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Inefficient and abortive classical complement pathway activation by the calcium inositol hexakisphosphate component of the *Echinococcus granulosus* laminated layer

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ABSTRACT

Persistent extracellular tissue-dwelling pathogens face the challenge of antibody-dependent activation of the classical complement pathway (CCP). A prime example of this situation is the larva of the cestode *Echinococcus granulosus sensu lato*, causing cystic echinococcosis. This tissue-dwelling, bladder-like larva is bounded by a cellular layer protected by the outermost acellular "laminated layer" (LL), to which host antibodies bind. The LL is made up of a mucin meshwork and interspersed nano-deposits of calcium inositol hexakisphosphate (calcium InsP_6). We previously reported that calcium InsP_6 bound C1q, apparently initiating CCP activation. The present work dissects CCP activation on the LL. Most of the C1 binding activity in the LL corresponded to calcium InsP_6, and this binding was enhanced by partial proteolysis of the mucin meshwork. The remaining C1 binding activity was attributable to host antibodies, which included CCP-activating IgG isotypes. Calcium InsP_6 made only a weak contribution to early CCP activation on the LL, suggesting inefficient C1 complex activation as reported for other polyanions. CCP activation on calcium InsP_6 gave rise to a dominant population of C3b deposited onto calcium InsP_6 itself that appeared to be quickly inactivated. Apparently as a result of inefficient initiation plus C3b inactivation, calcium InsP_6 made no net contribution to C5 activation. We propose that the LL protects the underlying parasite cells from CCP activation through the combined effects of inefficient permeation of C1 through the mucins and C1 retention on calcium InsP_6. This mechanism does not result in C5 activation, which is known to drive parasite-damaging inflammation.

1. Introduction

Pathogens that establish themselves in tissue sites of immune-competent mammals develop sophisticated immune evasion mechanisms. Helminths are the largest and most complex such pathogens (Díaz and Allen, 2007; Maizels and Smits, 2018). The larval stage of the cestode helminth *Echinococcus granulosus sensu lato* (*s.l.*) constitutes a prime example of this situation. This bladder-like structure (called hydatid cyst, or more correctly hydatid) can attain tens of cm in diameter within mammalian internal organs, most commonly liver or lungs. Strikingly, the hydatid usually grows surrounded by minimal host inflammation, in spite of eliciting detectable antigen-specific responses (Díaz et al., 2011a, 2016; Díaz and Sagasti, 2018).

An obvious component of host immunity that the hydatid needs to deal with is complement. This extracellular pro-inflammatory cascade composed mostly of soluble proteins can activate on both cellular and acellular surfaces (Merle and Church, 2015a; b). Complement can be activated *via* the classical, alternative or the lectin pathways (CCP, ACP and LCP respectively), which converge at the activation and covalent deposition of C3 on surfaces. The CCP, with which this work deals, is activated by antibodies bound to their antigen targets, but also by direct binding of its recognition component C1q to molecules or surfaces presenting certain arrays of negative charge (Almeda and Rosenberg, 1983; Kirschfink and Blase, 1997; Paidassi and Tacnet-Delorme, 2008;

Abbreviations: ACP, alternative complement pathway; CCP, classical complement pathway; fH, factor H; GL, germinal layer; HS, human serum; InsP_6, inositol hexakisphosphate; LCP, lectin complement pathway; VBS, veronal-buffered saline; VBS^{++}, VBS additionally containing Ca^{2+} and Mg^{2+}; LL, laminated layer

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Garlatti and Chouquet, 2010).

The most host-exposed structure of the hydatid is the so-called laminated layer (LL) (Díaz et al., 2011a; Díaz and Fernández, 2015). This is a thick (hundreds of μm to a few mm) acellular coat that protects the underlying cellular layer of the parasite, called germinal layer (GL); LL and GL together constitute the hydatid wall. The LL is permeable to macromolecules, which means that both its inner solvent-exposed surfaces and the outermost (tegumental) surface of the GL are potentially exposed to host complement (Díaz et al., 2011a; b; 2016). LL materials activate complement only weakly (Irigoín and Wurzner, 1996; Ferreira and Diaz, 2001); the pathway(s) involved have not been analyzed.

The LL is made up of two components: an aqueous gel formed by highly glycosylated mucins and interspersed nano-deposits of the calcium salt of *myo*-inositol hexakisphosphate (InsP_6) (Irigoín et al., 2002, 2004; Casaravilla et al., 2006; Díaz et al., 2009; Casaravilla et al., 2010; Lin et al., 2013). InsP_6 is a nucleo-cytosolic metabolite present in all eukaryotes (Raboy, 2003), which in *E. granulosus* has been recruited for contributing to an extracellular structure (the LL). InsP_6 is essentially insoluble under physiological extracellular pH and calcium concentrations (Veiga et al. 2006). The composition of calcium InsP_6, either synthetic or purified from the LL, is $Ca_5H_2L{\cdot}16H_2O$, where L represents fully deprotonated InsP_6 (Casaravilla et al. 2006; Veiga et al. 2006). Treatment of the LL with EDTA-containing buffers extracts InsP_6 as the sole detectable carbon-containing compound and eliminates the calcium InsP_6 deposits visible under the transmission electron microscope without altering the fibrillar mucin meshwork (Irigoín et al., 2002, 2004; Díaz et al., 2011b).

The LL mucin gel is permeable to many host macromolecules, as exemplified by IgG (Coltorti et al., 1974). Strongly bound host IgG is invariably associated with the LL, being found throughout its thickness (Varela-Díaz et al., 1973; Coltorti et al., 1974; Taherkhani et al., 2007; Breijo et al., 2008; Díaz et al., 2011a; Riesle et al., 2014). This IgG is in all likelihood bound to the antigenic mucin carbohydrates (Díaz et al., 2011a; Koizumi et al., 2011; Yamano et al., 2012; Díaz et al., 2015), as it remains bound after solubilization of calcium InsP_6 (Basika et al., 2012). On the other hand, calcium InsP_6, which has an enormous total surface area (Casaravilla et al., 2006), has major potential to interact with host molecules including complement components. We previously observed that synthetic calcium InsP_6 selectively binds C1q from human serum (HS) and deduced that calcium InsP_6 present in the LL triggers CCP activation (Irigoín et al. 2008). In the present work we have examined CCP activation on the LL in depth, taking into account both antibodies and calcium InsP_6 as possible CCP initiators. We have confirmed that calcium InsP_6 present in the LL activates the C1 complex. However, CCP activation initiated in this manner is inefficient in terms of C1 activation and it is controlled at the C3b level so that it does not result in C5 activation.

2. Materials and methods

2.1. Echinococcus granulosus s.l. materials

LL materials used in this work were from *E. granulosus s.l.* human and experimental mouse infections.

The human hydatid materials were obtained from four patients suffering from CE of the liver (2), lung (1) and spine (1). Liver and lung cysts were classified as CE3b according to the WHO Informal Working Group on Echinococcosis (WHO-IWGE) standardized sonographic classification (Brunetti et al., 2010), whereas the classification is not applicable to bone CE, where the larva grows by infiltration (Neumayr et al., 2013). The patients with abdominal and lung CE were born and living in Italy, while the one with bone CE was born and living in Uruguay. All patients received surgery as treatment for the disease, at the San Matteo Hospital Foundation, Pavia, Italy and Hospital Maciel, Montevideo, Uruguay respectively. All patients had signed an informed consent form for storage and scientific use of the biological samples. The materials were divided into two "samples" for experimental purposes, consisting respectively of pooled materials obtained from the liver and lung cysts and material from the spinal cyst.

Samples from mouse infection were retrieved from the peritoneal cavity of Balb/c mice inoculated i.p. with 2000 protoscoleces per mouse 12 months earlier (Mourglia-Ettlin et al., 2016). Hydatids from either host origin were incubated in 20 mM Tris/HCl buffer pH 7.4 containing 135 mM NaCl, 0.5 mM $CaCl_2$, 0.1% w/v Triton X-100 and 0.05% w/v NaN_3 w/v ("stock buffer") to help detach the GL from the LL. The LL was washed extensively with 2 M NaCl 0.5 mM $CaCl_2$ to remove adsorbed host proteins (Díaz et al. 2011a) and then taken back into stock buffer by centrifugation and re-suspension. The LL was converted into a suspension of particles (estimated size of 1 mm x 1 mm x 0.1 mm) with the help of an electric homogenizer (TissueRuptor, Qiagen). This format allows setup of complement binding reactions containing even amounts of LL material. The total dry mass content of the LL suspensions was determined by freeze-drying and weighing aliquots previously taken into water. LL suspensions were stored at 4 °C in stock buffer, and immediately before experiments taken into veronal-buffered saline (VBS) additionally containing 0.15 mM $CaCl_2$ and. 0.5 mM $MgCl_2$ (VBS^{++}) by an appropriate number of rounds of centrifugation and re-suspension.

LL calcium InsP_6 deposits were purified from hydatid walls from natural *E. granulosus s.l.* infections in cattle by alkaline digestion of the mucin component as in (Casaravilla et al. 2006); the composition of the calcium InsP_6 deposits does not vary with host species (Casaravilla et al., 2006).

2.2. Modifications to the LL

For specifically removing the calcium InsP_6 deposits, LL suspensions obtained as above were taken into 20 mM Tris − HCl buffer pH 8.0 containing 10 mM EDTA (1 mL per mg LL dry mass) and incubated for 15 min. Elimination of calcium InsP_6 was verified by the macroscopically obvious change of the LL from opaque to translucent (Irigoín et al. 2004). After treatment, LL suspensions were taken into VBS^{++}, ensuring that EDTA from the previous steps was diluted at least 10^6-fold in the procedure. This LL preparation was called "$LL_{No\ Ins P6}$".

In order to eliminate host antibodies, LL suspensions were subjected to proteolysis using pronase (Sigma P5147; 0.125 mg/mL final concentration) in 20 mM Tris − HCl pH 8.0), overnight at 37° with stirring (Casaravilla et al., 2010, 2014). Then particles were taken into VBS^{++} by centrifugation and re-suspension rounds sufficient to achieve a 10^{11}-fold dilution of pronase from the previous step. Absence of antibodies was verified by SDS-PAGE. This preparation was named "$LL_{No\ Ab}$". The pronase treatment was also applied to $LL_{No\ Ins P6}$ to obtain LL without either antibodies or calcium InsP_6 ("$LL_{No\ Ins P6\text{-}No\ Ab}$").

2.3. Complement reagents

Human serum (HS) depleted in C1q and purified human C1q were purchased from Complement Technologies Inc. In some experiments, C1q-depleted HS was compared with the same serum reconstituted with C1q at its mean physiological concentration (80 μg/mL; (Dodds et al., 1997)). For consistency, C1q-depleted HS reconstituted with C1q was also used in experiments not involving assessment of the contribution of C1q; experiments using non-depleted pooled HS from normal donors prepared in-house yielded similar results (data not shown). Both commercial and in-house prepared HS was flash-frozen in liquid nitrogen and stored at -70 °C until use.

2.4. SDS-PAGE and Western blotting

SDS-PAGE separation was carried out in 10% (analysis of antibodies, C1r, C1s, C4, C3) or 12.5% w/v acrylamide gels (analysis of C1q) under reducing conditions. Components eluted from LL particles (using 2 M NaCl, EDTA-containing buffer or hydroxylamine-containing

buffer as described below) were concentrated prior to analysis by precipitation with trichloroacetic acid (10% w/v final concentration) in the presence of lysozime as a carrier (40 µg per tube). The resulting pellets were washed with cold acetone and re-dissolved in SDS-PAGE sample buffer. For Western blot analyses, samples were transferred onto PVDF and probed with antibodies against human IgG and IgM (Dako P0214 and Sigma A6907 respectively; used together), mouse IgG and IgM (Invitrogen M30107 and M31507 respectively; used together), human C1q A chain (Abcam; ab155052), human C1r, C1s or C1 inhibitor (kindly donated by Robert B. Sim, Oxford, UK), human C4d (ProteinTech; 22233-I-AP), or human C3d (Dako; A0063), followed by peroxidase-coupled sheep anti-rabbit IgG (Calbiochem; 401,393). Blots were developed using ECL Supersignal West Pico chemiluminescent substrate (Thermo Pierce) in the G-Box F3 image capture equipment (Syngene). Quantitation of the integrated intensity of Western blot bands was carried out using the ImageJ software.

2.5. Characterization of antibodies bound to the LL

The IgG and IgM content of LL samples was analyzed by SDS-PAGE and western blotting. In addition, samples were analyzed by direct ELISA strategy allowing detection of antibody subclasses in addition to classes. In this strategy, LL samples were solubilized by sonication (Casaravilla et al., 2010), coated onto ELISA plates (10 µg total dry mass per well, in PBS), and probed with peroxidase-coupled antibodies against immunoglobulin classes/subclasses. For LL samples of human host origin, antibodies against the following human classes/subclasses were used: IgA (Sigma A0295, 1/5000), IgE (Dako P0295, 1/2000), IgM (Sigma A6907, 1/5000), IgG1 (Sigma B6775, 1/1000), IgG2 (Sigma B3398, 1/2500), IgG3 (Sigma B3523, 1/2000), and IgG4 (Sigma B3648, 1/10000). For LL samples of mouse host origin, antibodies against the following mouse classes/subclasses were used: IgA (Santa Cruz sc3793, 1/4000), IgE (Southern Biotec 1110-05, 1/2000), IgM (Invitrogen M31507, 1/3000), IgG1 (Santa Cruz sc2965, 1/6000), IgG2a (Invitrogen A10685, 1/4000), IgG2b (Santa Cruz, sc2971, 1/6000), and IgG3 (Santa Cruz, sc2972, 1/10000). Each antibody class/subclass was considered as present in a sample when the average OD resulting from probing triplicate wells coated with the sample was higher than the average OD + 3 SD of triplicate controls wells (treated only with PBS containing 1% v/v fetal calf serum as blocking agent). In addition, the relative abundances of antibody classes/subclasses present was very roughly estimated. For this purpose, OD values were normalized over OD values resulting from probing replica wells with *Ricinus communis* lectin (RCA 1); this lectin binds to the LL carbohydrates and thus acts as an LL loading control (Casaravilla et al., 2010). For each antibody class/subclass and sample, a ratio was calculated between its normalized OD value and the smallest value in the set; ratios approximately equal to 1, 2 and 3 were assigned the relative abundance indices +, + + and + + + respectively.

2.6. General setup of complement assays

Reactions were carried out in VBS^{++}, in 150 µl volumes in 1.5 mL polypropylene tubes. Reactions contained LL preparations (from human host origin unless indicated) and HS at the dilutions indicated in each case, or in a specified case, purified C1q. The final concentration of non-treated LL ("$LL_{control}$") was 6 mg/mL total dry mass. $LL_{No\ Ins P6}$, $LL_{No\ Ab}$ and $LL_{No\ Ab\text{-}No\ Ins P6}$ were assayed at the matching concentrations; this means that for example in assays comparing $LL_{control}$ and $LL_{No\ Ins P6}$, tubes contain the same concentration of LL mucins and mucin-bound antibodies. Analogously, since 1/3 of the LL dry mass is calcium $InsP_6$ (Casaravilla et al. 2006), purified calcium $InsP_6$ was assayed at 2 mg/mL. Reactions were started by passage of tubes from 0 °C to 37 °C. Tubes were shaken periodically during incubation at 37 °C. Reactions were stopped by passing back to 0 °C and then processed as detailed below for each component analyzed.

2.7. C1q binding

Reactions were set up as described above, using either HS or purified C1q. Then LL particles were washed with VBS^{++} so as to obtain a 10^5-fold dilution of any components not bound to the particles. Any bound C1q was eluted by incubation in 2 M NaCl for 30 min at 4 °C with stirring, followed by centrifugation to recover the corresponding supernatant.

2.8. C1r/C1s binding and activation in absence of C1-inhibitor activity

These reactions were set up as previously described but an additional 4 °C incubation step (30 min with stirring) to allow C1 binding without activation was included before the 37 °C incubation step. After incubation at 4 °C, particles were washed with cold VBS^{++} so that unbound or weakly bound proteins (including C1-inhibitor, C1-INH) would be diluted 10^6-fold. Thus, the subsequent incubation at 37 °C allowed activation of any bound C1 in the absence of C1-INH activity. Then bound proteins including C1 were eluted as described for C1q above and analyzed by Western blotting with C1r and C1s antibodies. C1r activation was calculated for each lane as the quotient between the integrated intensity of the band corresponding to the activated C1r light chain (the antibody used does not detect the heavy chain) and the total integrated intensity associated with C1r (both activated and non-activated). C1s activation was calculated similarly, except that the signal from the bands corresponding to the heavy and light chains in activated C1s (both detected by the antibody) was added together. Both C1r and C1s activation were expressed as percentages. For calculating C1r or C1s binding to the LL, the signal from all the bands associated with each component was added together.

2.9. C1s activation in serum

Reactions were set up and stopped in the general way previously described, SDS-PAGE sample buffer was added, and a representative portion of each reaction (containing both the soluble and insoluble components) was loaded for Western blot analysis. C1s activation was analyzed on the basis of formation of the covalent complex with C1-INH (Sim and Reboul, 1981). The complex, which under reducing conditions contains only the light chain of activated C1s, migrated at the expected molecular mass (137 kDa) and its identity was verified using an antibody against C1-INH (data not shown). A C1s activation index was calculated as the quotient between the integrated intensity of the C1s-C1INH complex band and the sum of the intensity of this band with the band corresponding to non-activated C1s; the index was expressed as a percentage.

2.10. C4 and C3 activation

Reactions were set up and stopped in the general way previously described. Supernatants were separated by centrifugation at 4 °C, added an equal volume of SDS-PAGE sample buffer and analyzed by Western blotting. An index of C4 activation was calculated as the quotient between the sum of the integrated intensities of the bands corresponding to the C4b α chain (85 kDa), iC4b α chain N-terminal fragment (70 kDa) and C4d (45 kDa) over the sum of the previous bands and the band corresponding to the intact C4 α chain (93 kDa). Of note, although iC4b is known to be fast inactivated in the presence of plasma regulators factor I and C4bp (Nagasawa and Ichihara, 1980), it was detected under our assay conditions, which correspond to a fairly high serum dilution (1:60). An index of C3 activation was calculated as the quotient between the integrated intensity of the band corresponding to the iC3b α chain N-terminal fragment (64 kDa) over the sum of the previous band and the band corresponding to the intact C3 α chain (104 kDa). C4 and C3 activation indices were expressed as percentages.

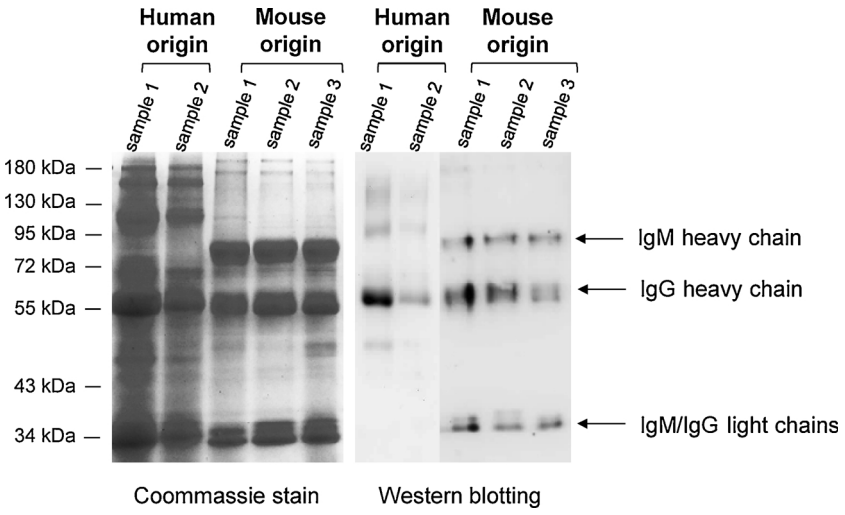

Fig. 1. The LL has strongly bound IgG and/or IgM antibodies. The presence of host antibodies in LL samples of human and mouse host origins used in this study was analyzed by SDS-PAGE (left) and by Western blot (right). Note that weakly adsorbed proteins were washed off the LL samples using 2 M NaCl prior to analysis. Also note that the antibodies against human IgG and IgM used (together) do not recognize the immunoglobulin light chain, unlike the antibodies against mouse IgG and IgM used.

2.11. C4 and C3 deposition and inactivation

Reactions were set up and stopped in the general way previously described. Then particles were washed with a low ionic strength solution (VBS^{++} diluted 1:10) so as achieve a 10^6-fold dilution of serum proteins except those strongly bound. Then C4/C3 products deposited on the LL were eluted in a sequential manner. First, C4/C3 deposited on calcium InsP_6 was recovered by treating with EDTA-containing buffer. Then, C4/C3 deposited on the mucin component was released by treating the remaining particles with hydroxylamine. In more detail, after the reactions pelleted LL particles were added 600 µL per tube of VBS containing 20 mM EDTA (VBS-EDTA) and centrifuged again. The resulting supernatants were added equal volumes of 375 mM hydroxylamine in Tris − HCl buffer pH 8.8 containing 135 mM NaCl and incubated for 45 min at 37 °C to release C4/C3 from any covalent acceptors solubilized by EDTA. The remaining particles were suspended in 150 µL of VBS-EDTA, added an equal volume of 375 mM hydroxylamine in Tris − HCl buffer pH 8.8 containing 135 mM NaCl and incubated for 45 min at 37 °C, followed by centrifugation to recover the supernatant. Indices of C4/C3 deposition were calculated as the sum of the integrated intensities of C4b/C3b and iC4b/iC3b bands (the C4d band was lost because gels were continued to run after exit of the dye front in order to separate well the intact C4 α chain from the α' chain in C4b). In order to be able to compare samples run on two different gels and thus transferred to two PVDF membranes, matching amounts of HS were run in both gels and the intensities of the C4/C3 bands in these lanes were used to normalize all other values across the gels/membranes. The fraction of deposited C4b or C3b that became inactivated at each time point was calculated as the quotient between the signal of the iC4b/iC3b band over the sum of this band with the band corresponding to C4b/C3b. C4b and C3b inactivation were expressed as percentages. In an indicated experiment, deposited C4/C3 were extracted in a single step by incubation in hydroxylamine-containing buffer in similar fashion to what was described above.

2.12. C5 activation

Reactions were set up and stopped in the general way previously described. Supernatants were separated by centrifugation at 4 °C and added PBS containing a protease inhibitor cocktail (Santa Cruz Biotechnology; sc-29130) and additional EDTA to achieve a final concentration of 6 mM EDTA. C5a was measured in supernatants by ELISA (R&D Systems; DY 2037).

3. Results

3.1. The LL has strongly bound antibodies of classes/subclasses that can activate the CCP

In agreement with previous reports (Varela-Díaz et al., 1973; Coltorti et al., 1974; Taherkhani et al., 2007; Díaz et al., 2011a; Riesle et al., 2014), the LL samples used in this work had host IgG even after the stringent washing applied (Fig. 1). In addition, the samples from mouse infections had IgM, consistent with published immunohistochemical data (Breijo et al., 2008). Since there is no evidence of IgM being bound to the LL in the context of natural infections, and not all IgG subclasses activate the CCP (Murphy et al., 2012; Vidarsson et al., 2014; Collins, 2016), we analyzed whether IgG subclasses capable of activating the CCP are bound to the LL. The human infection LL samples did not contain detectable IgG3 (Table 1), the human IgG subclass with highest CCP activation capacity (Vidarsson et al. 2014). However, they contained the moderately or weakly CCP-activating subclasses IgG1 and IgG2 (Table 1). Mouse infection samples contained the complement-activating subclasses IgG2a, IgG2b and IgG3 (Collins, 2016) in addition to IgM (Table 1). Thus, the LL can be expected to activate the CCP *via* bound antibodies.

3.2. Calcium InsP_6 in the LL can bind C1q and activate the C1 complex

We wished to know if native calcium InsP_6 present within the mucin fibrillar meshwork of the LL could bind C1q, as does synthetic calcium InsP_6 (Irigoín et al. 2008). The LL bound C1q from HS (Fig. 2A). This binding was selective, as the electrophoretic pattern of bound proteins was clearly different from that of whole HS (Fig. S1). In addition, binding was quantitatively important, as the three C1q chains were detectable by Coommassie staining (Fig. S1). At least 4/5 of the capacity to bind C1q in the LL was lost upon previous selective removal of calcium InsP_6 (Fig. 2 A; compare lanes marked $LL_{Control}$ and $LL_{No\ InsP6}$ within each host origin). The remaining, InsP_6-independent C1q binding is most probably accounted for by host immunoglobulins, consistent with the results previously shown. C1q was also selectively bound by calcium InsP_6 purified from the LL (Casaravilla et al. 2006) (Figs. 2A and S1). The LL bound C1q in an InsP_6-dependent manner also upon incubation with pure C1q (in the presence of excess irrelevant protein), and a coincident result was obtained using purified calcium InsP_6 (Fig. 2 B); this suggests a direct interaction between C1q and calcium InsP_6.

C1q could conceivably bind to calcium InsP_6 in a mode such that the C1 complex is dissociated. This was not the case, as purified calcium

Table 1
The LL has strongly bound antibodies belonging to classes/subclasses known to activate the CCP. Antibody classes and subclasses present in the LL samples used in the work were assessed by a direct ELISA strategy. For each antibody class/subclass, absence (-) or presence and abundance index (+/++/+++; calculated as explained in the "Materials and Methods" section) is given. Asterisks indicate those human or mouse antibody clases/subclases known to be capable of activating the CCP, the number of asterisks roughly denoting the relative activation capacity (Vidarsson et al., 2014; Collins, 2016).

Human origin		
	Sample 1	Sample 2
IgG1 (**)	+	+++
IgG2 (*)	+	++
IgG3 (***)	–	–
IgG4	+	–
IgA	+	+
IgE	+	+
IgM (****)	–	–

Mouse origin			
	Sample 1	Sample 2	Sample 3
IgG1	+	+	++
IgG2a (**)	+	+	++
IgG2b (**)	+	+++	+++
IgG3 (**)	+	++	+++
IgA	+	+	++
IgE	+	++	+++
IgM (****)	+	+	+

InsP_6 bound the two proteases of the C1 complex, C1r and C1s, upon incubation with HS (Fig. 3 A and B). In agreement, the LL also bound C1r and C1s, and most of the binding was dependent on the presence of calcium InsP_6 (Fig. 3; compare lanes marked $LL_{control}$ and $LL_{No\ InsP6}$ in both A and B). Physiologically, C1 complex activation is controlled by C1 inhibitor (C1-INH) (Sim and Reboul, 1981; Merle et al., 2015a). Under the conditions of the assay, in which C1-INH cannot act, most or all of the C1 complex bound became activated upon incubation at 37 °C (Fig. 3A, B). This suggests that the C1 complex binds calcium InsP_6 in a broadly "canonical" manner.

Previous proteolytic treatment of the LL with pronase eliminated bound immunoglobulins (Fig. S2) without causing solubilization of the mucin meshwork, in agreement with published observations (Casaravilla et al. 2014). Interestingly, the proteolytic treatment enhanced C1 binding detected in terms of bound C1r and C1s (Fig. 3 A, B; compare lanes marked $LL_{control}$ and $LL_{No\ Ab}$). The enhanced binding was entirely dependent on the presence of calcium InsP_6, since a LL sample treated as to eliminate both antibodies and InsP_6 bound no C1 (Fig. 3 A, B; lanes labeled "$LL_{No\ InsP6\text{-}NoAb}$". We reason that the enhancement of C1 binding observed after the proteolytic treatment is due to the loosening of the mucin meshwork by partial proteolysis (Casaravilla and Díaz, 2010), which likely facilitates access of the large C1 complex to the calcium InsP_6 deposits. Pronase-treated LL actually bound more C1 than the matching amount of purified calcium InsP_6 (Fig. 3 A, B; compare lanes marked "$LL_{No\ Ab}$" and "Calcium InsP_6"). This difference is most probably due to purification of the calcium InsP_6 deposits causing their clustering and hence reducing their combined surface area in relation to the deposits present in the native LL (Casaravilla et al., 2006). The previously mentioned observation that no C1 bound to the LL from which both immunoglobulins and calcium InsP_6 had been removed (Fig. 3 A, B; lane labeled "$LL_{No\ InsP6\text{-}NoAb}$") additionally suggests that the LL mucin glycans do not bind C1.

In sum, calcium InsP_6 found in the LL binds the C1 complex in a manner that can promote C1 activation, and this interaction accounts for most of the C1 binding capacity of the LL; the remaining capacity can be ascribed to host antibodies.

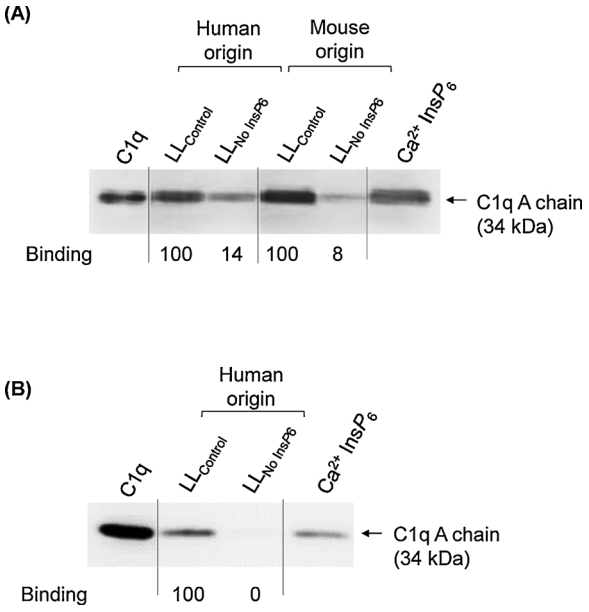

Fig. 2. The calcium InsP_6 component of the LL binds C1q selectively. (A) LL samples from either human or mouse host origin were untreated ("$LL_{control}$") or treated with EDTA to selectively dissolve away calcium InsP_6 ("$LL_{No\ InsP6}$"). These LL preparations or purified calcium InsP_6 were incubated with HS at 1:60 final dilution for 30 min at 37 °C, followed by washing; then the presence of C1q associating with the particles was analyzed by Western blot, with an antibody recognizing the C1q A chain, followed by densitometry. The protein loaded in each gel lane is the amount eluted from 900 µg dry mass of $LL_{control}$, or from matching amounts of $LL_{No\ InsP6}$ or purified calcium InsP_6 (Casaravilla et al. 2006); pure C1q (20 ng) was also loaded for comparison. (B) $LL_{control}$, $LL_{No\ InsP6}$ (both from human host origin) or purified calcium InsP_6 were incubated with purified C1q at the estimated concentration present in 1:60 HS (*i.e.* 1.3 µg/mL) in the additional presence of 1% w/v bovine serum albumin as an irrelevant protein, and samples were analyzed as described in part A. Estimates of the amount of C1q bound to $LL_{No\ InsP6}$ in relation to the amount bound to $LL_{control}$ are given below matched pairs of gel lanes.

3.3. Calcium InsP_6 contributes to CCP activation on the LL, but not in proportion to its contribution to C1 binding

We next tested whether calcium InsP_6 makes a contribution to CCP activation on the LL in HS. For this purpose, we chose a HS dilution (1:60) at which all complement activation took place *via* the CCP, as determined by the absence of C1q-independent C3 and C5 activation (Fig. S3). Under the chosen conditions, the LL caused activation of C1s (Fig. 4), C4 and C3 (Fig. 5). Previous removal of calcium InsP_6 did not cause a clear decrease in the activation of C1 (Fig. 4), and caused only minor decreases in activation of C4 and C3 that were mostly restricted to the later time-point measured (Fig. 5). These weak effects stood in contrast to the sharp decrease in C1 binding capacity previously observed upon the same treatment (Figs. 2 and 3). The considerable extent of calcium InsP_6-independent C1, C4 and C3 activation observed is in agreement with the previous detection of strongly bound IgG belonging to CCP-activating subclasses (Table 1).

In sum, calcium InsP_6 contributes to CCP activation on the LL but much less than it contributes to C1 binding.

3.4. C3b deposited on calcium InsP_6 after CCP activation appears to be inactivated very fast

We next analyzed the contribution of calcium InsP_6 to (CCP-dependent) covalent deposition of C4 and C3 on the LL. We first verified that C4 and C3 fragments released from the LL by hydroxylamine arose entirely from the *in vitro* incubation with serum, as opposed to being remnants from complement deposition *in vivo* (Fig. S4). The calcium

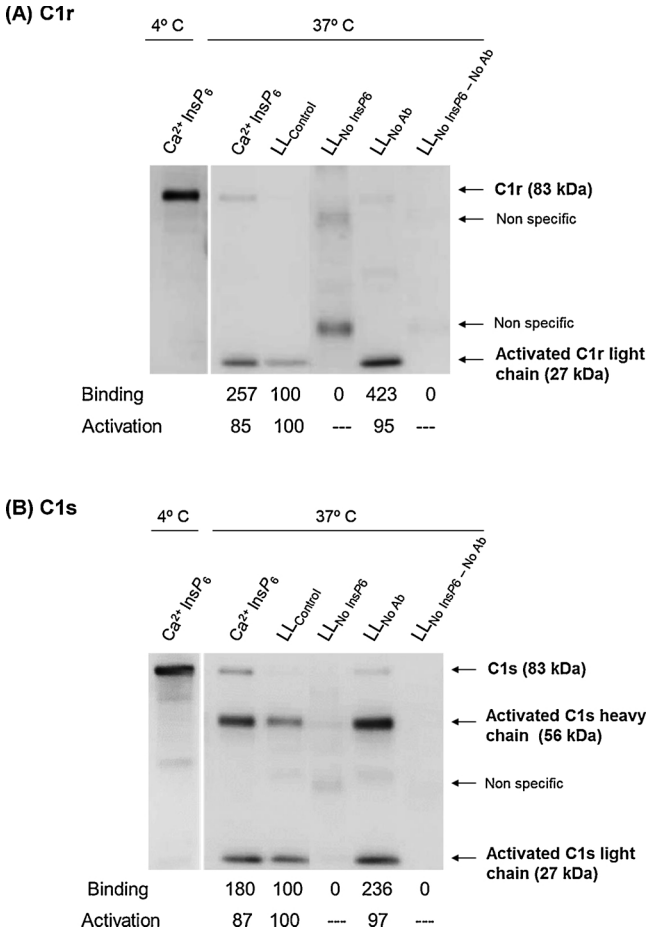

Fig. 3. The calcium InsP_6 component of the LL binds C1 in a manner that causes its activation in the absence of C1 inhibitor. LL samples (of mouse host origin) were untreated ("$LL_{control}$"), treated with EDTA to selectively dissolve away calcium InsP_6 ("$LL_{No\ InsP6}$"), treated with pronase to selectively digest away bound immunoglobulins and other non-mucin proteins ("$LL_{No\ Ab}$") or treated with both EDTA and pronase to remove both calcium InsP_6 and antibodies ("$LL_{NoInsP6\text{-}No\ Ab}$"). These four LL preparations or purified calcium InsP_6 were incubated with HS at 1:60 final dilution at 4 °C. Unbound proteins including C1-INH were washed off, and samples were further incubated at 37 °C to allow activation of any bound C1. Both binding and activation of C1r (A) and C1s (B) were analyzed by Western blot followed by densitometry. Binding is expressed relative to the $LL_{control}$ sample. Activation is expressed in terms of the fraction of antibody reactivity associated with activated C1r/C1s, as detailed in the Materials and Methods section. Note that the antibodies used recognize only the light chain of activated C1r but both chains of activated C1s.

InsP_6 deposits contain essentially no intrinsic covalent acceptors, as the phosphate group is not a nucleophile. However, the calcium InsP_6 deposits could support C4/C3 deposition by adsorbing proteins from serum (*in vitro*) or extracellular fluids (*in vivo*) that would act as covalent acceptors. In addition, CCP activation initiated on calcium InsP_6 could propagate to the mucin component of the LL. We therefore attempted to extract separately C4/C3 deposited on calcium InsP_6 from C4/C3 deposited on the mucins. For this purpose, after incubation with serum we first treated with EDTA to solubilize calcium InsP_6 and any associated proteins, and treated the resulting supernatant with hydroxylamine to release C4/C3 from acceptors. We then treated the insoluble remainder with hydroxylamine to release C4/C3 covalently bound to the LL mucin gel. Under the CCP-only conditions of the assay, approximately 2/3 of covalently bound C4 and C3 was deposited on calcium InsP_6 (Fig. 6 A, B; compare lanes marked "EDTA extraction" and "Post-EDTA hydroxylamine extraction", both corresponding to "$LL_{Control}$"). Elimination of calcium InsP_6 before complement activation

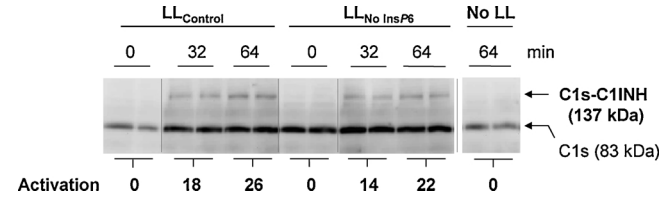

Fig. 4. The calcium InsP_6 component does not make a readily detectable contribution to C1 activation on the LL. LL samples (of human host origin) were untreated ("$LL_{control}$") or treated with EDTA to selectively dissolve away calcium InsP_6 ("$LL_{No\ InsP6}$"). HS at 1:60 final dilution was incubated with the LL preparations or with VBS^{++} only, for the indicated times at 37 °C. C1 activation was analyzed by Western blot and densitometry in terms of the formation of the covalent complex between activated C1s and C1-INH. Indices of C1s activation are given, calculated as detailed in the Materials and Methods section and averaged for each pair of duplicate reactions. The thin gray lines separate lanes that derive from different individual gels/PVDF membranes; data for $LL_{control}$ and $LL_{No\ InsP6}$ samples corresponding to the 32 and 64 min time-points all derive from the same gel/membrane.

markedly decreased deposition C4 and C3 on the mucins (Fig. 6; compare lanes "$LL_{Control}$" and "$LL_{No\ InsP6}$" within "Post-EDTA hydroxylamine extraction", particularly at the 15-min time-point). Thus, the calcium InsP_6 deposits contribute to CCP-dependent C4 and C3 deposition on the LL, and this C4 and C3 is deposited mostly on calcium InsP_6 itself, but also on the mucins.

We then quantified the proportion of deposited C4 and C3 found in the active C4b/C3b *vs* the inactive iC4b/iC3b forms. C4b deposited on calcium InsP_6 or on the mucins was inactivated similarly (Fig. 7 A). However, C3b deposited on calcium InsP_6 showed a clear trend towards faster inactivation in comparison to C3b deposited on the mucins (Fig. 7 B). As we have previously concluded that the rate of inactivation of C3b deposited on the LL mucins is fast in comparison with complement-activating surfaces (Irigoín et al. 2008), the present observation suggests a very fast rate of inactivation for C3b deposited on calcium InsP_6.

Thus, CCP activation by calcium InsP_6 results in C3b that is deposited mostly on calcium InsP_6 itself and is probably very rapidly inactivated.

3.5. Calcium InsP_6 makes no net contribution to C5 activation on the LL

We next assessed how the calcium InsP_6 deposits influence activation of the terminal steps of complement. Under CCP-only conditions, C5 activation was unaffected by previous elimination of calcium InsP_6 (Fig. 8A). This observation stands in contrast with the positive contribution of calcium InsP_6 to both activation and deposition of C4 and C3 (Figs. 5 and 6), but it is in line with the probable fast inactivation of C3b also detected (Fig. 7 B).

Under approximately physiological conditions (serum dilution 1:20), in which the ACP can amplify CCP-initiated activation, again calcium InsP_6 made no contribution to C5 activation (Fig. 8B). Similar to the previous observations for CCP-only conditions (Fig. 6 B), under CCP + ACP conditions calcium InsP_6 promoted C3b deposition onto itself, and to a lesser degree onto the mucins (Fig. S5; compare lanes marked "$LL_{Control}$" and "$LL_{No\ InsP6}$" both within "EDTA extraction" and within "Post-EDTA hydroxylamine extraction").

In sum, CCP activation on calcium InsP_6 is halted before it can make a net contribution to C5 activation, even under conditions in which it is amplified by the ACP.

4. Discussion

Our results show that the LL activates the CCP to some extent (Figs. 4, 5, 8, and S3). Most of this activation is independent of the calcium InsP_6 component, and is thus in all likelihood due to specifically bound host antibodies, which include isotypes known to activate

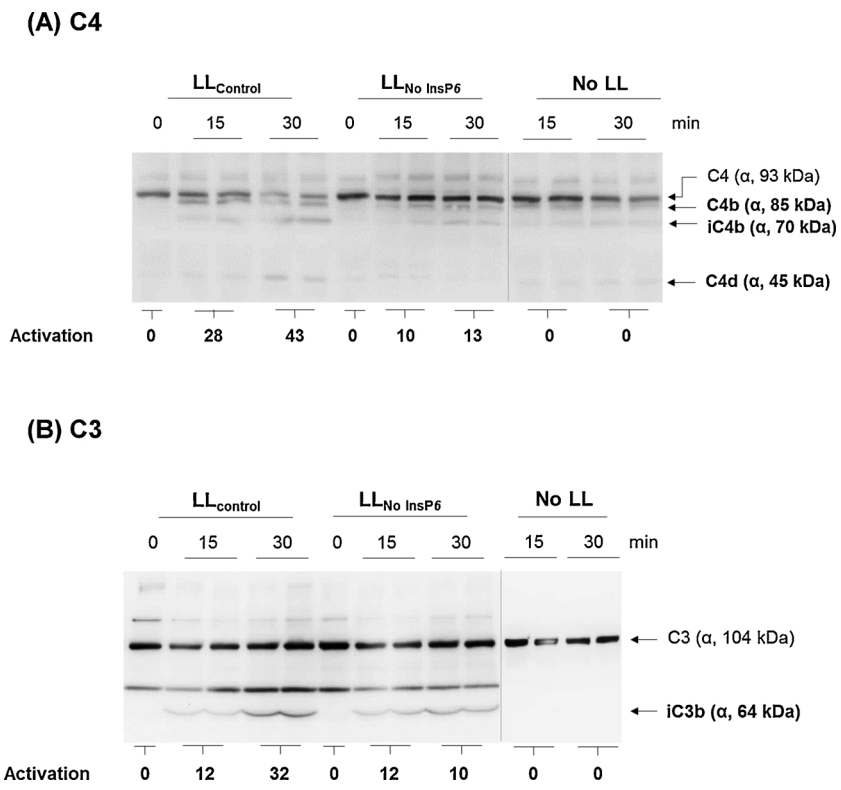

Fig. 5. The calcium InsP_6 component contributes to CCP-dependent C4 and C3 activation on the LL. LL samples (of human host origin) were untreated ("$LL_{control}$") or treated with EDTA to selectively dissolve away calcium InsP_6 ("$LL_{No\ InsP6}$"). HS at 1:60 final dilution was incubated with the LL preparations or with VBS^{++} only, for the indicated times at 37 °C. It had been previously verified that complement activation on the LL under these experimental conditions takes places *via* the CCP only. The activation of C4 (A) and C3 (B) was analyzed by Western blot and densitometry in reaction supernatants. Indices of C4 or C3 activation are given, calculated as detailed in the Materials and Methods section and averaged for each pair of duplicate reactions (except for zero time). The thin gray lines separate lanes that derive from different individual gels/PVDF membranes.

the CCP (Table 1). Analyses comparing $LL_{No\ InsP6}$ and $LL_{No\ InsP6\ -\ No\ Ab}$ showed the expected decrease in CCP activation after elimination of host antibodies, but considerable CCP activation remained, presumably due to natural antibodies in HS reactive with LL carbohydrates (data not shown).

Our results also suggest that the LL does not activate the LCP: using a HS dilution that precludes ACP activation, C3 deposition and C5 activation on (human origin) LL were entirely dependent on C1q (Fig. S3). This is in broad agreement with (cow) MBL not associating with the LL *in vivo* (Díaz et al., 1999) (although the ficolins have not been analyzed).

We had previously put forward the hypothesis that calcium InsP_6 in

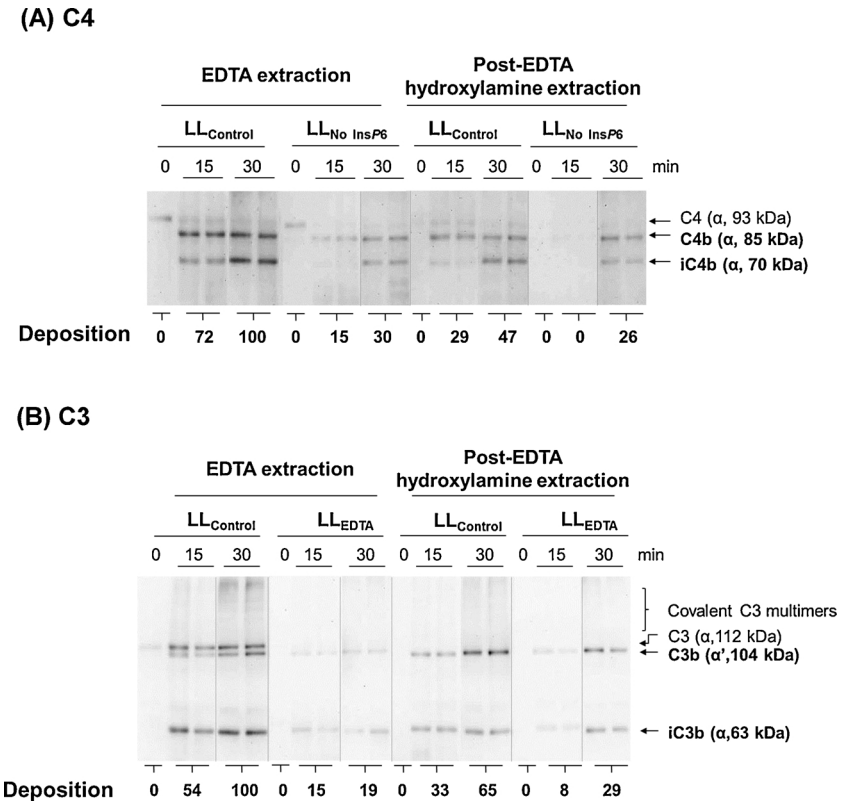

Fig. 6. Substantial proportions of the C4b and C3b deposited after CCP activation on the LL become associated with calcium InsP_6. LL samples (of human host origin) were untreated ("$LL_{control}$") or treated with EDTA to selectively dissolve away calcium InsP_6 ("$LL_{No\ InsP6}$"), and then incubated with HS at 1:60 final dilution for the indicated times at 37 °C. It had been previously verified that complement activation on the LL under these experimental conditions takes places *via* the CCP only. C4 and C3 products deposited through CCP activation were eluted from the LL particles so as to separately recover C4/C3 deposited on calcium InsP_6 ("EDTA extraction") and C4/C3 deposited on mucins ("Post-EDTA hydroxylamine extraction"), as detailed in the Materials and Methods section. Eluted C4 products (A) and C3 products (B) were analyzed by Western blot followed by densitometry. Indices of C4 or C3 deposition are given, calculated as detailed in Materials and Methods, averaged for each pair of duplicate reactions (except for zero time), and normalized over the highest value in the data series. The thin gray lines separate lanes that derive from different individual gels/PVDF membranes. Data for $LL_{control}$ and $LL_{No\ InsP6}$ samples obtained by both extraction procedures and corresponding to matching incubation times derive from the same gel/membrane. Also, data from the different gels/membranes were normalized using the quantitated C4/C3 bands from matching serum dilutions run on both gels. Note that the samples from the initial extraction contain some non-activated C3 carried over, which was obviously not taken into account for estimating C3 deposition.

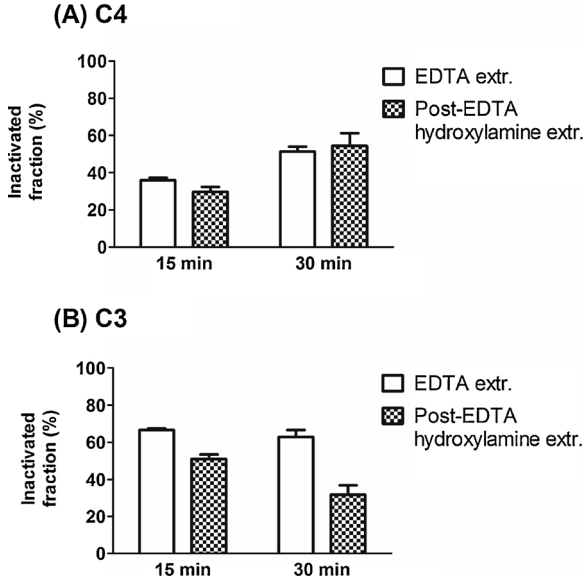

Fig. 7. C3b deposited on calcium InsP_6 is inactivated very fast. The inactivation of C4b to iC4b and of C3b to iC3b in the experiments shown in Fig. 6 and corresponding to $LL_{control}$ was quantified as detailed in the Materials and Methods section. The graph shows means +/- standard deviations of duplicate reactions for each experimental condition.

the LL could bind C1q and initiate CCP activation (Irigoín et al. 2008; Díaz et al. 2011a). This hypothesis was verified in the present article, in terms of C1q binding, C1 activation in the absence of C1-INH, and C1q-dependent C4 and C3 activation and their deposition in whole serum (Figs. 2, 3, 5, 6 and S3). However, it was also observed that whereas calcium InsP_6 makes a dominant contribution to C1 binding to the LL (Figs. 2 and 3), it makes a less important contribution to ensuing CCP activation (Figs. 4 and 5). Conversely, InsP_6-independent and presumably antibody-dependent sites in the LL make only a minor contribution to C1 binding but make important contributions to CCP activation. This is most probably explained by InsP_6 binding to the polyanion site in the C1q heads (distinct from the Fc binding site), and thus not efficiently causing the conformational change required for C1 activation (Garlatti et al. 2010) (Fig. 9 A). The InsP_6 species that forms the Ca^{2+} salt has 10 negative charges (Veiga et al. 2006), which are fully neutralized by Ca^{2+} in the bulk solid, but not at its surface.

The contribution of calcium InsP_6 to CCP activation on the LL, barely detectable in terms of C1s activation (Fig. 4), becomes nonetheless clear in terms of C4 and C3 deposition (Fig. 6). However, in contrast to the net positive contribution to C3 deposition, the calcium InsP_6 component does not contribute to C5 activation on the LL. This contrast holds true under CCP-only conditions (Figs. 6 B and 8 A) and under conditions in which the ACP is additionally operative (Figs. 8 B and S5). Thus, CCP activation driven by calcium InsP_6 is aborted between the step of C3 deposition and the step of C5 activation. This may be explained by CCP activation started on calcium InsP_6 resulting in C3b that is mostly deposited on calcium InsP_6 itself (probably *via* adsorbed proteins), where it appears to be very rapidly inactivated (Figs. 6 B and 7; model summarized in Fig. 9A). Since there is significant "overspill" of CCP activation initiated on calcium InsP_6 to the mucins (Fig. 6 B), it may be expected that calcium InsP_6 still contributes to some extent to overall C5 activation. The reason why this does not happen is most likely competition for C1: calcium InsP_6 can be expected to compete some C1 away from antibodies and therefore to decrease the amount of C5 activation that LL-bound antibodies ultimately give rise to.

As all the factor I-cofactor activity associated with LL is accounted for by host factor H (fH) (Díaz et al., 1997), the proposed fast C3b inactivation on calcium InsP_6 would imply that fH is operative on the surface of this compound (Fig. 9A). fH shows affinity for solid InsP_6 salts in surface plasmon resonance experiments (Irigoín et al. 2008). This affinity is not high enough for fH to be a major serum protein retained on synthetic calcium InsP_6 (Irigoín et al., 2008). However fH is functionally recruited to surfaces in the context of ternary complexes, fH-surface-C3b (Carreno et al., 1989; Morgan et al., 2011; Merle et al., 2015a). Thus, it is likely that fH localizes to the surface of calcium InsP_6 in contexts in which there is complement activation, *in vitro* or *in vivo* (see Fig. 9 A). In this reasoning, at least part of the fH found in association with the LL *in vivo* would be bound to calcium InsP_6, in agreement with its extraction by EDTA-containing buffers (Díaz et al., 1997).

The population of C3b/iC3b deposited on calcium InsP_6 *via* adsorbed serum proteins would have been undetectable in our previously published experiments, in which we washed off with high ionic strength solution those proteins non-covalently bound to the LL before extracting covalently bound C3 fragments (Irigoín et al., 2008). The conclusion reached at in the previous work that calcium InsP_6 does not influence C3b inactivation (Irigoín et al., 2008) was confirmed in the course of the present work (data not shown), but it applies only to C3b bound to the mucins.

The LL is freely permeable to IgG (150 kDa) (Coltorti et al. 1974), but apparently not to IgM (950 kDa) (Breijo et al., 2008). Our observation that treatment of the LL with pronase increases the amount of C1 complex retained in the LL (Fig. 3) suggests that the mucin gel imposes a steric restriction to the access of the C1 complex (766 kDa). This effect in combination with C1 binding on calcium InsP_6 can be expected to result in C1 concentrations reaching the GL tegument that are much lower than those in the extracellular fluid surrounding the parasite. In other words, the LL may act as a combined molecular permeation and affinity matrix hampering the access of C1 to the GL (Fig. 9 B). This is biologically relevant, as specific IgG antibodies including complement-fixing isotypes can localize to the GL tegument, as observed in cattle infections (Butler, 1983; Riesle et al., 2014). The calcium InsP_6 deposits are absent from the innermost 3 μm of the LL, next to the GL (Richards and Arme, 1983): it is tempting to speculate that this may constitute an adaptation to avoid bystander damage to the GL tegument from CCP activation on calcium InsP_6.

In summary, calcium InsP_6 in the LL binds C1q and causes CCP activation that is inefficient in term of C1 activation and that additionally is mostly aborted after C3b deposition so that no net C5

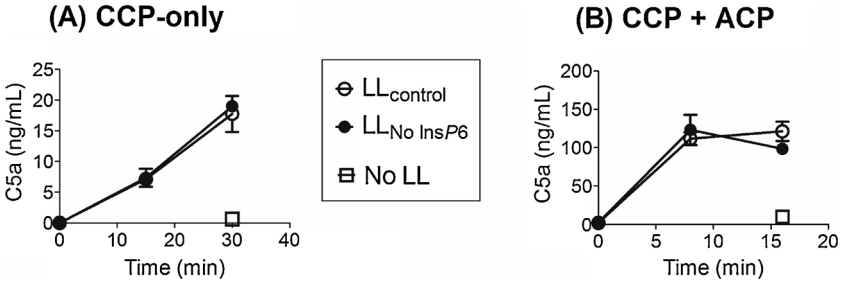

Fig. 8. The calcium InsP_6 component makes no net contribution to C5 activation on the LL. HS at final dilution of 1:60 (A) or 1:20 (B) was incubated with LL preparations or VBS^{++} alone for the indicated times at 37 °C. These HS dilutions allow the activation of complement on the LL through the CCP only (1:60) or through the CCP and ACP (1:20). C5a was quantified in reaction supernatants. The graphs show means +/- standard deviations of triplicate (A) or duplicate reactions (B).

activation results. This is reminiscent of the mode of complement activation displayed by certain components of the mammalian extracellular matrix (Sjoberg et al., 2005, 2009). The mechanism probably benefits the parasite by limiting activation on the GL tegument, without incurring in the cost of additional C5 activation; C5 activation is known to be detrimental to the parasite *via* eosinophil recruitment (Ferreira et al., 2000). In addition, it is conceivable that C1q binding by the LL has CCP-independent immune-regulatory effects, given the multiple interactions and activities described for C1q (Clarke et al., 2015; Kouser et al., 2015; Lu et al., 2017; Minutti et al., 2017; Thielens et al., 2017; Reid, 2018).

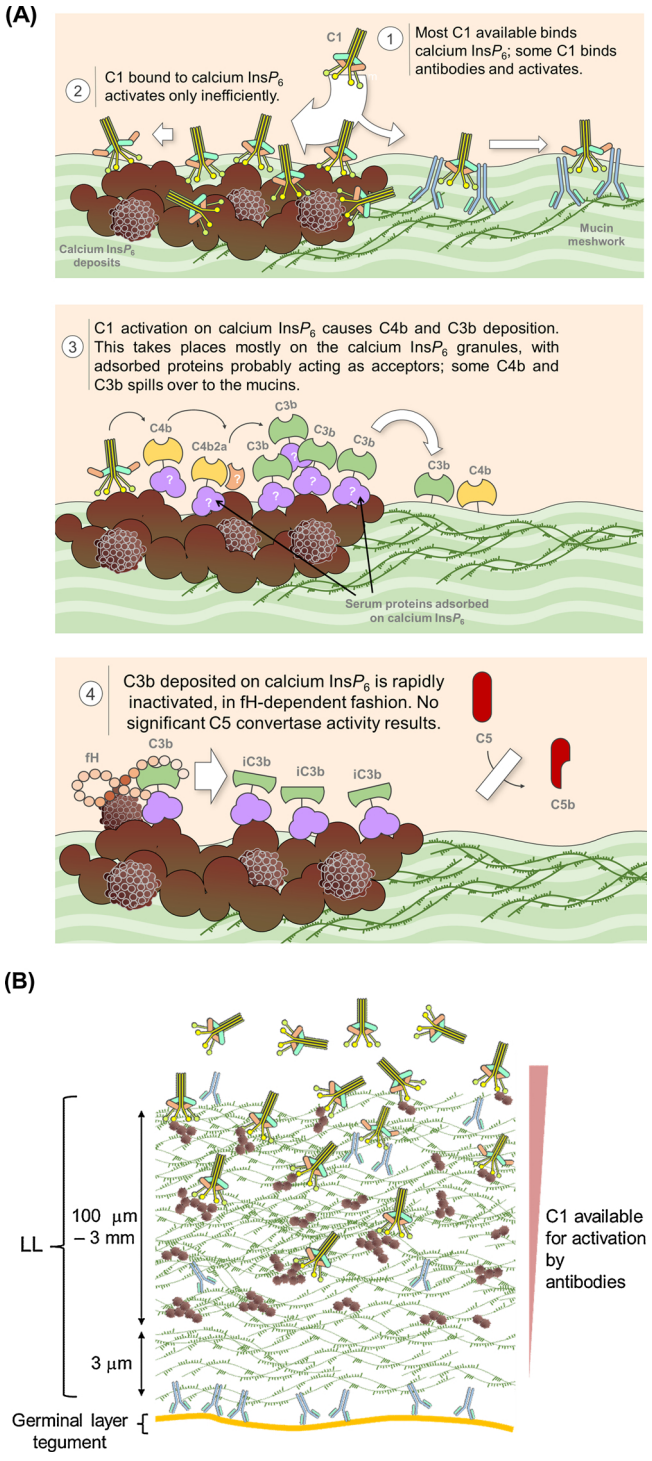

Fig. 9. Models of the proposed effects of the calcium InsP_6 component on CCP activation on the LL and the hydatid wall. (A) Calcium InsP_6 in the LL binds C1 complex through its C1q component. Although inefficiently, this causes C1 activation and initiates the CCP pathway. This results mostly in C4b and C3b deposited on calcium InsP_6 itself, in a manner bridged by adsorbed serum proteins. C3b deposited on calcium InsP_6 would be very rapidly inactivated in fH-dependent fashion. The overall process does not contribute to C5 activation on the LL. Although not depicted, it is likely some C5 is activated as a consequence of CCP activation of calcium InsP_6 but this is compensated by less CCP activation on antibodies because calcium InsP_6 competes C1 away from antibodies. (B) The high binding capacity of calcium InsP_6 deposits for C1q, combined with a restriction to the access of C1 imposed by the LL mucin gel would prevent the binding of functional C1 to the germinal layer tegument. This would avoid CCP activation triggered by antibodies bound to the germinal layer tegument. The previously reported absence of calcium InsP_6 deposits in the innermost 3 µm of the LL would prevent bystander damage to the germinal layer tegument by CCP activation initiated on calcium InsP_6.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.05.009.

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