow through the LL, which is hardly compatible with a thick, stable, and probably cross-linked LL like that of *E. granulosus*.

## 3. Advances on the LL structure: glycome

The LL is a massive and extraordinarily carbohydrate-rich structure right at the interface with the host (Díaz et al., 2011a, 2011b). Throughout biology, carbohydrates, with their extraordinary structural diversity, are central in the relaying of information between cells, as well as between extracellular matrices and cells. Carbohydrates convey information through receptors called lectins (Gabius et al., 2011). Lectins normally bind to the terminal mono- or disaccharides of glycans, recognizing not only identity of these saccharide residues (glucose, galactose, sialic acid, etc.), but also the way in which they are linked (α1–4, β1–3, etc.) (Gabius et al., 2011). Importantly, effective binding requires multivalency, and thus the spacing of the motifs bound, within an individual glycan chain, and more commonly between different chains, is critical. The recognition of pathogen carbohydrates by host lectins is a central determinant of how the innate immune system perceives pathogens (real of potential) and therefore the type of instructive signals that are relayed to the adaptive immune system (Osorio and Reis e Sousa, 2011). In fact, many of the major inflammatory triggers for innate immunity present in bacteria, fungi and protozoans are glycoconjugates (Gowda, 2007; Richardson and Williams, 2014; Royet et al., 2011). In addition, the receptors of adaptive immunity (antibodies but also the T-cell receptor) can recognize glycoconjugates, making these molecules important as vaccine components (Avci, 2013; Avci et al., 2013). Specifically in the larval echinococcoses, host antibody responses are notoriously biased toward carbohydrate epitopes (Díaz et al., 2011a, 2011b). For all these reasons, the detailed structure of the LL carbohydrates is a major piece of information for understanding host–parasite relationships in the larval echinococcoses.

It appears that an overwhelming proportion of the carbohydrates present in the LL are the *O*-glycans decorating the mucins (Del Puerto et al., 2015; Díaz et al., 2009). Animal mucin-type *O*-linked glycans are synthesized in the Golgi apparatus by sequential addition of monosaccharide units to serine or threonine side chains of

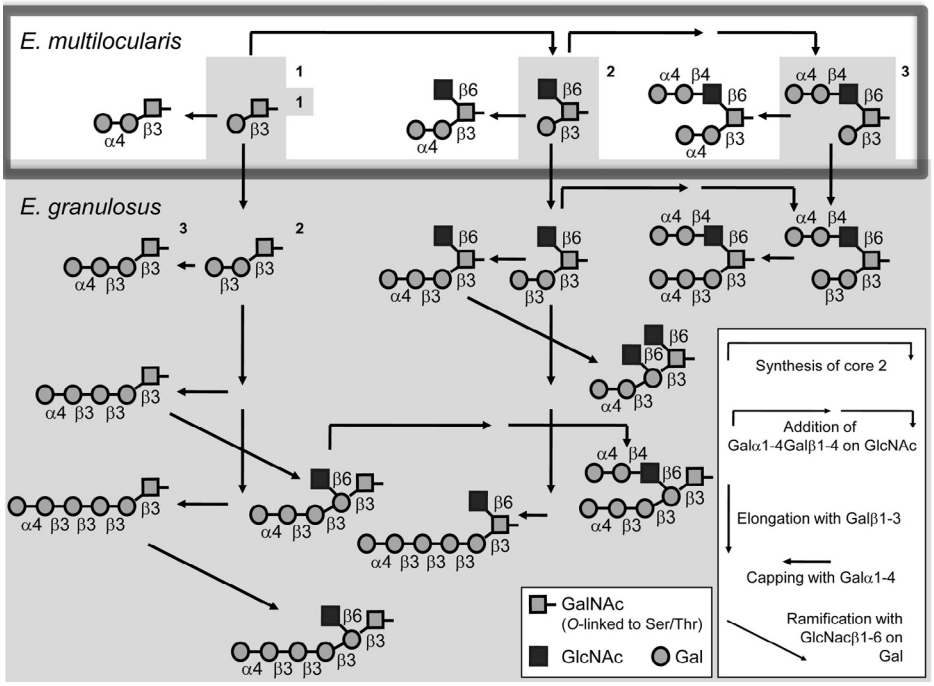


**Fig. 1.** Differences in LL glycome between *E. granulosus* and *E. multilocularis*. Glycans on light gray background are found in the *E. granulosus* LL and glycans within the gray rectangle are found in the *E. multilocularis* LL. The numbers in bold indicate the first three glycans in molar abundance in each species; these as given on light gray and white and backgrounds for *E. granulosus* and *E. multilocularis* respectively. The glycans shown have been elucidated in full (Del Puerto et al., 2015; Díaz et al., 2009; Lin et al., 2013) except for *E. multilocularis* glycans Galα1-4Galβ1-3GalNAc, Galα1-4Galβ1–3(GlcNAcβ1–6)GalNAc and Galα1-4Galβ1–3(Galα1–4 Galβ1-4GlcNAcβ1–6)GalNAc, which were deduced from the partial data (Del Puerto et al., 2015; Hülsmeier et al., 2002). The glycan Galβ1-3Galβ1-3GalNAc, shown as being present in *E. granulosus* only, is also present in *E. multilocularis* as a trace component (Del Puerto et al., 2015). In the *E. granulosus* LL many further glycans are present, which can be deduced from MS data to correspond to variants of those shown bearing additional Galβ1–3 units and/or GlcNAcβ1–6 or Galα1-4Galβ1-4GlcNAcβ1–6 branches on Galβ1–3 residues (Díaz et al., 2009; Lin et al., 2013). Those intermediates between successive biosynthetic steps that are not shown are not found in significant abundance in the assembled LL. Additional biosynthetic relationships to those shown are possible. Note that a deficit in the activity catalyzing the elongation with Galβ1–3 suffices to restrict LL glycan biosynthesis to the structures within the gray rectangle, i.e. those found in *E. multilocularis*. The structures found in this species but not in *E. granulosus* may arise when the capping enzyme does not suffer from competition by the elongation enzyme for common substrates.

the peptide backbones (apomucins). The first two or three biosynthetic steps are common to many different glycan structures, which thus share common "cores" (Brockhausen et al., 2009). The most common such cores, namely cores 1 and 2, are the basis of all the detected LL glycans (Del Puerto et al., 2015; Díaz et al., 2009; Hülsmeier et al., 2002) (see Fig. 1). In *E. granulosus*, the two cores can be elongated with a variable number of galactose (Gal) β1–3 units, and this chain can be capped with a single Galα1–4 residue (Díaz et al., 2009). In addition, the core 2 *N*-acetylglucosamine (GlcNAc) residue can be decorated with the Galα1-4Galβ1–4 disaccharide. This gives rise to Galα1-4Galβ1-4GlcNAc motif, which is the cause of antibody cross-reactivity between the LL and human red blood cells of the $P_1$ blood group (Russi et al., 1974). This set of structural features suffices to describe many of the detected *E. granulosus* glycans but could not describe other glycans (especially large ones) that were known, from mass spectrometry data, to bear more than two HexNAc (*N*-acetylhexosamine, usually either GlcNAc and/or *N*-acetylgalactosamine, GalNAc) residues. Recent structural work (Lin et al., 2013) ascertained that the non-core HexNAc residues are GlcNAcβ1–6 ramifications on the Galβ1–3 chain previously mentioned (including the Gal residue that is part of the cores). These GlcNAcβ1–6 residues can be decorated with the Galα1-4Galβ1–4 disaccharide previously mentioned, thus reproducing the blood $P_1$-antigen motif in a molecular context different to that previously known. The number of different mucin *O*-glycan structures in the *E. granulosus* LL glycans is very large, as judged by the complexity of chromatographic profiles, which increases notably with increasing glycan size (Díaz et al., 2009; Lin et al., 2013). When the information from the structures fully elucidated and from those glimpsed in terms of MS data only (Díaz et al., 2009; Lin et al., 2013) is put together, the scenario that arises is the following: (i) all the glycans are built from the small number of structural motifs previously summarized; (ii) different combinations of use of this set of motifs give rise to a large number of individual glycans. In other words, we are confident that the larger glycans (7–18 residues) detected in terms of monosaccharide composition only (Díaz et al., 2009) must be similar to the smaller structures that we elucidated but bear longer Galβ1–3 chains and correspondingly higher numbers of GlcNAcβ1–6 and/or Galα1-4Galβ1-4GlcNAcβ1–6 branches. As within the glycans fully elucidated, once the Galβ1–3 chain reaches a length of 3 residues the Galα1–4 cap is invariably present, we predict that the larger glycans must be always capped. It must be however cautioned that LL glycobiology may have further surprises, as the proposed apomucins have glycosylphosphatidylinositol anchors (GPI anchors, see discussion later), yet mannose, a component of all known GPI anchors, has not been detected (Del Puerto et al., 2015; Díaz et al., 2009).

Lin et al. (2013) also determined that the gross glycan composition of the LL is maintained over a range of host species and infection locations. Finally they determined that the monoclonal antibody E492 (Baz et al., 1999) binds the $P_1$ trisaccharide. This explains the antibody's reactivity with *E. granulosus* protoscoleces, which have N-glycans that carry a trisaccharide probably corresponding to this motif (Khoo et al., 1997), as well as with the *E. multilocularis* LL (Walker et al., 2004) (see discussion later).

In addition to pioneering work in the 1960s and 1970s (Kilejian and Schwabe, 1971; Kilejian et al., 1962; Korc et al., 1967), a crucial advance for LL glycomics was the partial elucidation, by Peter Kohler's group in 2002, of the glycans of a mucin fraction from the

*E. multilocularis* larva (Hülsmeier et al., 2002). This fraction was purified with the monoclonal antibody Em2(G11), which only binds the *E. multilocularis* LL (Barth et al., 2012). The set of *E. multilocularis* glycans was clearly related to that later described by us in the *E. granulosus* LL, but biased toward simpler structures. While in the *E. granulosus* LL, as mentioned, glycans of up to 18 monosaccharide units are detected, the largest glycans detected in the Em2(G11) fraction have 5–6 units (Hülsmeier et al., 2002). In particular, the Galβ1–3 chain, which in *E. granulosus* can, as mentioned, encompasses 4 units and most probably more, is limited in the *E. multilocularis* Em2(G11) fraction to the single Galβ1–3 residue of the cores. Associated with this, the core Galβ1–3 residue can be directly capped with Galα1–4, while in *E. granulosus* such capping only takes place for longer chains. Given that purification, with a monoclonal antibody and/or the previous solubilization step, could well have biased the mucin composition, it was not known if the set of glycans in the Em2(G11) fraction was representative of the *E. multilocularis* LL. Recent work (Del Puerto et al., 2015) shows that it was indeed representative, and additionally contributes a quantification of the relative abundances of the different structures. Thus the contrasting thickness of the *E. granulosus* and *E. multilocularis* LLs correlates with a difference in the size of their mucin glycans. The quantification also shows that structures terminated in Galβ1-4GlcNAc (*N*-acetyl-lactosamine), which are biosynthetic intermediates, are excluded from the final set of glycans, almost to the same extent in *E. multilocularis* as in *E. granulosus*. Further, the quantification shows that it is not the capping that prevents Galβ1–3 chain elongation in *E. multilocularis*, as only a quantitatively minor proportion of the structures is capped. It must be instead the activity of the enzyme transferring Galβ1–3 units onto Galβ1–3 acceptors (the elongation enzyme) that differs between the two species. Although very weak, the activity is apparently not absent from *E. multilocularis*, as traces of Galβ1-3Galβ1-3GalNAc are detected. The difference in activity of the elongation enzyme suffices to explain the glycomic differences between the LL of the two species, as illustrated on Fig. 1. Galβ1–3 chains were previously only known in nature in amphibian egg-jellies (Guerardel et al., 2000; Morelle and Strecker, 1997; Strecker et al., 1995), and are therefore not a conserved structural motif in animals, or even in platyhelminths. Analysis of genomic and transcriptomic data identified a likely candidate for the *Echinococcus* elongation enzyme, on the basis of: (i) similarity with mammalian group GT31 glycosyltransferases (which catalyze the addition of β1–3 Gal or β1–3 GlcNAc), (ii) highest relative expression in the GL among all *E. granulosus* tissues/stages, (iii) higher relative expression in the GL of *E. granulosus* than in that of *E. multilocularis* (Del Puerto et al., 2015). According to phylogenetic analysis, the enzyme appeared within the taenid lineage, as part of a strong clade-specific expansion of the GT31-family (Del Puerto et al., 2015; Tsai et al., 2013). The enzyme is also expressed (always at the mRNA level) in *E. granulosus* protoscoleces, and its ortholog is expressed in the cisticercus of *Taenia solium*, which does not have an LL. This predicts that certain carbohydrates of larval taenids, other than those making up the *Echinococcus* LL, may also bear Galβ1–3 chains. Except for the LL, the larval taenid *O*-glycans in particular have not been studied. In the earlier reasoning, Galβ1–3 chains would have been a pre-existing structural motif in taenids that was recruited to the formation of the LL in *Echinococcus*.

One of the glycans found in the *E. multilocularis* but not the *E. granulosus* LL, Galα1-4Galβ1-3GlcNAc, was found to be the dominant antigen with respect to antibody responses of infected humans against the Em2(G11) fraction (Yamano et al., 2012). This is interesting in the light of the finding that this glycan has a low relative abundance (Del Puerto et al., 2015). The Galα1-4Galβ1-3GlcNAc glycan is also recognized by antibodies from humans infected with *E. granulosus*, much of this reactivity being eliminated by previous adsorption with the Galα1-4Gal substructure (Yamano et al., 2012). This is ascribable to reactivity, in *E. granulosus* infections, against longer capped glycans, i.e. structures terminated in Galα1-4Galβ1-3Gal. We previously proposed such structures to be immunogenic (Díaz et al., 2011a, 2011b), on the basis of data predating the carbohydrate structural analyses (Russi et al., 1974). In sum, the new data support the possibility that Galα1-4Galβ1-3Gal/GalNAc motifs, more than the related Galα1-4Galβ1-4GlcNAc motif, may be dominant antigens for antibody responses, both in *E. multilocularis* and in *E. granulosus* larval infections.

## 4. Advances on the interaction with innate immunity and evolutionary considerations

As exposed previously (Díaz et al., 2011a, 2011b), the immune cells of *Echinococcus* intermediate hosts can be expected to make contact not only with the LL proper but also with particles shed from the LL as a consequence of metacestode growth. Such particles were detected, with the help of the Em2(G11) antibody mentioned, in tissue of infected patients (Barth et al., 2012). These particles, less than 1 μm in diameter, are not only abundantly detected close to the parasite, but also detected mm away from it and to some extent in draining lymph nodes. As mentioned, lectins are central players in innate immune decoding of pathogens. A screen among lectins known to be expressed in mammalian macrophages identified only the mouse Kupffer cell (liver macrophage) receptor (KCR; CLEC4F) as a lectin able to bind the *E. granulosus* LL unequivocally (Hsu et al., 2013). The receptor also bound to a range of synthetic LL carbohydrates (Koizumi et al., 2011), as well as to the *E. multilocularis* LL, and the interactions observed were in line with the known specificity of the KCR (Coombs et al., 2006). Although the result was obtained using recombinant soluble dimeric receptors, it will most likely hold true when re-tested using mouse Kupffer cells, on which the KCR is expressed as a trimeric cell-surface receptor (Fadden et al., 2003). *Echinococcus* larvae show marked preference for the liver, as further discussed later. *In situ* antigen priming in the liver parenchyma, and through Kupffer cells in particular, is known to be tolerogenic, as opposed to conventional priming in the lymph nodes draining the organ (Thomson and Knolle, 2010). Thus the new data are consistent with the hypothesis that the LL carbohydrates are evolutionarily optimized for ensuring the (non-immunogenic or tolerogenic) clearance of shed LL particles by Kupffer cells. This hypothesis (Díaz et al., 2011a, 2011b) includes the possibility that KCR engagement favors the release of anti-inflammatory mediators, although there is currently no precedent of signaling via KCR, other than in the restricted context of internalization. The hypothesis also encompasses the possibility of interaction with, and conditioning of, macrophages recruited into the liver, as these can also express the KCR (Yang et al., 2013). Although other *Echinococcus* stages (certainly protoscoleces, although there are no glycobiological data on oncospheres) do express Gal-rich carbohydrates (Khoo et al., 1997) and are bound by Kupffer cells (Walbaum et al., 1994), we do not believe an interaction with the KCR determines a larval preference for the liver. Indeed, route of entry into the body, rather than any active homing process, seems to determine infection location, at least for *E. granulosus* (Heath, 1971).

The KCR is, as far as it is known, only found at the genomic level in rodents (Fadden et al., 2003), and only expressed in liver macrophages (Yang et al., 2013). Some light on the possible significance of the interaction of the LL with this receptor is gained from putting together the proposed evolutionarily relationships among *Echinococcus* species (Nakao et al., 2013a, 2013b) with their preferred intermediate host species and metacestode anatomical locations (Fig. 2). Rodents are the probable ancestral hosts for the genus *Echinococcus*, as well as for the whole of the taenids (Hoberg et al., 2000). In agreement, the two most basal *Echinococcus* species (*E. vogeli* and *E. oligarthra*) have rodents as intermediate hosts (D'Alessandro and Rausch, 2008; Thompson and Jenkins, 2014). After the divergence of the remaining species, extensive diversification of intermediate

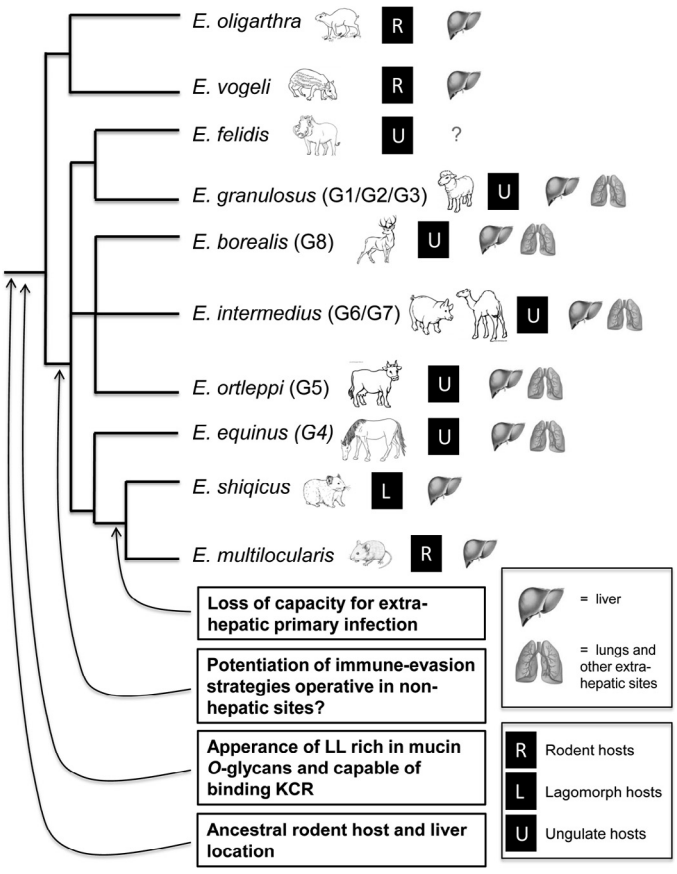


**Fig. 2.** Hypotheses in relation to the interaction of the LL with host immunity in the context of the evolution of the genus *Echinococcus*. The evolutionary tree shown represents topological relationships only, and is based on that built by Nakao et al. from selected nuclear genes (Nakao et al., 2013a, 2013b), with the following changes: (i) a node with bootstrap value under 50 was collapsed; (ii) *E. canadensis* genotypes G6 and G7 have been given the denomination *E. intermedius* (Thompson and Jenkins, 2014); (iii) *E. canadiensis* genotype G8 was given the denomination *E. borealis* (Lymbery et al., 2014). *E. canadiensis* (genotype G6) is omitted, as it was not represented in the original tree, but on the basis of mitochondrial genome phylogeny, it can be placed in the *E. borealis*/*E. intermedius*/*E. ortleppi* clade (Nakao et al., 2013a, 2013b). Preferred intermediate host species and anatomical locations of the larvae are shown. The ancestral condition of the liver location is supported by the fact that the liver is also the most frequent infection site for the larva of *Versteria mustelae* (Loos-Frank, 2000), considered to be the sister genus of *Echinococcus* within the taenids (Nakao et al., 2013a, 2013b). The appearance of the LL in the *Echinococcus* lineage after the split with the *Taenia* branch is supported by: (i) the carbohydrate-rich surface structures of *Taenia* metacestodes being based on N-glycans, and therefore not homologous to the LL (Díaz et al., 2011a, 2011b), and (ii) no homologs of the deduced LL apomucins being found in the *T. solium* or *H. microstoma* genomes (Tsai et al., 2013).

host species appears to have occurred, including the colonization of various ungulates. Within this group of species, only the line leading to the sister species *E. multilocularis* and *E. shiqicus* would have switched back to small intermediate hosts (rodents and lagomorphs respectively) (Gottstein and Hemphill, 1997; Xiao et al., 2006). With respect to anatomical location, the whole genus has the liver as the preferred infection site (Brunetti and White, 2012; D'Alessandro and Rausch, 2008; Gottstein and Hemphill, 1997; Thompson and Jenkins, 2014; Xiao et al., 2006). For the two most basal species (*E. vogeli* and *E. oligarthra*), the liver is essentially the only infection site, and extra-hepatic locations are almost anecdotal (D'Alessandro and Rausch, 2008). In contrast, infection of large mammals, and especially of ruminants (in *E. granulosus sensu lato*), is associated with substantial proportions of extra-hepatic sites (Tenhaeff and Ferwerda, 1935; Thompson, 1995). This may relate to the larger diameter of the lymphatic vessel (lacteal) of the jejunal villi in ruminant than in non-ruminant species, which makes *E. granulosus* oncospheres more likely to be carried in the lymph, instead of the portal circulation (Heath, 1971). Consistent with this general picture, in the two species that would have adapted back to infecting small mammals, extra-hepatic primary infections are again very rare (although *E. multilocularis* can from the liver, invade other organs) (Gottstein and Hemphill, 1997; Xiao et al., 2006). In sum, the most likely scenario is that the liver is the ancestral infection site, and concomitant with adaptation to large mammals there was adaptation to extra-hepatic sites in addition to the liver.

The LL-KCR interaction is surely present in *E. vogeli*, as the LL of this species binds strongly the lectin PNA (Ingold et al., 2001), with specificity for non-decorated core 1 (Gabius et al., 2011), a known KCR target (Coombs et al., 2006). Hence in all likelihood it was very early in the evolution of the genus *Echinococcus* that an *O*-glycan-dominated LL capable of binding liver-specific host receptors developed. In this context, the interaction between the LL sugars and the KCR would have been conserved in species using non-rodent intermediate hosts because: (i) constraints associated with LL structure would have made drastic changes in the glycans unfavorable and (ii) the promiscuity of the KCR with respect to Gal/GalNAc-terminated structures (Coombs et al., 2006) meant that slight changes in glycan structure (as those that separate *E. granulosus* and *E. multilocularis*) did not abrogate the interaction. As complementary or alternative explanations, it cannot be ruled out that non-rodent liver macrophages express (non-KCR) lectins that also bind Gal/GalNAc-terminated glycans (Falasca et al., 1996) and/or that LL materials interact with hepatocytes via the asialoglycoprotein receptor, expressed throughout mammals (Grewal, 2010). Of course, the KCR-LL interaction is probably operative in the extant species that infect rodents, making the analysis of *E. multilocularis* infection in KCR gene-deficient mice worthwhile.

While the pattern of response of Kupffer cells to LL materials is not known, our group is studying the response of "non-liver" macrophages and dendritic cells (DCs). For this purpose, we are mainly using bone marrow-derived cells, as well as the *in vivo* response of peritoneal cells to injected particles. The main initial conclusions (Casaravilla et al., 2014) are that the LL particles do not elicit cytokines from macrophages or DCs, indicative of absence of TLR agonists in them, and that they bias the responses to added TLR agonists. Although the cytokine bias can be said to be anti-inflammatory, as IL-10 is increased and IL-12 is decreased, in terms of co-stimulatory molecules it is a mixed bag, as CD86 expression is increased (and to some extent, induced by LL in the absence of TLR agonists). Thus the phenotypes elicited are best described as "semi-mature" (Lutz, 2012). The effects on cells depend on contact, and they are redundant with those of the actin polymerization inhibitor cytochalasin D. Our unpublished data indicate that the effects on DC and macrophages are wide ranging in terms of cellular functions; we are currently analyzing impacts on cellular signaling, cell proliferation, and energy metabolism. The effects on cytokines and co-stimulatory molecules were not abrogated by oxidation of carbohydrates in the LL particles using periodate (Casaravilla et al., 2014). This fits well with the picture drawn by the lectin-binding experiments mentioned earlier (Hsu et al., 2013), suggesting that non-liver DCs/macrophages may not have lectin receptors that bind the LL sugars. This would of course complement an affinity for the KCR in terms of localizing LL particles to Kupffer cells and away from conventional antigen-presenting cells. It must however be stressed that this is only a suggestion that requires experimental testing. As further notes of caution, the lectin MGL (CLEC10A; expressed in non-liver DCs and macrophages (van Kooyk et al., 2014)) gave borderline binding to the LL (Hsu et al., 2013), soluble recombinant lectins may not mimic the specificity of the native cell-surface receptors, and periodate does not oxidize internal monosaccharide residues with 1–3 linkages, as those in the Galβ1–3 chains

of the *E. granulosus* LL. Removal of the calcium Ins$P_6$ deposits also does not appear to make a difference in terms of the cellular responses (our unpublished results).

In contrast with the observation using periodate, reduction of disulphide bonds in the LL material did cause a decrease in all the effects observed on DCs (Casaravilla et al., 2014). This treatment was previously known to loosen the mucin meshwork (without *per se* causing its solubilization) (Casaravilla and Díaz, 2010), and we reason that it must target inter-molecular bonds between N-terminal extensions of the cysteine-containing MUC-2 family members mentioned. The possibility that this N-terminal extension (as homodimerized peptide) was an agonist engaging a receptor on host cells was not supported by experiments employing synthetic peptides (our unpublished observations). We now favor the idea that the LL conditions DCs and macrophages in contact-dependent fashion by virtue of properties at the supramolecular (material) level. This is in line with various observations that indicate that phagocytic cells sense a range of physical properties of their targets, in addition to clues conveyed via conventional ligand–receptor interactions (Flach et al., 2011; Underhill and Goodridge, 2012).

The evolutionary context previously given also suggests that colonization of large mammals by *Echinococcus* should have been accompanied by expansion of immune-evasion strategies useful in extra-hepatic sites. We see the conditioning of non-liver-specific DCs and macrophages by *E. granulosus* LL materials (Casaravilla et al., 2014) as belonging to this category of adaptation (Fig. 2). As non-organ-specific immune evasion mechanisms would be useful also in the liver, they may well be found (though possibly in less powerful versions) in the rodent-infecting species, both phylogenetically basal (e.g. *E. vogeli*) and non-basal (e.g. *E. multilocularis*). Finally, these non-liver-specific evasion mechanisms certainly include LL-independent elements: for example, soluble secretions from the *E. multilocularis* larva condition DCs in a tolerogenic direction (Nono et al., 2012).

## 5. Conclusions and outlook

Important insights on the LL have been made possible by the genomic and transcriptomic data newly available. Further insights, including inter-species contrasts with respect to Ins$P_6$ synthesis/translocation, will be possible once higher quality, more thoroughly annotated, and/or more easily searchable data sets become available. This includes a desirable homogenization/correspondence between the two gene nomenclatures now existent for *E. granulosus* (Tsai et al., 2013; Zheng et al., 2013). Proteomic data, which are a need, represent a challenge that may only be surmounted with participation of the best expertise available in mucin proteomics.

The clarification of the phylogeny of the genus *Echinococcus* (Lymbery et al., 2014; Nakao et al., 2013a, 2013b; Thompson and Jenkins, 2014) contributes importantly to understanding the LL. Additional data on the *E. vogeli* LL (Ingold et al., 2001) would be of much help, allowing in particular inference of characteristics of the ancestral LL. Another important advance has been to place the LL, and consequently larval *Echinococcus* as a whole, in the context of the host organ they evolved to be adapted to, i.e. the liver. Although the results that lead to this realization are only biochemical (Hsu et al., 2013), we believe that there is much promise in studying effects of the LL on liver-specific immune cells. In addition, our current/planned studies on the (possibly receptor-independent) effects of the LL on non-liver-specific immune cells can be anticipated to lead to very interesting insights.

## Acknowledgements

This work was funded by Wellcome Trust Project Grant 092752 (to AD and JEA). JEA is also funded by the MRC (MR/K01207X/1) (U.K.).

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