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C-type lectins on macrophages participate in the immunomodulatory response to *Fasciola hepatica* products

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Summary

Fasciola hepatica releases excretory–secretory products (FhESP), and immunomodulatory properties have been described for the carbohydrates present in these parasite products. The interaction of FhESP with the innate immune cells, such as macrophages, is crucial in the early stage of infection. In this work we observed that peritoneal macrophages from naive BALB/c mice stimulated *in vitro* with FhESP presented: an increased arginase activity as well as Arginase I expression, and high levels of transforming growth factor-β and interleukin-10. A similar macrophage population was also observed in the peritoneum of infected mice. A partial inhibition of the immunomodulatory effects described above was observed when macrophages were pre-incubated with Mannan, anti-mannose receptor, Laminarin or anti-Dectin-1, and then stimulated with FhESP. In addition, we observed a partial inhibition of these effects in macrophages obtained from mice that were intraperitoneally injected with Mannan or Laminarin before being infected. Taken together, these results suggest the participation of at least two C-type lectin receptors, mannose receptor and Dectin-1, in the interaction of FhESP with macrophages, which allows this parasite to induce immunoregulatory effects on these important innate immune cells and may constitute a crucial event for extending its survival in the host.

Keywords: Dectin-1; *Fasciola hepatica*; macrophages; mannose receptor

Introduction

C-type lectin receptors (CLRs) are a large superfamily of soluble and membrane-bound proteins that share a common domain structure, the carbohydrate recognition domain, which is required for binding to specific carbohydrate structures of endogenous self-molecules as well as specific pathogens and pathogen-derived ligands.[1–3] Interestingly, not all interactions between CLRs and pathogenic ligands have beneficial outcomes, with some pathogens appearing to have evolved immunoevasive activities through these receptors.[4,5]

Among the CLRs, the mannose receptor (MR) is mainly expressed in alveolar and peritoneal macrophages (MΦ), and derived from blood monocytes.[6] It is able to bind branched sugars with terminal mannose, fucose or *N*-acetyl-glucosamine,[7] and mediate both endocytosis and phagocytosis, hence facilitating the clearance of both particulate and soluble ligands. In addition, it binds numerous microorganisms and microbial products including *Mycobacterium tuberculosis*,[8] *Trypanosoma cruzi*,[9] *Streptococcus pneumoniae*, *Klebsiella pneumonia*,[10] human immunodeficiency virus gp120,[11] and helminths such as *Trichinella spiralis*[12] and *Trichuris muris.*[13] However, the importance of MR in pathogen recognition is unclear because mice lacking this receptor show normal immune responses to *Candida albicans*,[14???] *Pneumocystis carinii*[15] and *Leishmania* sp.,[16] despite all these pathogens possessing structures that bind to the receptor. Therefore, MR could be considered to be an important molecule of the innate immune system, which may be exploited to influence the activation of the acquired immune system during pathological conditions.

Dectin-1 is another CLR normally expressed on monocytes, MΦ, dendritic cells, neutrophils and a subset of splenic T cells,[17] which recognizes particulate and soluble β-1,3-linked and β-1,6-linked glucans from fungi, bacteria and plants, and is the principal receptor for these carbohydrates on leucocytes.[18,19] This receptor can also recognize the following ligands: an unidentified endogenous

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ligand on T cells, which interacts with Dectin-1 through a different binding site to β-glucans,[20,21] and an unidentified molecule on mycobacteria, which does not express β-glucans.[22–24] Recently it has also been implicated in the recognition and uptake of apoptotic cells and the cross-presentation of cellular antigens.[25] Of all the known CLRs, only Dectin-1 has been conclusively demonstrated to function as a signalling pattern recognition receptor (PRR),[26] by regulating the expression of innate response genes, including those encoding co-stimulatory molecules, and pro-inflammatory cytokines and chemokines.[27–29] Paradoxically, the recognition of fungal β-glucans by Dectin-1 also triggers the production of non-protective cytokines, such as IL-23 and IL-10, and although the reasons for this are not fully understood, it is likely that Dectin-1 plays a central role in the immunomodulatory activities of these carbohydrates.[27,29,30]

Helminth parasites, on the other hand, are able to interfere in MΦ activation or induce the development of regulatory MΦ, which results in killing defects in cells, and enhanced survival and spread of parasites in the host.[31] *Fasciola hepatica* releases excretory–secretory products (FhESP) that have different immunomodulatory effects,[32–40] with the interaction of FhESP with the innate immune cells, such as peritoneal macrophages (pMΦ), being crucial in the early stage of the infection for the establishment of this parasite in the host. In addition, different experimental models have demonstrated the induction of alternatively activated MΦ by the parasite-released products.[41–45]

The way in which helminth infections drive polarized T helper type 2 and anti-inflammatory responses is likely to be due in part to the nature and quantity of the parasite pathogen-associated molecular patterns (PAMPs) present on their surfaces or in the excretory/secretory products, among which glycans seem to modulate the anti-parasite immune response.[46–49]

Although little is known about the recognition of extracellular parasite PAMPs by antigen-presenting cells, such as MΦ, or about the subsequent immunoregulatory effects, numerous immunomodulatory properties have been described for helminth carbohydrates, which are well-known components of FhESP.[32,43,50] Therefore, the aim of this study was to evaluate the participation of CLRs, particularly of MR and Dectin-1, in the interaction of FhESP with pMΦ, and in the immunomodulatory effects induced by these parasite products on these innate immune cells.

Materials and methods

Reagents

For cell cultures, RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 50 μg/ml gentamycin (Sigma-Aldrich Co., St Louis, MO) were used. Mannan, Laminarin, Polymixin B, anti-mouse IgG horseradish peroxidase and lipopolysaccharide (LPS) were also acquired from Sigma-Aldrich. The anti-mouse Dectin-1 antibody and the Syk inhibitor were bought from R&D Systems (Minneapolis, MN) and Calbiochem (Merck, Darmstadt, Germany), respectively. Anti-mouse MR (CD206), anti-mouse arginase I, anti-mouse β-actin, anti-mouse α-tubulin and goat-anti-rabbit IgG horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phycoerythrin-conjugated anti-mouse F4/80 was bought from Invitrogen (Carlsbad, CA). Transforming growth factor-β (TGF-β) Cytoset and the IL-10 kit were obtained from Biosource (Camarillo, CA) and e-Bioscience (San Diego, CA), respectively.

FhESP preparation

The FhESP were prepared according to a procedure described by Diaz *et al.*[51] with some variation. Briefly, live adult worms of *F. hepatica* were obtained from the bile ducts of bovine livers and then washed with PBS, pH 7·4, before being incubated (1 worm/2 ml PBS) for 3 hr at 37°. Then, the supernatant was centrifuged (16 000 ***g***, 30 min, 4°) before being concentrated using a high-flow YM 10 membrane filter (Millipore-Amicon Corp., Billeria, MA), and stored at −20° until used. The protein concentration (500 μg/ml) was measured using a Bradford protein assay (Bio-Rad, Hercules, CA), and the quantity of contaminating LPS present in FhESP was determined using the *Limulus* amoebocyte lysate test (Endosafe Times; Charles River Laboratories, Wilmington, DE), resulting in endotoxin levels < 90 units/ml of FhESP, and < 3·6 endotoxin units/ml (or 0·36 ng/ml) in the final culture conditions. Also, cultures were carried out by adding 10 μg/ml polymixin B (Sigma-Aldrich), and as the effects exerted by FhESP on pMΦ were the same (data not shown), this made it unlikely that the results attributed to FhESP stimulation were actually the result of LPS contamination.

Mice and purification of macrophages from PECs

Six- to 8-week-old female BALB/c mice were purchased from the Ezeiza Atomic Centre (CNEA, Buenos Aires, Argentina), and housed and cared for in the animal resource facilities of the Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Cordoba, following institutional guidelines. All experimental protocols were approved by the Animal Experimentation Ethics Committee, Faculty of Chemical Sciences, National University of Cordoba. To obtain pMΦ, the peritoneal cavity was washed with ice-cold PBS containing 0·1% FBS and 5 mM EDTA. After determination of viability by trypan blue exclusion (cell viability was > 95%), peritoneal exudate cells were re-suspended in RPMI supplemented with 10% FBS, 2 mM glutamine and 50 μg/ml

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gentamycin, and adjusted to 2×10^6 cells/well in 24-well plates for Western blot assays or 5×10^5 cells/well in 48-well plates for the remaining assays. The remaining adherent cells were highly enriched for MΦ, with flow cytometry analysis revealing 90% F4/80$^+$ cells.

Culture of naive pMΦ

Macrophages obtained from non-infected BALB/c mice were stimulated with FhESP at a concentration of 20 μg/ml for 48 hr. To investigate the role of receptors in the FhESP effects, we pre-incubated the cells with Mannan (1 mg/ml), Laminarin (0·5 mg/ml), Syk inhibitor (20 μM), anti-Dectin-1 (0·5 μg/ml) or anti-MR (10 μg/ml) for 30 min at 37° in 5% CO_2. Then, after being washed the cells were stimulated with FhESP at a concentration of 20 μg/ml for 48 hr.

Culture of pMΦ derived from infected animals

Mice were orally infected with 10 metacercariae of *F. hepatica* (Baldwin Aquatics Inc., Monmouth, OR). Forty-eight hours after being infected, we carried out peritoneal lavages and the adherent cells were cultivated for a further 48 hr.

To evaluate the participation of MR and Dectin-1 during the infection, mice were intraperitoneally injected with Mannan (1 mg/ml), Laminarin (0·5 mg/ml) or PBS 4 hr before being infected.[52] The blocker concentrations used were based on *in vitro* experiments, and we used a volume of 2 ml for the peritoneal cavity.

Cytokine assays

Culture supernatants were collected after 48 hr in the *in vitro* as well as in the *in vivo* experiments and assayed for the presence of TGF-*β* and IL-10 according to the manufacture's protocol, using a capture ELISA kit purchased from Biosource and e-Bioscience, respectively.

Arginase activity assay

Briefly, the arginase activity was measured as previously described by Corraliza *et al.*[53] After lysis of the cells in Triton X-100 containing 5 μg pepstatin, 5 μg aprotinin and 5 μg antipain as protease inhibitors, the mixture was stirred for 30 min at room temperature. Then, 50 μl of 10 mM $MnCl_2$ and 50 mM Tris–HCl were added to lysed cells to activate the enzyme by heating for 10 min at 56°. The arginine hydrolysis was initiated by the addition of 25 μl 0·5 M L-arginine, pH 9·7, at 37° for 45 min. The reaction was stopped with 400 μl H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, volume/volume/volume). Then, after the addition of 25 μl α-isonitrosopropiophenone, followed by heating at 95° for 45 min, the urea concentration was measured at 540 nm, with the results expressed as μg of urea.

SDS–PAGE and Western blot analysis

For Western blot analysis, 20 μg pMϕ lysate were separated on 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane (Amersham Bioscience, Amersahm place, Little Chalfont, UK). Non-specific binding was blocked with 5% non-fat dry milk in Tris–HCl saline buffer containing 0·01% Tween 20 (TBS-T) for 60 min at room temperature. The membranes were incubated overnight with specific primary antibodies at 4°, washed three times with TBS-T, and then incubated with secondary horseradish peroxidase-conjugated antibodies for 1 hr at room temperature. The specific bands were revealed by a chemiluminescence reaction (Amersham Biosciences) with autoradiographic or maximum performance light films (Kodak, Rochester, NY) and quantified by densitometric analysis using IMAGE software (SCION Version free online, Scion Corporation, Frederick, MD).

MR and Dectin-1 binding assays

To demonstrate binding of FhESP to MR and Dectin-1, we performed inhibition assays. Total peritoneal cells were pre-incubated with Mannan (1 mg/ml), Laminarin (0·5 mg/ml), Galactose (1 mg/ml) or specific blocking antibodies to MR (10 μg/ml) and Dectin-1 (0·5 μg/ml) for 30 min at 37°, and washed before being incubated with FITC-labelled FhESP for 1 hr (different time-points were used, and the most representative was chosen). The reaction was terminated by the addition of cold PBS, and the cells were washed and finally fixed with 1% *p*-formaldehyde solution after being analysed by flow cytometry. To discriminate the specific population of pMΦ, we considered the F4/80$^+$ cells, which corresponded to the pMΦ population.

Statistical analysis

Data are expressed as means ± SEMs. The two-tailed Student's *t*-test was used, and a one-way analysis of variance with Tukey–Kramer's post-hoc test was used to determine the statistical significance for all pairwise multiple-comparison procedures. A *P*-value of 0·05 was considered significant. All experiments were performed in triplicate and equivalent results were obtained in each experiment.

Results

Products from *F. hepatica* are able to induce immunomodulatory effects on pMΦ in the early stage of infection as well as after *in vitro* stimulation

To evaluate the behaviour of pMΦ during the early stage of the infection we challenged BALB/c mice with *F. hepatica* metacercariae for 48 hr, and for the *in vitro* studies we

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stimulated pMΦ derived from naive mice with FhESP for 48 hr. It was observed that high levels of arginase activity and Arginase I (Arg I) expression with respect to the control group were induced in both cases, after the infection (Fig. 1a, $P < 0{\cdot}018$ and $P < 0{\cdot}000035$, respectively) as well as in the *in vitro* stimulation with FhESP (Fig. 1c, $P < 0{\cdot}001$ and $P < 0{\cdot}020$, respectively). In addition, high levels of IL-10 and TGF-β production with respect to the basal group were observed in pMΦ derived from infected mice (Fig. 1b, $P < 0{\cdot}020$ and $P < 0{\cdot}027$, respectively), as well as in pMΦ stimulated *in vitro* with FhESP (Fig. 1d, $P < 0{\cdot}00061$ and $P < 0{\cdot}028$, respectively).

These results suggest that products from *F. hepatica* are able to induce immunomodulatory effects on pMΦ during the early stage of infection as well as after *in vitro* stimulation.

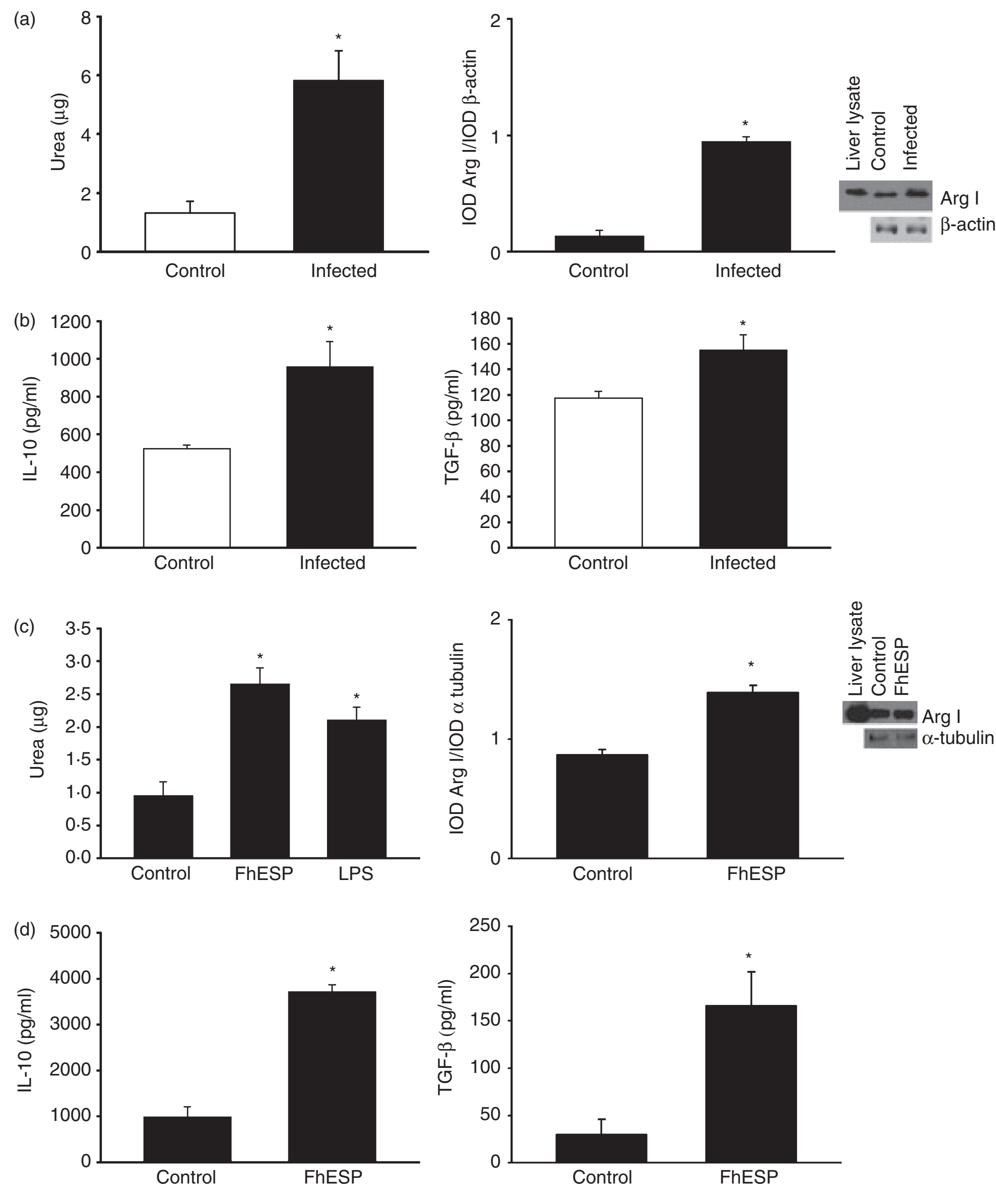

Figure 1. Products from *Fasciola hepatica* are able to induce immunomodulatory effects on peritoneal macrophages (pMΦ) in the early stage of infection as well as after *in vitro* stimulation. Expression levels of Arginase I (Arg I) by Western blot and arginase activity in pMΦ derived from infected BALB/c mice, 48 hr after metacercariae challenge and further cultivation for 48 hr, as well as in naive mice derived pMΦ stimulated with *F. hepatica* excretory–secretory products (FhESP; 20 µg/ml) for 48 hr (a, d). Transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) levels were measured by ELISAs in culture supernatants in the respective pMΦ of the *ex vivo* (b) and *in vitro* (d) experiments. In the infection experiment, four mice/group were analysed. Data are mean ± SEM of three independent experiments, analysed in triplicate. *$P < 0{\cdot}05$ respect to pMΦ from non-infected mice (a–c) or pMΦ in medium alone (c–d).

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Mannose receptors participate in the interaction of FhESP with pMΦ *in vitro*

To investigate the role of MR in the FhESP effects induced on pMΦ, these cells were pre-incubated with Mannan or with a specific blocking antibody to MR for 30 min at 37° before being stimulated with FhESP for 48 hr. As shown in Fig. 2(a), a partial inhibition in the increased arginase activity was detected in pMΦ pre-incubated with Mannan or anti-MR and then stimulated with FhESP ($P < 0{\cdot}01$ and $P < 0{\cdot}05$ anti-MR, respectively) compared with cells stimulated with FhESP. Similar results for Arg I expression were obtained in pMΦ pre-incubated with Mannan and then stimulated with FhESP with respect to cells stimulated with FhESP (Fig. 2b, $P < 0{\cdot}00032$). Also, a partial reduction of the high levels of IL-10 and TGF-β induced by FhESP when pMΦ were pre-incubated with Mannan or anti-MR was found compared with cells stimulated with FhESP alone ($P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively, employing Mannan; $P < 0{\cdot}05$ employing anti-MR) (Fig. 2c).

To demonstrate the specific binding of FhESP to MR, we performed an inhibition assay in which cells were pre-incubated with Mannan or anti-MR for 30 min at 37°

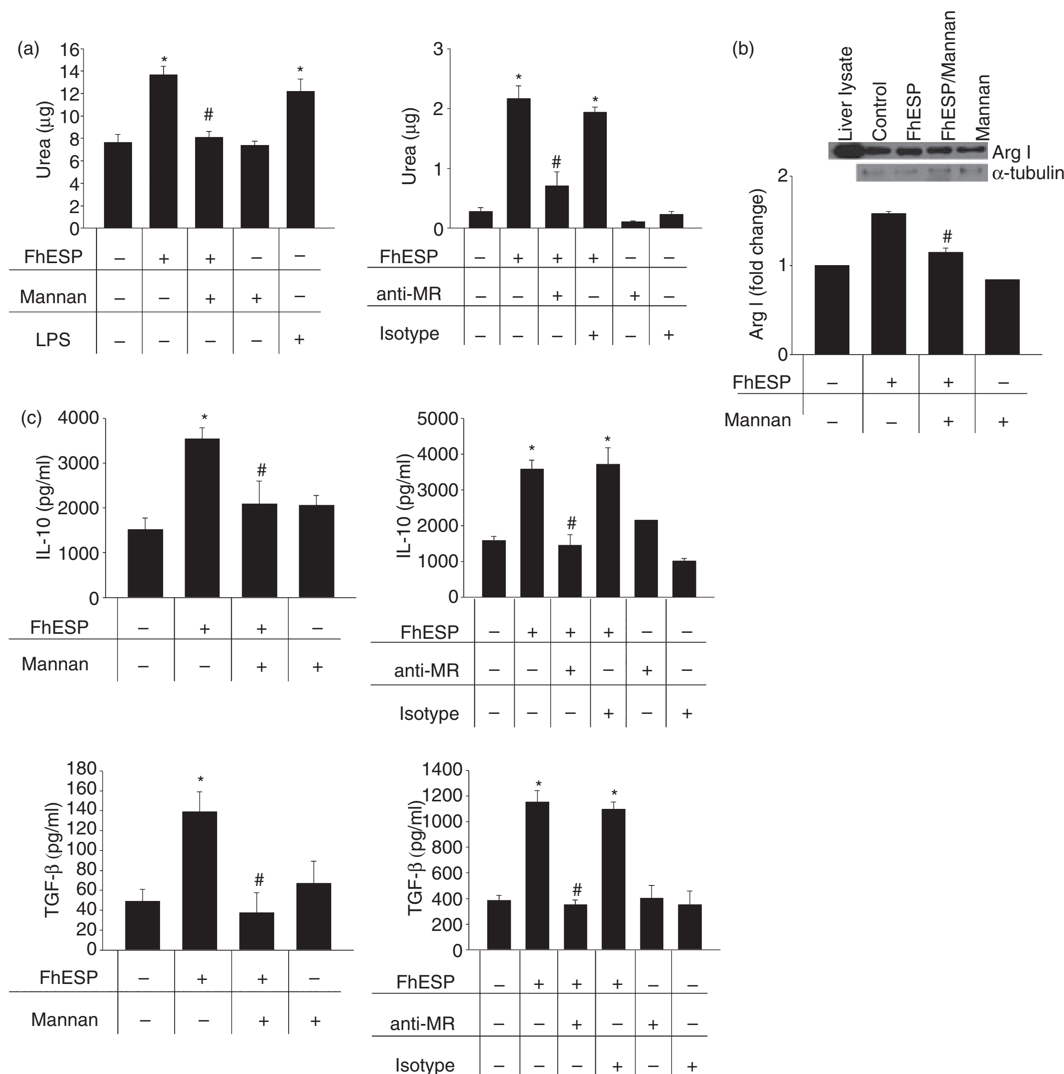

Figure 2. Mannose receptor (MR) participates in the induction of the increased arginase expression and activity, as well as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) production exerted by *Fasciola hepatica* excretory–secretory products (FhESP) on peritoneal macrophages (pMΦ). Arginase activity (a), Arginase I (Arg I) expression (b), as well as TGF-β and IL-10 production (c), were determined in pMΦ pre-incubated with mannan (1 mg/ml) or anti-MR for 30 min at 37° before being stimulated with FhESP (20 µg/ml) for 48 hr. Data are mean ± SEM of three independent experiments, analysed in triplicate. *$P < 0{\cdot}05$ respect to pMΦ in medium alone; #$P < 0{\cdot}05$ respect to pMΦ stimulated with FhESP.

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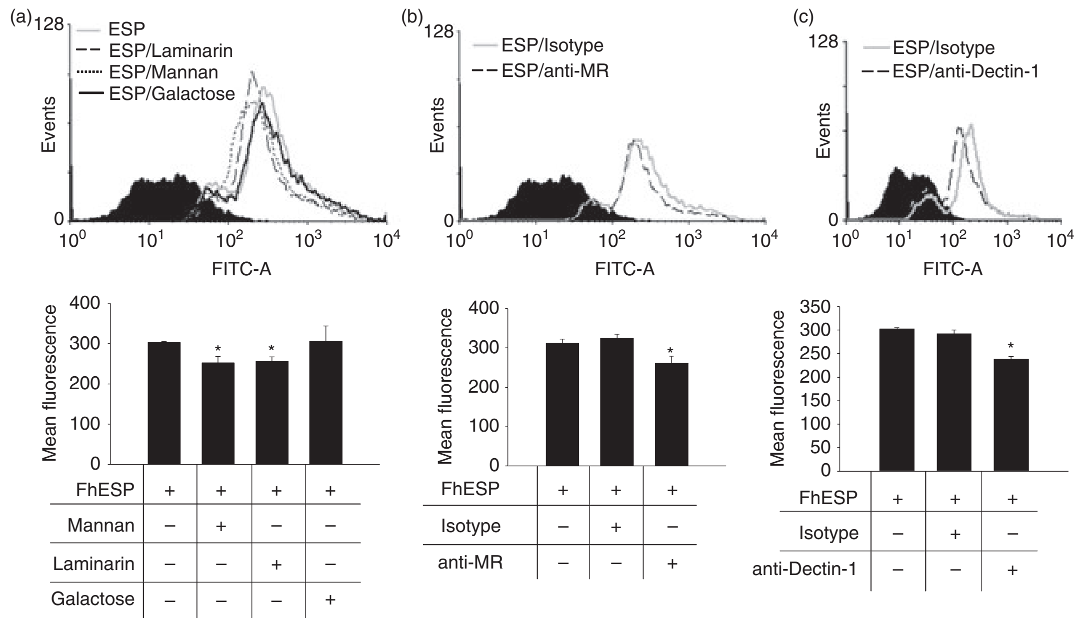

Figure 3. Mannose receptors (MR) and Dectin-1 participate in the interaction of *Fasciola hepatica* excretory–secretory products (FhESP) with peritoneal macrophages (pMΦ). Binding assays to determine the union of FhESP–FITC to MR and Dectin-1 on pMΦ were performed by employing blockers to the respective receptors. The binding was evaluated by flow cytometry in cells gated on F4/80$^+$ cells, with the histograms showing control (solid histogram); FhESP–FITC stimulated cells and isotype controls (solid grey line); pre-treatment with Mannan (1 mg/ml) or Laminarin (0·5 mg/ml) (a), anti-MR (10 μg/ml) (b), anti-Dectin-1 (0·5 μg/ml) (c) (open line); pre-treatment with galactose (1 mg/ml) (a) (solid black line). Data are mean ± SEM of three independent experiments, analysed in triplicate. *$P < 0{\cdot}05$ with respect to pMΦ stimulated with FhESP–FITC.

before being incubated with FITC-labelled FhESP for 1 hr. As shown in Fig. 3(a,b), the binding of FhESP to pMΦ was significantly less than that of the respective controls ($P < 0{\cdot}0011$ and $P < 0{\cdot}046$, respectively). Based on these above findings we suggest that MR participates in the interaction of FhESP with pMΦ.

Dectin-1 participate in the interaction of FhESP with pMΦ *in vitro*

Although there is no previous evidence suggesting the involvement of Dectin-1 in parasite product recognition by innate immune cells, in our preliminary inhibition experiments employing blockers to different receptors we found that, in a similar way to Mannan, Laminarin also inhibited the effects exerted by FhESP on pMΦ. As shown in Fig. 4(a), when we performed the experiments in the presence of Laminarin or of a specific blocking antibody to Dectin-1 for 30 min at 37°, we observed a partial inhibition in the high levels of arginase activity induced by FhESP respect to cells stimulated with FhESP alone ($P < 0{\cdot}001$ and $P < 0{\cdot}05$, respectively). In addition, an inhibition of the increased Arg I expression was observed when cells were pre-incubated with anti-Dectin-1 (Fig. 4b, $P < 0{\cdot}0003$). When the involvement of Dectin-1 was evaluated in the production of regulatory cytokines, we observed a reduction of the high levels of IL-10 and TGF-β induced by FhESP in pMΦ pre-incubated with Laminarin or anti-Dectin-1 compared with cells stimulated with FhESP alone (Fig. 4c, $P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively, employing Laminarin; $P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively, employing anti-Dectin-1).

Dectin-1 signals through a novel pathway, including an interaction with Syk (spleen tyrosine kinase) which then activates downstream signalling components, including the transcription factor nuclear factor-κB.[27,29,54] When we evaluated the role of Syk by employing an inhibitor of this kinase, a partial inhibition of the increase in arginase activity (Fig. 4a, $P < 0{\cdot}05$) as well as in the IL-10 and TGF-β levels (Fig. 4c, $P < 0{\cdot}001$ and $P < 0{\cdot}01$, respectively) induced by FhESP was observed with respect to cells stimulated with FhESP alone.

To demonstrate the specific binding of FhESP to Dectin-1, we performed an inhibition assay in which cells were pre-incubated with Laminarin or anti-Dectin-1 for 30 min at 37° before being incubated with FITC-labelled FhESP for 1 hr. As shown in Fig. 3(a,c), the binding of FhESP to pMΦ was significantly less than for the respective controls ($P < 0{\cdot}036$ and $P < 0{\cdot}017$, respectively). Based on the above observations we suggest that Dectin-1 is involved in the interaction of FhESP with pMΦ.

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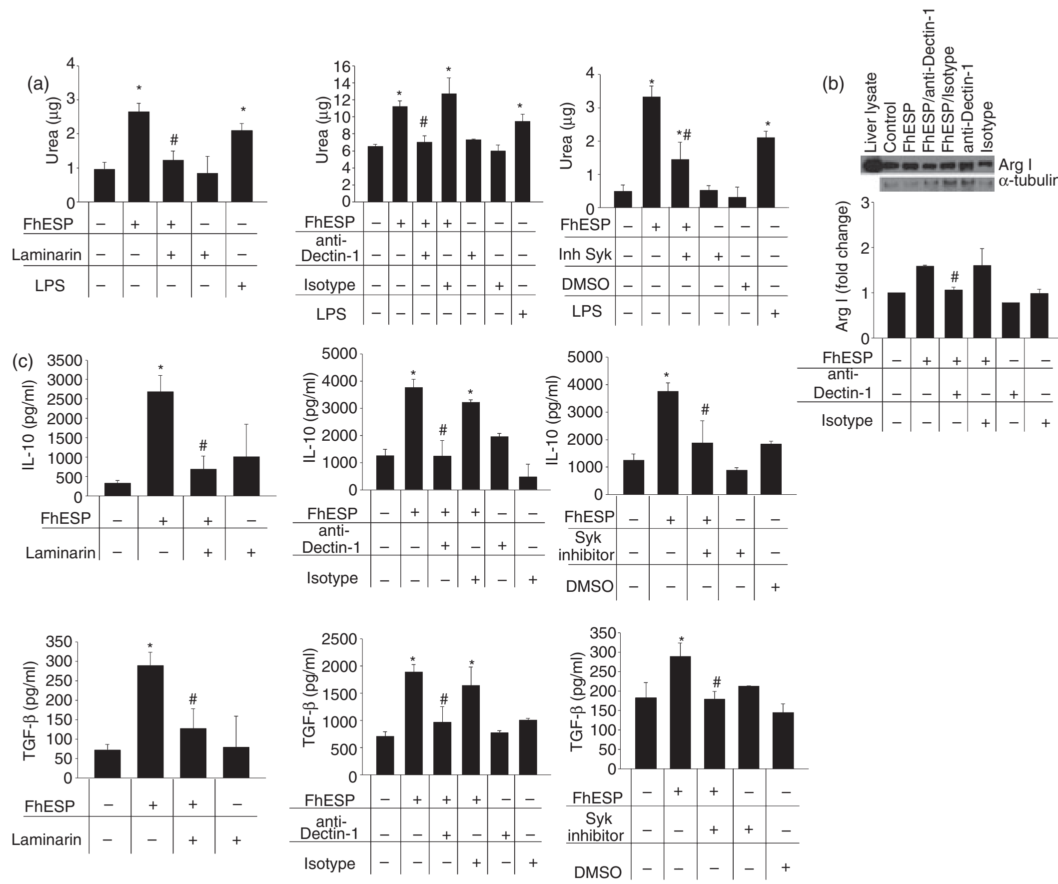

Figure 4. Dectin-1 participates in the increased arginase expression and activity, as well as in the higher Transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) production induced by *Fasciola hepatica* excretory–secretory products (FhESP) on peritoneal macrophages (pMΦ). Arginase activity (a), Arginase I (Arg I) expression by Western blot (b), as well as TGF-β and IL-10 levels (c) were determined in pMΦ pre-incubated with Laminarin (0·5 mg/ml), anti-Dectin-1 specific antibody (0·5 μg/ml), or Syk inhibitor (20 μM) for 30 min at 37° before being stimulated with FhESP (20 μg/ml) for 48 hr. Data are mean ± SEM of three independent experiments, analysed in triplicate. *$P < 0{\cdot}05$ respect to pMΦ in medium alone; #$P < 0{\cdot}05$ respect to pMΦ stimulated with FhESP.

MR and Dectin-1 participate in the phenotypic changes observed in the pMΦ during the early stage of *F. hepatica* infection

To determine if the participation of MR and Dectin-1 observed in the *in vitro* experiments may be extrapolated to *in vivo* events, mice were intraperitoneally injected with Mannan or Laminarin 4 hr before infection. Forty-eight hours after infection, peritoneal lavages were carried out and the adherent cells were cultivated for 48 hr. As shown in Fig. 5, there was a partial inhibition in the increased arginase activity and Arg I expression ($P < 0{\cdot}05$ and $P < 0{\cdot}00045$, respectively, with intraperitoneal Laminarin injection; $P < 0{\cdot}05$ and $P < 0{\cdot}0000039$, respectively, with intraperitoneal Mannan injection), as well as in the high levels of IL-10 and TGF-β production ($P < 0{\cdot}05$ and $P < 0{\cdot}05$, respectively, with intraperitoneal Laminarin injection; $P < 0{\cdot}05$ and $P < 0{\cdot}001$, respectively, with intraperitoneal Mannan injection). Therefore, taking into account the *in vitro* as well as the *in vivo* results, we can conclude that the participation of at least two CLRs, MR and Dectin-1, occurred in the interaction of *F. hepatica* products with pMΦ.

Discussion

Macrophages are innate immune cells that can respond to endogenous and exogenous stimuli. These cells are rapidly generated following injury or infection, by recognizing danger signals through receptors capable of inducing specialized activation programmes, which finally influence their physiology and functions.[55–58] Some micro-organisms can interfere with MΦ activation or induce the development of regulatory MΦ, resulting in killing defects in cells and enhanced survival and spread of parasites in the host. In agreement with previous results in different experimental models,[41–45] we have observed that *F. hepatica*-released products induced a pMΦ population with

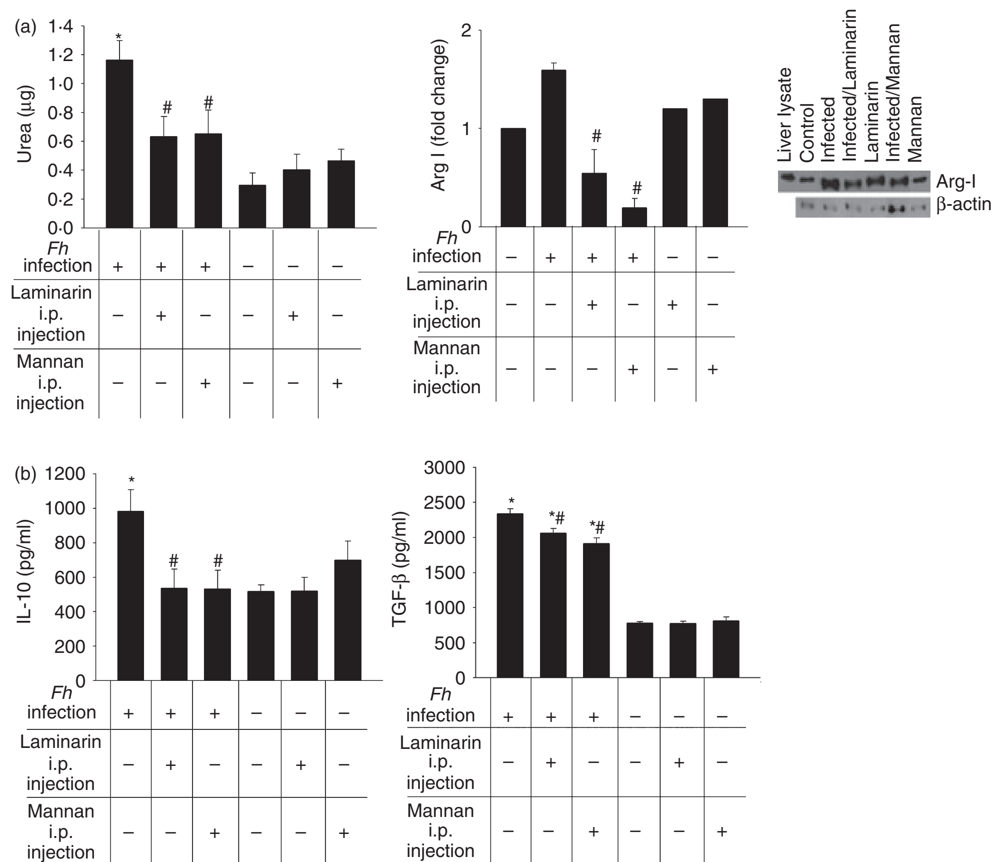

Figure 5. Mannose receptor (MR) and Dectin-1 participate in the *in vivo Fasciola hepatica*-induced effects on peritoneal macrophages (pMΦ) during the early stages of the infection. Mice were intraperitoneally injected with Mannan (1 mg/ml) or Laminarin (0·5 mg/ml) 4 hr before being challenged with *F. hepatica* metacercariae. Forty-eight hours after the infection, pMΦ were cultivated for 48 hr more. Arginase activity and Arginase I (Arg I) expression were determined in the respective cell lysates (a), and levels of transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) were measured in the culture supernatants (b). Four mice/group were analysed, and data are mean ± SEM of two independent experiments, analysed in triplicate. *$P < 0{\cdot}05$ with respect to pMΦ from non-infected mice; #$P < 0{\cdot}05$ respect to pMΦ from infected mice.

immunoregulatory properties during the early stage of infection as well as after *in vitro* stimulation. In this regard, we observed increased levels of arginase activity and Arg I expression, and high levels of IL-10 and TGF-β production. In addition, we observed an increased MR expression, low expression of MHC class II molecules, and a down-modulated response to the activating molecule LPS in pMΦ stimulated with FhESP (unpublished data, L. Guasconi and D. T. Masih).

Immunomodulatory properties have been described for carbohydrates present in the FhESP. For example, previous studies in our group have indicated a possible role of carbohydrate components of FhESP in the induction of eosinophil apoptosis, because the treatment of these products with sodium metaperiodate markedly diminished their effects.[50] Furthermore, the oxidation of FhESP suggests that glycan residues played a role in the parasite-MΦ interactions evaluated by Flynn and Mulcahy[43] in a bovine model. Nevertheless, little is known about the PRRs involved in the recognition of *F. hepatica* PAMPs by MΦ or the subsequent immunoregulatory effects induced on these cells. Considering that some pathogens have evolved immunosuppressive activities through their interaction with CLRs (a group of PRRs which are best known for their ability to recognize specific pathogen-associated carbohydrate structures[4,5]) together with previous evidence suggesting a possible role of glycan residues in the interaction of FhESP with innate immune cells such as eosinophils and MΦ, we decided to evaluate the role of the CLRs expressed in pMΦ in the interaction with parasite products. These results demonstrated the participation of MR in the interaction of FhESP with pMΦ, and related to this, when these cells were pre-incubated with Mannan as a competitive ligand or a specific blocking antibody to MR before being stimulated with FhESP, the increase in arginase activity or Arg I expression as well as the high levels of TGF-β and IL-10 were partially inhibited. Similar effects were observed when mice were intraperitoneally injected with Mannan before being infected.

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Among the numerous microorganisms recognized by MR are other helminth parasites such as *Trichinella spiralis*[12] and *Trichuris muris.*[13] According to Matthew *et al.*,[13] components of *T. muris* bind to the MR in a redundant way, hence generating an effective immune response that leads to MΦ activation, parasite expulsion and tissue repair, in a similar way to the results observed in mice lacking this receptor and infected with *Candida albicans*,[14???] *Pneumocystis carinii*[15] or *Leishmania* sp.,[16] which all showed a normal immune response. On the other hand, the carbohydrate components of *Taenia crassiceps* may favour a Th2 response, with MR being a candidate receptor in this effect.[59] In this regard, the engagement of MR with certain fungal pathogens and selected ligands or antibodies may result in an immunosuppressive function characterized by a profile of anti-inflammatory and tolerogenic cytokines, which could prevent the generation of Th1-polarized responses.[60,61] Therefore, we support the idea that MR can be exploited by *F. hepatica* to influence the activation of the immune system, specifically by modulating the pMΦ phenotype and function.

Dectin-1, on the other hand, has been conclusively demonstrated to function as a signalling PRR,[26] which regulates the expression of innate response genes including those encoding co-stimulatory molecules, and pro-inflammatory cytokines and chemokines.[27–29] Paradoxically, the recognition of fungal β-glucans by Dectin-1 also triggers the production of non-protective cytokines, such as IL-23 and IL-10, although the reasons for this are not fully understood. However, it is likely that Dectin-1 plays a central role in the immunomodulatory activities of these carbohydrates.[27,29,30] Recent studies suggest that internalization of Dectin-1 following interaction with ligand leads to attenuation of the signalling pathways involved in innate gene induction. This may be particularly relevant for understanding the role of innate immune receptors such as C-type lectins, which play a dual role in microbe phagocytosis and in inducing inflammation.[62,63] The findings presented in this work are novel results because Dectin-1 has still not been associated with an innate immune response to any parasite, and are in agreement with reports describing an immunomodulatory response for this receptor. In this regard, when pMΦ were pre-incubated with Laminarin as a competitive ligand or a specific blocking antibody to Dectin-1 before being stimulated with FhESP, the increase in arginase activity or Arg I expression was partially inhibited, and the high levels of TGF-β and IL-10 were lowered, with similar effects being observed when mice were intraperitoneally injected with Laminarin before being infected.

Dectin-1 signals via a novel hemITAM motif that becomes phosphorylated by Src family kinases on receptor engagement,[27,64] permitting recruitment and activation of the spleen tyrosine kinase (Syk). Then, Syk couples to downstream pathways and, via the adaptor CARD9, to the activation of nuclear factor-κB,[27,29,54] which together with nuclear factor of activated T cells and the transcription factors activated by mitogen-activated protein kinases downstream of Syk, regulates the expression of the innate response genes.[27–29] To evaluate the role plays by Syk employing an inhibitor of this kinase, we observed that the increase in arginase activity was partially inhibited and the TGF-β and IL-10 levels were lowered. This effect observed in the IL-10 production is in agreement with that reported in the bibliography, which demonstrated that IL-10 induction through the ligand/Dectin-1 interaction is Syk-dependent.[27,65]

Based on the above findings, together with the results that show the interaction of FhESP with MR and Dectin-1 observed in the binding assays, we propose that these CLRs would be highly employed by *F. hepatica*. We suggest that both CLRs are important in the modulation of pMΦ activation, which are among the first innate immune cells to come into contact with the parasite on entering the host. However, we considerer that the pathways used by these receptors for immunomodulation are not exactly the same. In relationship with this, in preliminary assays we observed that the suppressor phenomenon induced by pMΦ stimulated with FhESP in the naive T-cell proliferative response, is Dectin-1-dependent but not MR-dependent (*in vitro* assays, unpublished data, L. Guasconi and D. T. Masih). In this regard, we have begun to evaluate potential signalling pathways involved on each receptor's effects. Finally, we considered that both CLRs cooperate for the same purpose: modulating the anti-parasite immune response. The summary of the effects of MR and Dectin-1 would be part of the strength that finally prevails over the immunostimulatory signals from other PRRs, which ultimately determine the survival of this parasite in the host.

However, considering that little is known about the extracellular parasite PAMPs, their recognition by antigen-presenting cells or the link to initiate the Th2 response and cytokine production, further studies are required to improve the understanding of host defence against such microorganisms. Ultimately, identification of the molecules which interact with these CLRs could help to clarify how they direct the immune response.

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Disclosures

The authors have no conflict of interest to disclose.

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