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Short communication

The surface carbohydrates of the *Echinococcus granulosus* larva interact selectively with the rodent Kupffer cell receptor

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ABSTRACT

The larvae of the cestodes belonging to the genus *Echinococcus* dwell primarily in mammalian liver. They are protected by the laminated layer (LL), an acellular mucin-based structure. The glycans decorating these mucins constitute the overwhelming majority of molecules exposed by these larvae to their hosts. However, their decoding by host innate immunity has not been studied. Out of 36 mammalian innate receptors with carbohydrate-binding domains, expressed as Fc fusions, only the mouse Kupffer cell receptor (KCR; CLEC4F) bound significantly to the *Echinococcus granulosus* LL mucins. The receptor also bound the *Echinococcus multilocularis* LL. Out of several synthetic glycans representing *Echinococcus* LL structures, the KCR bound strongly in particular to those ending in Galα1-4Galβ1-3 or Galα1-4Galβ1-4GlcNAc, both characteristic LL carbohydrate motifs. LL carbohydrates may be optimized to interact with the KCR, expressed only in liver macrophages, cells known to contribute to the tolerogenic antigen presentation that is characteristic of this organ.

The larvae of the cestodes belonging to the genus *Echinococcus* colonize mammalian internal organs, primarily the liver. Larval *Echinococcus granulosus*, the causative agent of cystic echinococcosis (hydatid disease), which is the main focus of this article, can develop in other organs in addition to liver. It infects many ungulate species (including in particular livestock animals) and accidentally humans. This larva (hydatid) is a fluid-filled, bladder like, unilocular structure. Larval *Echinococcus multilocularis*, the causative agent of alveolar echinococcosis, infects rodents (and accidentally, humans) and develops almost exclusively in the liver as a labyrinth of interconnected vesicles. In all cases, *Echinococcus* larvae are bounded by a larval wall comprising a thin inner layer of cells (germinal layer) and an outer acellular, carbohydrate-rich coat called the laminated layer (LL) (reviewed in [1,2]). The LL, which is specific to the genus *Echinococcus*, is essentially a meshwork of highly glycosylated mucins [3]. For *E. granulosus*, the glycans decorating the LL mucins have been studied [4,5]. They are mucin-type *O*-glycans composed only of galactose (Gal), N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc), and based on either core 1 (Galβ1-3GalNAc1α-Ser/Thr) or core 2 (Galβ1-3(GlcNAcβ1-6)GalNAc1α-Ser/Thr). The core Gal residue can be substituted with a variable number of Galβ1-3 residues, thus giving rise to a $(Gal\beta1\text{-}3)_n$ "main chain" (defined as comprising the mentioned core residue). This chain can be capped with a single Galα1-4 residue. In addition, the core 2 GlcNAc residue can be decorated with the Galα1-4Galβ1-4 disaccharide, thus giving rise to the P_1 blood antigen motif (Galα1-4Galβ1-4GlcNAc). Also, the main chain can be ramified with GlcNAcβ1-6 residues, which in turn can be decorated with Galα1-4Galβ1-4, thus again giving rise to P_1 motifs. Glycans related to the above are found in a LL fraction from *E. multilocularis* [2,6]. Comparison of the two sets of glycans suggests an apparent species difference, as capping with Galα1-4 appears to take place in *E. multilocularis* directly on the core 1 Gal residue, while in *E. granulosus* it happens only on chains comprising at least two Galβ1-3 residues. Thus the LL glycans in *E. granulosus* are longer than those in the *E. multilocularis* fraction [2,4–6].

Abbreviations: ASGR, asialoglycoprotein receptor; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; IIRs, innate immune receptors; KCR, Kupffer cell receptor; LL, laminated layer; MGL, macrophage galactose-specific lectin.

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Under normal conditions in *E. granulosus* infections (i.e. excluding the cases of hydatid rupture), the parasite components in contact with the host immune system are only the soluble secreted molecules (so far largely uncharacterized) and the LL. The LL mucins have only very short non-glycosylated domains [7], and the LL is apparently devoid of non-mucin proteins [3]. In consequence, the relatively simple glycome of the LL constitutes the major part of the "identity card" that this parasite presents to the host immune system. Decoding of pathogen- and, more generally, microorganism-derived molecules by the innate immune system shapes the type of response mounted by the adaptive immune system in each case. The interaction of microorganism-derived carbohydrates with host lectin receptors is an important part of this decoding process. This applies to platyhelminth pathogens such as the well-studied schistosomes [8]. Further, in the genus *Taenia* of larval taeniids, carbohydrates are known to deliver signals to innate immunity [9]. Hence, the identity of the host innate lectin receptors that bind the LL is a central question in the immunology of larval *Echinococcus* infections. Importantly, these infections are characterized by the induction of tolerogenic circuits in the hosts' immune systems [1,10–12], through mechanisms that largely remain unexplored at the molecular level. We have commenced to address the decoding of the LL carbohydrates by host innate lectins, with the help of a substantial panel of recombinant innate immune receptors (IIRs), expressed as human IgG_1 Fc fusions, and therefore in dimeric presentation [13].

A soluble preparation containing the whole of the *E. granulosus* LL mucins was coated onto ELISA plates and probed with the recombinant IIRs. A total of 40 IIRs were assayed, of which 36 contain a known carbohydrate recognition domain, and 31 belong to the C-type lectin family (Fig. 1). All the receptors are of human origin, except for one murine receptor (Supplementary Table S1). The monoclonal antibody E492, which recognizes the P_1 motif mentioned above [5], was used to confirm adsorption of the LL mucins to the plates, while human IgG1 was used to estimate the level of non-specific binding. Strikingly, among all the IIRs tested, only the mouse Kupffer cell receptor (mKCR; systematic name CLEC4F) gave a signal that was clearly in excess of that of IgG1 (OD_{450} ~1.1 vs ~0.3) (Fig. 1). Another five lectins, namely asialoglycoprotein receptor isoform 1 (ASGR-1; CLEC4H1), macrophage galactose-specific lectin (MGL; CLEC10A), FCN2 (L-Ficolin), NCR2 (NKp44) and NCR3 (NKp30), gave borderline signals (OD_{450} ~ 0.25–0.3). The possible biological significance of these interactions should be further analyzed using the native receptors and/or cells expressing them, as our recombinant system cannot be expected to imitate avidity effects, potentially very important in recognition of the LL mucins. In contrast to the results with the LL mucins, 9 out of 17 IIRs show clear binding to the polysaccharides extracted from Reishi, a fungus often used as dietary supplements, in the first application of the same profiling system [13].

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As expected, KCR binding to the LL mucins was calcium-dependent (Fig. 2a). The binding was specific, as it could be outcompeted by excess solubilized LL mucins. LL material subjected to strong non-specific proteolysis, which leaves only the highly glycosylated mucin domains [3], was as effective as a competitor as untreated material (Fig. 2b), confirming that the KCR binds the mucin glycans. The KCR bound to the LL mucins of *E. multilocularis* at least as strongly as to those of *E. granulosus* (Fig. 2c). In fact, the estimated apparent affinity was higher for *E. multilocularis* LL than for *E. granulosus* LL (Kd ~4 vs Kd ~13, respectively). Again, previous proteolysis caused very little change in KCR binding, for both species. Given the well-known affinity of the KCR for sugars terminated in Gal (and GalNAc) [14,15], it is not surprising that the receptor bound to the Gal-rich *Echinococcus* LL glycans. Further, sugars presented as *O*-glycopeptides are better KCR targets than the same sugars carried on artificial spacers, suggesting that mucin *O*-glycans (endogenous or not) are likely KCR counterparts *in vivo* [14]. In addition, as glycans on the LL mucins are probably presented in a very dense array [2,7], bivalent interactions with the chimeric KCR in this study probably contribute to the overall avidities observed. Multimeric and very high avidity interactions are likely with the native KCR, which is trimeric [16], and further presented in multiple copies on the cell surface.

The *O-glycans* present in the *E. multilocularis* LL mucin fraction mentioned above have been synthesized (with either a 2-(trimethylsilyl)ethyl group or a spacer and biotin tag derivatizing the reducing end anomeric hydroxyl) [17,18]. *E. multilocularis* LL *O*-glycans share most non-reducing terminal structures with the *E. granulosus* ones, or in cases are identical to them [2]. We used this tool to study the recognition of the specific carbohydrate motifs present in the LL by the KCR. In a direct binding format using the biotin-tagged glycans, strong signals were obtained for glycans "H" and "I" (Fig. 2d), which share the sequence Galα1-4Galβ1-3GalNAc. A third glycan displaying similar binding strength was glycan "J", which lacks the Galα1-4 "cap" on the main chain but instead carries a Galβ1-4 residue on the core 2 GlcNAc residue, so that a terminal N-acetyl-lactosamine (LacNAc) motif results. Glycan "K", which carries the P_1 motif, showed slightly weaker but still strong binding. Substantially weaker binding was observed for glycan "G", corresponding to non-decorated core 2. A slightly different panel of (nonbiotinylated) glycans was tested in a competition format using plates coated with solubilized *E. granulosus* LL (Fig. 2e). The strongest competitor was glycan "D", corresponding to the same carbohydrate structure as biotinylated glycan "J" already mentioned as a strong binder. The glycans "A" and "F", corresponding to biotinylated glycans "G" and "K", respectively, were weaker competitors (the order of binding strength being inverted with respect to the previous assay format). Finally, only very weak competition was displayed by glycan "L" (Galβ1-3Galβ1-3GalNAc). Although the two assay formats evidently did not measure exactly the same parameters (bivalent interactions can be expected to contribute to the overall signal in the direct binding format only), and not all glycan structures were represented in both (biotin-tagged and non-tagged) panels, an overall picture results as follows. Out of the *Echinococcus* LL glycan motifs assayed, the KCR binds most strongly to terminal LacNAc and to the Galα1-4Galβ1-3GalNAc sequence. The Galα1-4Galβ1-4GlcNAc trisaccharide (P_1 motif) is a slightly weaker target, while terminal Galβ1-3Galβ is a very weak target. This scenario is in broad agreement with published data obtained by glycan array [14,15] showing that (i) both *O*-glycans and *N*-glycans carrying single or repeated terminal LacNAc motifs are among the best KCR targets identified, (ii) the disaccharide Galβ1-3Galβ is not a KCR target. However, the binding to the glycan carrying the P_1 trisaccharide observed by us contrasts with the lack of binding observed previously [14] to the same trisaccharide present in a different context (i.e. directly bound to a spacer, as opposed to being part of a core 2-based *O*-glycan). The previous glycan array data additionally indicate that KCR binds to glycopeptides carrying the non-decorated core 1, which is an abundant *Echinococcus* LL glycan [2,4,6], but is not represented in our panel. Conversely, the glycan arrays against which KCR was previously tested do not include structures with the Galα1-4Galβ1-3GalNAc sequence, identified as a good KCR ligand by our data. In sum, the binding of the KCR to the native *Echinococcus* LL mucins must be based on the recognition of: (i) non-decorated core 1 (as suggested from the mentioned glycan array data), (ii) the P_1 motif, and (iii) capped main chain structures, terminated in Galpα1-4Galβ1-3GalNAc/Gal. With respect to this third contribution, it is worth noting that our