Inflammatory potential of particles from the Echinococcus granulosus laminated layer

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Abstract

Cystic echinococcosis is caused by the tissue-dwelling larva (hydatid) of *Echinococcus granulosus sensu lato*. A salient feature is this larva is being protected by the acellular laminated layer (LL), made up of mucins and calcium inositol hexakisphosphate (Ins P_6). As the parasite grows, the LL sheds abundant particles that can accumulate in the parasite's vicinity. Although foreign particles accumulating in tissues usually cause inflammation, the LL displays adaptations for minimising various host responses, and *in vivo* evidence of inflammation induced by LL particles is essentially lacking. In this work, we show that LL particles injected i.p. at a dose of 225 μg dry mass per mouse cause infiltration of eosinophils, neutrophils and monocytes/macrophages as well as disappearance of resident (large peritoneal) macrophages. The calcium Ins P_6 component was dispensable for these responses. Oxidation of the mucin carbohydrates caused decreased recruitment of neutrophils but the carbohydrate-oxidized particles caused cell influx nonetheless. The control of local granulomatous inflammation is key for survival of this larva. Therefore, our results suggest that *E. granulosus* must deploy mechanisms to avoid excessive local build-up of LL particles (such as targeting particles to liver Kupffer cells; *Infect Immun* 91:e0003123) and/or to condition the recruited cells towards immune-regulatory phenotypes.

Introduction

The larval stage of the cestode parasites in the *Echinococcus granulosus sensu lato* species cluster cause cystic echinococcosis (CE) in humans and livestock species (1, 2). The larva (hydatid) usually grows within host organ's parenchymae and is outwardly protected by the massive acellular laminated layer (LL) (3–6). The LL is composed of a mucin meshwork and deposits of the calcium salt of inositol hexakisphosphate (InsP_6) (5, 7–10). Larval growth is accompanied by shedding of LL particles, which in natural infections are present in large amounts in the host tissues in the parasite's vicinity (11). In experimental intraperitoneal infections in mice, the shed parasite materials are observed in the peritoneal cavity (i.e. the infection site) and also in liver Kupffer cells, implying that the materials circulate systemically (12). LL material uptake by Kupffer cells is mostly dependent on the lectin receptor Clec4F, the only innate immune receptor known to bind the LL mucin glycans (12, 13).

The local inflammatory reaction in CE is usually subdued, and vigorous inflammation when present (especially with a granulomatous profile) harms the parasite (14). The properties of the LL are thought to contribute to local inflammatory control (2–4, 15–19). Indeed, LL particles used as sole stimulus do not elicit inflammatory cytokines from dendritic cells or macrophages, and they moderate the responses to TLR agonists used as co-stimuli (16, 18). LL particles also inhibit macrophage proliferation induced by exogenous IL-4 or M-CSF (19). However, LL particles (freed of calcium InsP_6) injected i.p. at a dose of 30 μg per mouse cause considerable disappearance of resident peritoneal cavity macrophages together with marginal recruitment of inflammatory macrophages (18), i.e. two responses typical of inflammatory particulate stimuli (20–22). Therefore, LL particles may have the potential to elicit inflammation, especially at higher doses than those used in our previous study (18). In this work, we have assessed cell recruitment after i.p. injection

of LL particles at a dose 5-fold higher than the highest dose used in our previous work, and determined the contributions to the process of the major components of the LL, namely calcium Ins$P_6$and mucin glycans.

Materials and Methods

LL particles. Aqueous suspensions of LL particles in the tens to hundreds of μm (largest dimension) range were prepared from hydatid walls obtained from cattle naturally infected with *E. granulosus sensu stricto* from Uruguay. Parasite samples were genotyped by amplification and sequencing of mitochondrial cytochrome c oxidase according to (23); all samples in the study corresponded to genotype G1. Particles were prepared as in (16), using freeze-drying for the dehydration step and applying the modification described next. $CaCl_2$ (0.5 mM) was included in the initial washes and in the rehydration step (omitting EDTA) in order to preserve the calcium InsP_6 deposits (9, 24). This yielded a preparation that contains all the components found in the native LL, which we called "wpLL" (from "*w* hole *p* articulate*l* aminated *l* ayer"). A fraction of wpLL was treated with EDTA-containing buffer so as to obtain the particulate preparation that contains the mucin meshwork but lacks calcium InsP_6, which we previously termed pLL (16). A fraction of pLL was treated with sodium periodate in order to destroy the terminal monosaccharide residues of the mucin glycans followed by sodium borohydride to reduce to alcohols the aldehyde groups formed ("pLL periodate"), or treated with sodium borohydride only ("pLL mock") (16). All final materials were kept as suspensions in sterile PBS (with 0.5 mM $CaCl_2$ in the case of wpLL) containing antibiotic/antimycotic, at 4°C for 6 months at most (16). The dry mass content of the final preparations was determined as in (16). Precautions to exclude endotoxin contamination were applied as in (16). The final materials were assumed not to contain biologically significant levels of endotoxins on the basis of extensive experience with previous batches in terms of negative LAL tests and lack of elicitation of inflammatory cytokines from bone marrow-derived dendritic cells (16, 18, 25).

Mice and particle injections

Female C56BL/6 mice were obtained from DILAVE (MGAP, Uruguay) and kept at the Instituto de Higiene animal house. Animals aged 8 – 12 weeks were inoculated intraperitoneally (i.p.) with 225 μg or wpLL or 150 μg of pLL, pLL periodate or pLL mock diluted into 200 μL of sterile endotoxin-free saline, or injected with vehicle only. The doses of wpLL and pLL were chosen so as to correspond to equal dry masses of the mucin component, since calcium InsP_6 represents one-third of the LL dry mass (9). Mice were killed 24 h later and peritoneal cavity cells retrieved by lavage for their analysis by flow cytometry. The procedure was approved by the Universidad de la República's Animal Experimentation Committee (CHEA; protocol 101-900-000441-16).

Flow cytometry.

Peritoneal lavage cells were surface stained with the antibodies listed in Supplementary Table 1. Flow cytometry data were acquired in a FACS Canto II Cytometer (BD), at the Instituto de Higiene, Universidad de la República, and then analysed using the FlowJo software package. Single cells were selected on the basis of FSC-H and FSC-A, and then viable cells selected using the Live/Dead Green^{TM} viability probe. Within live cell singlets, eosinophils and neutrophils were defined as SiglecF^{hi} and Ly6G^{+} cells respectively. B cells (CD19^{+}) were excluded from SiglecF^{-} Ly6G^{-} cells. SiglecF^{-} Ly6G^{-}CD19^{-} cells were divided into monocytes (Ly6C^{hi}) and non-monocytes (Ly6C^{-}). The second category was further divided into small peritoneal macrophages and dendritic cells (SPM/DC; MHCII^{+}F4/80^{-}), MHCII^{-}F4/80^{med} macrophages, and large peritoneal macrophages (LPM; MHCII^{-} F4/80^{high}) (26, 27). The gating strategy is summarised in supplementary Figure S1.

Statistical analysis .

Data were analysed by the non-parametric method of Kruskal-Wallis (28), followed by the post-hoc multiple comparison test described by Conover (29) and the Benjamini-Hochberg correction for controlling the false discovery rate (30). The symbols *, ** and *** represent p-values less than 0.05, 0.01 and 0.001 respectively.

Results

In order to obtain information on the inflammatory potential of LL particles and the impact of the calcium $InsP_6$component on this potential, we injected i.p. LL particles containing calcium $InsP_6$ (wpLL) or freed from this component (pLL). Injection of the LL materials at the dose under study brought about only a trend towards higher total numbers of cells in the cavity, but did cause significant influxes of eosinophils, neutrophils and monocytes (Fig. 1a - d). An increase in the number of SPM/DC was also observed, which reached significance for pLL but was only a trend for wpLL (Fig. 1 e). In all likelihood, this increase was mostly accounted for by SPM, which on the basis of previous studies of macrophage recruitment to the body cavities (20–22, 26, 31) likely arose from recruited monocytes. We also detected significant increases in MHCII$^-$ F4/80^{med} macrophages (Fig. 1 f). These cells are deduced to derive from SPM on the basis of previous studies on inflammatory stimuli injected into the peritoneal cavity (26). In agreement, this population did not express the long-term resident macrophage marker Tim4 whereas approximately 4/5 of the major resident macrophage population (the F4/80^{hi} LPM) expressed this marker as expected (data not shown) (32). LPM numbers decreased sharply in response to LL materials (Fig. 1 g), as expected when an inflammatory stimulus is injected into the peritoneal cavity (22, 33, 34). Notably, the presence of the calcium $InsP_6$ component did not enhance cell recruitment nor disappearance of LPM, and it actually generated trends towards weaker recruitment of all the inflammatory cell types studied (Fig. 1 a – f).

We next tested the contribution of the mucin glycans to the inflammatory cell recruitment and disappearance of resident macrophages observed. For this purpose, we injected pLL, which was untreated, treated with periodate to destroy the terminal monosaccharides (pLL periodate), or subjected to a mock treatment (pLL mock). In comparison to mock-treated particles, periodate-treated particles tended to generate weaker cellular influx, a trend that reached significance only for neutrophils (Fig. 2 a – e). Periodate-treated particles were nonetheless able to cause significant increases in eosinophils, neutrophils and MHCII$^-$ F4/80^{med} macrophages. LPM disappearance was unaffected by periodate treatment (Fig. 2 f).

Discussion

This work shows that LL particles can elicit cell recruitment. We do not know how the dose used in this work compares with the amounts of particles accumulating locally in the infection setting. However, since this infection can last for years or decades (limited by the host's lifespan), high local doses of LL particles must eventually be reached unless the materials find their way into systemic circulation and are captured elsewhere in the body. Circulation of LL materials and their capture by Kupffer cells do take place, on the basis of our results in intraperitoneal mouse infections (12). Therefore, our present results suggest that at least part of the evolutionary advantage of LL materials being mopped up efficiently by Kupffer cells may be to avoid the excessive build-up of the materials and consequent cell recruitment to the parasite's vicinity. Complementarily, it is possible that a certain level of host cell recruitment is acceptable or beneficial to the parasite, especially if the recruited cells are induced to adopt suppressive phenotypes. Indeed, cells recruited to the parasite's vicinity in intraperitoneal mouse infections display predominantly suppressive phenotypes (27); the possible role of LL particles in this phenomenon is under study in our group.

It is noteworthy that the calcium $InsP_6$component, which represents 1/3 of the mass of the LL, makes no contribution to cell recruitment, and it actually tends to diminish it. In terms of the generation of the major complement anaphylatoxin C5a by LL particles exposed to normal human serum, calcium $InsP_6$ has no net effect (15), although the analogous experiment in mouse serum has not been carried out. The present results suggest that in the mouse tissue milieu *in vivo* , calcium $InsP_6$ also has no net effect or has a weak inhibitory effect on cell recruitment in general.

LL particles elicited cellular influx and resident macrophage disappearance even if their mucin glycans were oxidised so as not to be able to engage carbohydrate receptors (15, 16). In possible agreement, out of a panel of mammalian innate immune lectin receptors, only Clec4F (which in rodents is expressed only in Kupffer cells (35, 36)) bound clearly to the LL mucins (13). The elicitation of host responses by the LL independently of its major molecular-level motifs ($InsP_6$ and mucin glycans, as well as a predicted major exposed peptide sequence) was previously observed by our group during *in vitro* work with DC (16, 18). Together with other results, this led us to propose that LL particles may impact on DC and other innate cells (except by Kupffer

cells) by receptor-independent mechanisms (3, 18, 25). The present results are in broad agreement with this previous proposal.

The diminished capacity to recruit neutrophils (and possibly additional cell types) shown by the periodate-treated material may be explained by natural anti-carbohydrate antibodies binding the LL mucins and causing some degree of classical complement pathway (CCP) activation. In broad agreement, we previously observed that LL particles (free of calcium Ins$P_{\ 6}$ and of infection-induced antibodies) cause some CCP activation in normal human serum that must be ascribed to natural antibodies (15). The LL mucin glycans may play a stronger role in promoting cell recruitment in the infection context, in which they are targets of specific antibodies that may further activate the CCP (3, 15).

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Figure legends

Figure 1. LL particles can give rise to cell recruitment *in vivo* and the calcium InsP_6 component does not contribute to the response. Mice were injected i.p. with LL particles containing their intrinsic components mucins and calcium Ins$P_{\ 6}$ (wpLL) at a dose of 225 μg per mouse or with LL particles containing only their mucin component (pLL) at a dose of 150 μg per mouse (which corresponds to the mass of mucins present in a 225 μg dose of wpLL), or with vehicle only (saline). Total cells and selected cell populations in the peritoneal cavity were quantitated by a combination of cell counter and flow cytometry 24 h later.

Figure 2. The intact mucin glycans are mostly dispensable for inflammation induced by LL particles. Mice were injected i.p. with LL particles containing only their mucin component at a dose of 150 μg per mouse or with vehicle only (saline). The LL particles were untreated (pLL), subjected to a mock treatment (pLL mock) or treated with sodium periodate for oxidation of terminal monosaccharide residues (pLL periodate). Total cells and selected cell populations in the peritoneal cavity were quantitated by a combination of cell counter and flow cytometry 24 h later.

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