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Research Article

Fasciola hepatica ESPs Could Indistinctly Activate or Block Multiple Toll-Like Receptors in a Human Monocyte Cell Line

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Abstract

Fasciola hepatica is a parasitic helminth that induces Th2/Treg responses in its mammalian host. Some reports have suggested that ESPs achieve these polarized immune responses by delaying the activation of dendritic cells and macrophages during the early stages of innate immunity, a process that is mediated by TLR4. The present study aimed to investigate whether TLRs other than TLR4 could also be targeted by *F. hepatica* ESPs.

To achieve this aim a screening system was optimized using THP1-Blue CD14 cells. ESPs were first separated based on their molecular weight and according their net charge by ion exchange chromatography (IEC). Results demonstrated that *F. hepatica* ESPs mainly cathepsin, serpin and endopin are capable of activating TLR2, TLR4, TLR8 and likely also TLR5 and TLR6. In contrast, fatty acid binding protein strongly suppressed the stimulation induced by various TLR-ligands. Further studies are needed to understand how these apparent contradictory effects of molecules of the same protein mix complement each other in the context of an active infection resulting in the polarized Th2-immune response that characterize *F. hepatica* infections

ABBREVIATIONS

ESPs: Excretory-Secretory Products; TLRs: Toll-Like Receptors; FABP: Fatty Acid Binding Protein; CatL: Cathepsin-L, FPLC: Fast Protein Liquid Chromatography; IEC: Ion Exchange Chromatography; ELISA: Enzyme-Linked Immunosorbent Assay; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; MALDI-MS/MS: Matrix Assistant Laser Desorption / Ionization With Tandem Mass Spectrometry; NF-kB: Nuclear Factor-kB; SEAP: Secreted Embryonic Alkaline Phosphatase; PMB: Polymixyn-B; FLA: Flagellin; OxPAC: oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine; CL075: Thiazoloquinoline Compound; LPS: Lipopolysaccharide; HKLM: Healt Killed Listeria Monocytogenes

INTRODUCTION

Fasciola hepatica excretory-secretory products (ESPs) are believed to play an important role in tissue penetration, and immune evasion [1-3]. ESPs are capable of scavenging free radical production, lowering phagocytic activity, affecting cell recruitment to the infection site and inducing the alternative activation of macrophages that favors the polarization of host immune response to the Th2 phenotype [4,5]. This produces an ideal immunological environment that permits the parasite survival into the host and guarantees the development of chronic infections. These polarized Th2 responses are only possible with an efficient suppression of Th1 cytokines, which result detrimental for hosts in cases of co-infections with *Bordetella pertusis* or *Mycobacterium tuberculosis* that require of Th1 immunity for their complete eradication [6-8]. It has ben suggested that such Th2 polarized immune response is achieved because *F. hepatica* poorly activate the cells of innate immune system, specially dendritic cells and macrophages and that this effect can be mimicry by ESPs [9-11]. Studies performed with cathepsin-L1 (CatL1) and glutathione *S*-transferase (GST) considered major components of the ESPs demonstrate that the immunomodulatory effect of these molecules is mediated by TLR4 [11]. The present study aimed to investigate whether TLRs other than TLR4 could be targeted by *F. hepatica* ESPs and identify these molecules.

MATERIALS AND METHODS

Fractionation of *F. hepatica* excretory-secretory products (ESPs) and their reactivity with sera infection

ESPs from *F. hepatica* adult flukes were prepared by maintenance in vitro techniques as previously described [12]. ESPs were fractionated based on molecular weight (MW) by a molecular sieving ultra filtration system in which ESPs were

sequentially passed through YM-100, YM-30, YM-10 and YM-3 membranes (AMICON Corp., Lexington, Massachusetts). Proteins retained on each of these membranes were designated as ESPs ≥100kDa, ESPs ≥ 30-100kDa, ESPs ≥10-30kDa and ESPs ≥3-10kDa, respectively. Proteins were desalted against PBS using PD-10 columns (GE Healthcare), lyophilized, re suspended in 2-ml of sterile distilled water and analyzed by 12% SDS-PAGE stained with Coomassie-blue. Indirect ELISA assay previously described [13] was used to assess the reactivity of all these proteins against pools of sera from rabbits with 3 or 10 weeks of *F. hepatica* infection [14].

Ion exchange chromatography and protein identification

ESPs ≥10-30kDa was selected for fractionation by ion exchange chromatography (IEC) using a Mono Q 5/50 GL column (Amersham-Bioscience) connected to an AKTA FPLC System. Separation of proteins was achieved by a stepwise elution with 10mM Tris-HCL pH 8.0, containing 1M NaCl. Recovered IEC-fractions were desalted against PBS, concentrated and stored at -20°C until use. Protein concentration of each IEC-fraction was measured using the Pierce™ BCA kit (Thermo Fisher, USA) [15] and analyzed by 12% SDS-PAGE. Major protein bands were manually excised from the electrophoresis gel, analyzed by MALDI-MS/MS and identified by comparison with molecules of the Swiss-Prot and NCBInr databases using the MASCOT search engine (Matrix-Science, London, UK) as described elsewhere [16,17].

Endotoxin removal

Endotoxins were removed from all molecules using PMB-columns and the presence of endotoxins was assessed prior to and after removing endotoxins as previously described [18].

Screening system using THP1-Blue CD14 cell

THP1-Blue™-CD14 cells (InvivoGen, San Diego, CA, USA) expressing all toll-like receptors (TLRs) and genes involved in the corresponding signaling cascades were used. Cells were transected with a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of the NF-κB promoter. Cells ($2x10^6$ cells/ml) were seeded in sterile endotoxin-free flat-bottomed 96 well plates (Costar) within RPMI 1640 (Invitrogen) supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate with 10% fetal bovine serum, containing 50 U/ml Pen-Strep, Blasticidin 10µg/ml and Zeocin 200µg/ml using. Plates were incubated at 37°C, 5% CO_2 for 3hrs in the presence of ESPs at concentrations ranging among 5 to 60µg/ml. Cells stimulated with TLR-agonists at the concentration suggested by manufacturer were used as positive activation control. The agonists used in this study were heat killed *Lysteria monocytogenes* (HKLM, $5x10^7$ cells/ml), lipopolysaccharide (LPS, 1µg/ml), flagellin (FLA, 100 ng/ml), and thiazoloquinoline compound (CL075, 0.5 µg/ml). After 19 h of incubation 20µl of solution from each well was transferred to a clean 96-well micro plate and mixed with 150µl of the QUANTI-Blue™ reagent (QB) (Invivogen). After an additional incubation of 7 h, the absorbance was measured at a wavelength of 655nm (A_{655}). In the inhibition experiments cells were first exposed to 30µg of oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC) (inhibitor of TLR2 and TLR4 pathways), 100µg of Polymixyn B (PMB) (inhibitor of TLR4) or 100µM of chloroquine (Chlor) (inhibitor of TLR-3, 7, 8, 9) and after 30 min of incubation at 37°C, 5% CO_2 were exposed to ESP-fractions or ligands. Incubation was prolonged for 19h, followed by the addition of the QB reagent as described above. As a positive control, cells were activated with a known specific TLR-ligand. For the negative control, cells were exposed to a specific TLR-inhibitor. The reduction in the absorbance value was used as criteria of specific activation for a given TLR and was calculated using the formula R (%) = (C-E) / C x 100, where C represents the mean A_{665} of three replicate obtained when cells are stimulated with ESPs or ligands and E represents the mean A_{665} value obtained when cells were first exposed to the TLR-inhibitors and then stimulated with the ESPs or ligands.

NF-κB activation in TLR4-transfected HEK cells

Human Embryonic Kidney 293 cells expressing exclusively TLR4 (Invivogen) were co-transfected with genes encoding (CD14), myeloid differentiation protein–2 (MD2), and a SEAP reporter gene were maintained in DMEM and seeded at a density of 2.52×10^4 cells/well in 96-well flat-bottom plates as previously described [18]. Cells were cultured with each IEC-fraction (15µg/ml) or LPS (1µg/ml), and incubated at 37°C, 5% CO_2 for 19 h. In the inhibition experiments, cells were cultured with PMB (100mM) for 30 min before LPS (1µg/ml) or IEC-fraction stimulation. The percent of reduction of the absorbance values was calculated as described above.

Statistical analysis

All determinations were done in triplicate and each experiment was repeated three times. The results expressed as the mean A_{655} values ± SD for each determination. Statistical significance among different experimental determinations was performed using unpaired Student *t*-test using GraphPad Prism software (Prism 6) a p-value ≤ 0.05.

RESULTS AND DISCUSSION

F. hepatica is a large extracellular helminth that has evolved numerous mechanisms to avoid the immune response of the host. Mammalians are usually infected by ingestion of aquatic plants that harbor the infectious metacercariae. Newly excysted juveniles (NEJs) immediately penetrate the host intestine wall and migrate within peritoneal cavity for approximately 3 days [19]. Mammalian hosts display no clinical signs at this time and pathological findings are rare [20,21], which suggest that parasite possesses mechanisms that suppresses immune activation within this tissue. Experiments in laboratory animals have demonstrated that at only 24 h of infection, a large amount of alternative activated macrophages are recruited in the peritoneal cavity of infected animals [10,22,23] and that flukes induce an apoptotic effect on peritoneal immune cells [24,25]. Thus, the control that *F. hepatica* exerts on the immune system of its host likely begins from the earliest stages of infection. Between 4-6 days after the infection, NEJs have penetrated the Glisson's capsule and established them firmly within the parenchymal

tissue where they migrate for approximately 5-6 weeks before reaching the bile ducts and develop into their adult form after 8-10 weeks of infection [26]. During its entire trajectory into the mammalian host, *F. hepatica* secretes a milliard of molecules termed excretory-secretory products (ESPs), which, it is believed are responsible for the parasite's immunomodulation. ESPs interact with antigen presenting cells, specifically monocytes / macrophages and dendritic cells, exerting on these cells a strong suppressive effect that determines the ultimate outcome of *F. hepatica* infection. Cathepsin-L1 (CatL1) and glutathione S-transferase (GST), considered major components of the ESPs, play an essential role in such immunomodulatory effect through the interaction with TLR4 [11].

The present study aimed to identify whether, in addition to TLR4, other TLRs of monocytes could also be targeted by *F. hepatica* ESPs. To address this aim, ESPs were separated into four fractions of molecular weight (MW) ≥3-10kDa, ≥10-30kDa, ≥ 30-100kDa and ≥100kDa and each fraction was tested in its capacity to stimulate TLRs in THP1-Blue CD14 cells, a human monocyte cell line that expresses multiple TLRs. The fractions ≥3-10kDa and ≥100kDa were recovered very low protein concentration (<0.2mg/ml) and consequently, they were no longer used in subsequent experiments. ESPs ≥10-30kDa and ESPs ≥ 30-100kDa had at average protein concentrations of 1.426 ± 0.06 mg/ml and 1.576.4 ± 0.03 mg/ml, respectively. ESPs ≥10-30kDa showed to contain polypeptides of ~12-14kDa as major components as well as a minor component of 26-28kDa. ESPs ≥30-100kDa contained a single major polypeptide band of ~26-28kDa and a weak band of ~55kDa (Figure 1a). The protein band of ~26-28kDa observed in ESPs ≥10-30kDa and ESPs ≥30-100kDa corresponds to a mixture of GST and CatL isoforms, which was confirmed by MALDI-MS/M analysis (data not shown). This is consistent with previous proteomic studies reporting that ~80% of the *F. hepatica* ESPs are CatL and ~4% are GST is o forms [27]. Both, ESPs ≥10-30kDa and ESPs ≥ 30-100kDa showed to be immunoreactive with sera from rabbits with 3 or 12 weeks of active *F. hepatica* infection (Figure 1b), which is consistent with the fact that *F. hepatica* ESPs are highly antigenic molecules that strongly react with sera from animals and humans with acute or chronic fascioliasis [14, 28-30]. Next, we wanted to ascertain whether in the absence of GST the reactivity of the ESPs ≥10-30kDa or ESPs ≥30-100kDa with infection sera could be affected. GST molecules were removed by using GSTrap 5/5 HP column, which was assessed by enzymatic activity determination using the CDNB-assay [31]. The removal of GST does not produce visible change in the reactivity of ESP-fractions with sera infection, indicating that the contribution of GST to the antigenicity of these ESPs is minimal.

Next, we proceeded to determine whether ESPs ≥10-30kDa or ESPs ≥30-100kDa could stimulate TLRs of THP1-Blue CD14 cells. We found that both antigens significantly induced the secretion of high levels of SEAP, which is indicative of TLR-activation. The presence or absence of GST in the ESPs ≥10-30kDa or ESPs ≥30-100kDa made no differences in the results. The amount of SEAP stimulated by ESPs ≥10-30kDa or ESPs ≥30-100kDa was similar to those observed by stimulation of cells with specific agonists for TLR2, TLR4, TLR5, TLR6 or TLR8 and were directly proportional to the concentration of ESPs added to culture, with 15μg being the minimal protein concentration of both antigens rendering maximal SEAP secretion. To identify the TLRs that are activated by these ESP-fractions, independent experiments were conducted in which cells were first cultured with a TLR-inhibitor and 30 min later were stimulated with the ESPs ≥10-30kDa or ESPs ≥30-100kDa. Results demonstrate that the levels of SEAP produced by ESPs ≥10-30kDa were significantly reduced both by OxPAC (inhibitor of TLR2 /TLR4) 96.2% (p<0.0001) and PMB 87.3% (p <0.001) (Figure 2a). Results suggest that ESPs ≥10-30kDa possess components that could indistinctly stimulate TLR2 and TLR4. When the experiment was performed with the ESPs ≥30-100kDa, the SEAP levels in the presence of OxPAC were reduced by 93.5% and in the presence of PMB were reduced by 43.2% (Figure 2b), which suggest that ESPs ≥30-100kDa could preferentially activate TLR2 rather than TLR4. TLR4 and TLR2 are receptors localized on the surface of antigen presenting cells that are typically activated by lypolysaccharide and lipopeptides, respectively. Preparations containing phosphatidylserine from *Schistosoma mansoni* and *Ascaris lumbricoides* activate TLR2 [32]. Due to the unavailability of specific antagonists for TLR5 or TLR6, we were unable to conclusively determine under our experimental conditions whether ESPs ≥10-30kDa or ESPs ≥30-100kDa could stimulate TLR5 or TLR6. However, based on the observation that OxPAC or PMB do not suppress 100% the stimulation of TLR2 or TLR4 induced by both ESP-fractions, this would not be unlikely.

An unexpected result from this study was the observation that chloroquine, an antagonist of endosomal TLR3, TLR7 and TLR8, reduced the levels of SEAP induced by ESPs ≥10-30kDa or ESPs ≥30-100kDa by 53.4% and 46.5%, respectively suggesting that these antigens could contain RNA species capable to activate endosomal TLRs. Since THP1-CD14 cells express low levels of TLR3 and TLR7 and these receptors were unresponsive to their corresponding agonists even in an excess of ligand (data not shown), it was possible to assume that ESPs ≥10-30kDa or ESPs ≥30-100kDa are targeting TLR8. Identical results were obtained after treating the antigens with RNAse. A feasible explanation to this finding is that parasite ssRNA species encapsulated into exosomes, and therefore, not susceptible to RNAse degradation, are activating TLR8. The presence of exosomes containing RNA was recently reported in *F. hepatica* [33], and is consistent with previous report of endosomal TLR3 activation of DC with antigens from the parasitic helminth *S. mansoni* [34].

Next we focused our attention on ESPs ≥10-30kDa, removed GST and fractionated the antigen by IEC. Chromatographic separation produced four different fractions, named P1, P2, P3 and P4, which were eluted with a NaCl gradient with molarity of 0.15M, 0.3M, 0.5M and 1M, respectively. The protein concentration of each fraction was 3,181 ± 0.06 μg/ml, 2,534 ± 0.1 μg /ml, 1,166 ± 0.4 μg /ml and 1,677 ± 0.5 μg /ml respectively and their electrophoretic pattern is shown in (Figure 3). MALDI-MS/MS analysis identified cathepsin, serpins and endopins proteins in the fractions P1 to P3. Fatty acid binding protein (FABP) was identified as main component of fraction P4 (Table 1). Considering that a diverse range of helminth products have shown to be recognized by TLR4 [32,35-37], we screened P1, P2, P3 and P4 in their capacity to stimulate TLR4 in HEK293-TLR4 cells. Results demonstrate that the levels of SEAP produced by fractions P1, P2 and P3 was suppressed by more than 76% (Figure 4a) in

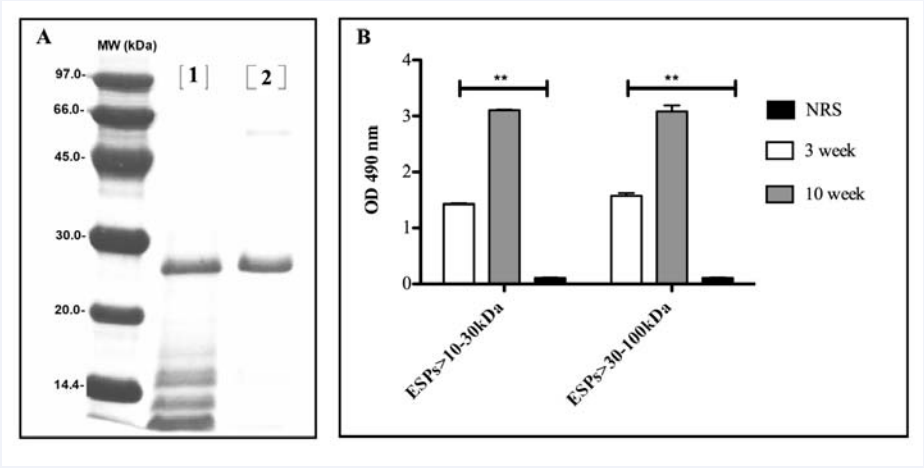

Figure 1 ***Fasciola hepatica*** **ESPs of different ranges of molecular weights are reactive with sera infection.** *F. hepatica* ESPs were separated based on their molecular weights int fractions of ≥10-30kDa ≥30-100kDa. **(A)** Proteins were analyzed by 12% SDS-PAGE stained with coomassie blue. Lanes-1 and 2 shows the protein composition of ESPs ≥10-30kDa and ESPs ≥30-100kDa, respectively. **(B)** Reactivity of ESPs ≥10-30kDa and ESPs ≥30-100kDa with a pool of sera from negative rabbit sera (NRS) and pools of sera from rabbits with acute (3 weeks) or chronic (10 weeks) infection were tested. Dashed line indicates the cut-off value previously determined above which all samples are considered positive. ** Indicate statistical differences $p < 0.001$ between NRS and sera from 3 or 10 week of infection obtained with each antigen.

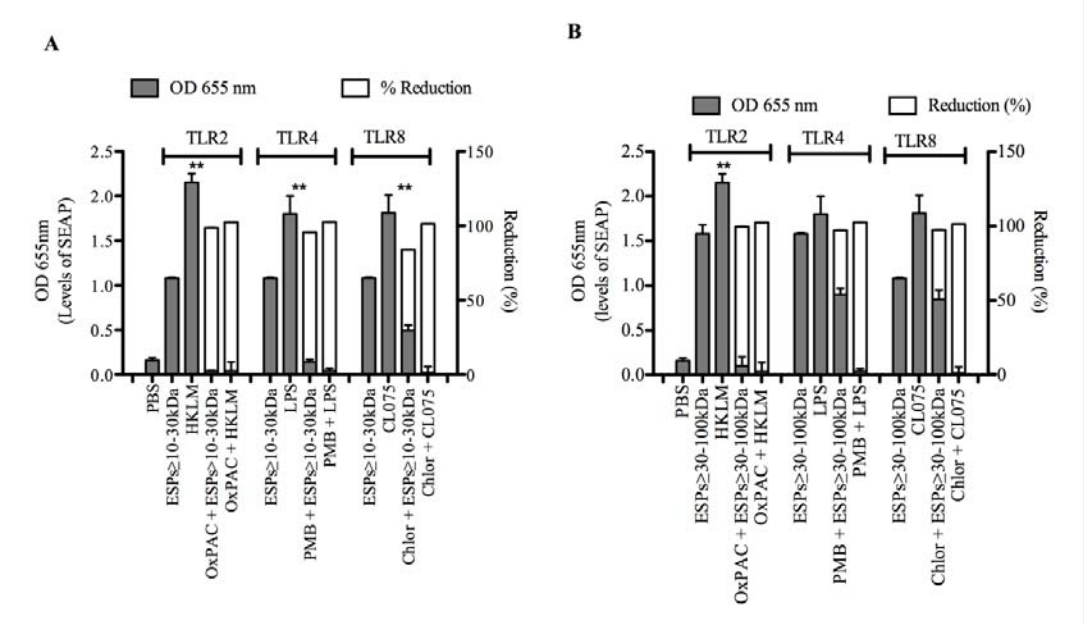

Figure 2 (**Screening system to evaluate the capacity of ESPs≥10-30kDa or ESPs≥30-100kDa to stimulate toll-like receptors of THP1-Blue CD14 cells:** THP1-Blue™-CD14 cells were exposed to ESPs≥10-30kDa **(A)** or ESPs ≥ 30-100kDa **(B).** In both experiments, cells treated with specific agonist for TLR2 (HKLM), TLR4 (LPS) or TLR8 (CL075) was used. After 19 h of incubation at 37°C, 5% CO_2 the QB reagent was added and 7 h later readings at 655nm were made. Grey bars indicate levels of secreted embryonic alkaline phosphatase (SEAP) measured by readings at 655nm indicating specific TLR-stimulation. White bar represent percentage (%) of reduction in the absorbance values obtained when cells were first exposed to OxPAC, PMB or Chlor (inhibitors of TLR2/TLR4, TLR4 and TLR8, respectively) prior stimulation with ESPs≥10-30kDa, ESPs≥30-100kDa fractions, HKLM, LPS or CL075. Reduction % was calculated by the formula: R% = (C-E) / C x 100, where C is the mean absorbance of three replicate obtained when cells are stimulated with antigen or agonists and E is the mean absorbance value obtained when cells are first exposed to the antagonist and then stimulated with the antigen or agonists. ** Indicate significant reductions $P < 0.001$.

the presence of PMB, which confirm that cathepsins, serpin or endopin proteins target TLR4. Cathepsins are lysosomal cysteine proteinases of the papain super family involved in the catabolism of mammalian cell proteins. It has been shown that these enzymes can disrupt immune defense mechanisms directed against parasites by facilitating the migration of parasites through the host tissues and the acquisition of nutrients from the host [38]. Our finding that fractions containing CatL1 are able to stimulate

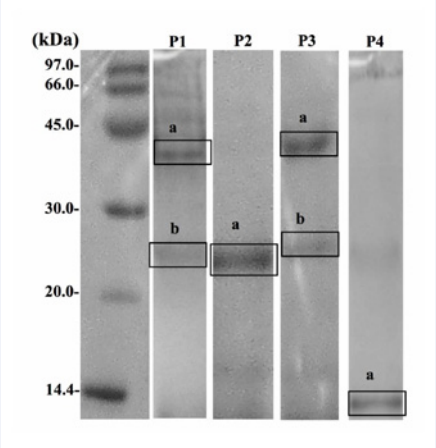

Figure 3 Electrophoretic analysis of fractions separated by ion exchange chromatography: ESPs≥10-30kDa was separated by ion exchange chromatography (IEC) using a Mono Q-HR5/50 column in an FPLC system. Four fractions were collected and termed P1, P2, P3 and P4. IEC-fractions were analyzed by 12.5% SDS-PAGE stained with coomassie blue. Major protein bands from P1, P2, P3 and P4 were excised from gel, designated with letter (a) and (b) and submitted to MALDI-MS/MS analysis.

Table 1: Proteins identified by MALDI-MS/MS infractions recovered when *F. hepatica* ESP ≥10-30kDa were separated by ion exchange chromatography.

Band ID	MW (kDa) (exp. /theor)	Theor. Ip	Pep. Count	Total Ion Score	Description	Specie	Accession No.
P1a	40. 46.3	6.9	9	77	Serpin peptidase inhibitor	*Bos taurus*	DAA17343
P1b	25.0 / 35.0	6.1	9	322	Chain A crystal structure of pro-cathepsin L1	*F. hepatica*	206X-A
P2a	25.0 / 35.1	5.9	8	358	Cathepsin L	*F. hepatica*	AAM11647
P3a	43.0 / 46.2	5.67	12	310	Muscle Endopin 1A	*Bos taurus*	Q9TTE1
P3b	28.0 / 37.0	5.43	10	303	Secreted Cathepsin L2	*F. hepatica*	ABQ95351
P4a	12.0 / 14.7	5.91	7	230	Fatty acid binding protein	*F. hepatica*	Q7M4M0

MALDI-MS/MS: Matrix Assistant Laser Desorption / Ionization With Tandem Mass Spectrometry; MW: Molecular Weight; kDa: kilodalton; Exp./ Theor: Experimental / Theoretical; Pep: Peptide; Ip: Isoelectric Point

TLR4 is consistent with previous studies that have demonstrated this molecule stimulates the secretion of IL6, IL12p40 and MIP2 from dendritic cells and enhanced the expression of CD40 via TLR4 [11]. Serpin and endopins [39-43] are serine protease inhibitors that play key physiological roles in numerous biological systems such as blood coagulation, complement activation, and inflammation. Our findings demonstrating that these molecules can stimulate various TLRs suggest these molecules could have any role in the parasite immunomodulation. The observation that the fraction P4, which contained FABP as a unique component suppressed significantly the activation induced by LPS (Figure 4a) was not surprising because we had already demonstrated that a native FABP purified from a crude extract of *F. hepatica* adult fluke exerted strong suppressive effects in the activation status and inflammatory response from murine macrophages exposed to LPS [18]. However, FABP also suppressed the stimulation induced by HKLM (TLR2-ligand) and CL075 (TLR8-ligand) in THP1-Blue CD14 cells (Figure 4b), which suggests a broader spectrum of action for FABP than those previously reported [18]. Interestingly, such suppressive effect was only evidenced when FABP is partially purified and not when it is mixed with other ESP-components as it was noted by the high activation levels induced by the unpurified ESPs >10-30kDa. Likely this occurs because FABP is a minor component in the ESPs and its effect is minimized in the presence of major components such as CatL, which exert strong immunomodulatory effect by targeting TLR4 [11]. However, if this assumption were correct, would this mean that the suppressive effect of FABP would be irrelevant for the parasite immunomodulation during the *F. hepatica* infection? Studies are in progress to respond this interrogates.

CONCLUSION

Results of the present study suggest that whereas *F. hepatica* cathepsins and protease inhibitors activate TLR4, TLR2, TLR8 and likely also TLR5 and TLR6 other ESP components like fatty acid binding protein can exert an inhibitory effect on all these receptors. Studying how all molecules identified here exert their immunomodulatory effect individually, and in conjunction with other parasite molecules will help understand the immune mechanisms that *F. hepatica* uses to avoid the host's immune response and this knowledge will let us to improve vaccines and to develop novel drugs.

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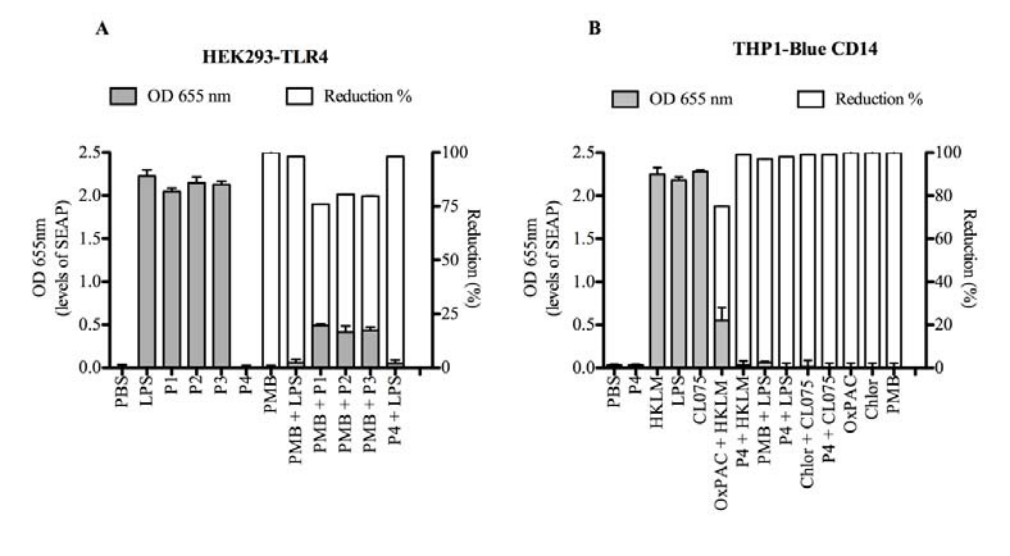

Figure 4 Capacity of ESPs ≥ 10-30kDa fractions eluted from IEC to stimulate or inhibit the activation of various TLRs: (A) In the activation experiments performed with HEK293-TLR4 cells were exposed to 15µg/ml of each IEC-fraction (P1, P2, P3 or P4). Cells treated with LPS (1µg/ml) were used as activation control and cells treated with PMB (100µm) or PBS was used as antagonist or negative control, respectively. In the inhibition experiment cells were first treated with PMB and then stimulated with LPS, P1, P2 or P3. Cells were also treated with P4 and then stimulated with LPS. **(B)** In the experiments performed with THP1-Blue CD14 cells were first cultured with P4 (15µg/ml) and then stimulated with HKLM, LPS or CL075. Cells only stimulated with agonist were used as activation control and cells treated with OxPAC, PMB or Chlor. Were used as antagonist control. Grey bars indicate levels of secreted embryonic alkaline phosphatase (SEAP) measured by readings at 655nm indicating specific TLR-stimulation. White bar represent percentage (%) of reduction in the absorbance values. Reduction % was calculated by the formula: R% = (C-E) / C x 100, where C is the mean absorbance of three replicate obtained when cells are stimulated with antigen or agonists and E is the mean absorbance value obtained when cells are first exposed to the antagonist and then stimulated with the antigen or agonists.

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REFERENCES

1. Berasain P, Carmona C, Frangione B, Dalton JP, Goni F. Fasciola hepatica: parasite-secreted proteinases degrade all human IgG subclasses: determination of the specific cleavage sites and identification of the immunoglobulin fragments produced. Exp. Parasitol. 2000, 94: 99-110.
2. Berasain P, Goni F, McGonigle S, Dowd A, Dalton JP, Frangione B, et al. Proteinases secreted by Fasciola hepatica degrade extracellular matrix and basement membrane components. J Parasitol. 1997; 83: 1-5.
3. Dalton JP, Brindley PJ, Knox DP, Brady CP, Hotez PJ, Donnelly S, et al. Helminth vaccines: from mining genomic information for vaccine targets to systems used for protein expression. Int. J. Parasitol. 2003; 33: 621-640.
4. Harnett W, Harnett MM. Helminth-derived immunomodulators: can understanding the worm produce the pill? Nature Rev. Immunol. 2010; 10: 278-284.
5. Hewitson JP, Grainger JR, Maizels RM. Helminth immunoregulation: the role of parasite secreted proteins in modulating host immunity. Mol Biochem Parasitol. 2009; 167: 1-11.
6. Brady MT, O'Neill SM, Dalton JP, Mills KH. Fasciola hepatica suppresses a protective Th1 response against Bordetella pertussis. Inf Immu. 1999; 67: 5372-5378.
7. Flynn RJ, Mannion C, Golden O, Hacariz O, Mulcahy G. Experimental Fasciola hepatica infection alters responses to tests used for diagnosis of bovine tuberculosis. Inf Immu. 2007; 75: 1373-1381.
8. O'Neill SM, Brady MT, Callanan JJ, Mulcahy G, Joyce P, Mills KH, et al. Fasciola hepatica infection downregulates Th1 responses in mice. Parasite Immunol. 2000; 22: 147-155.
9. Donnelly S, O'Neill SM, Stack CM, Robinson MW, Turnbull L, Whitchurch C, et al. Helminth cysteine proteases inhibit TRIF-dependent activation of macrophages via degradation of TLR3. J. Biol. Chem. 2010; 285: 3383-3392.
10. Donnelly S, Stack CM, O'Neill SM, Sayed AA, Williams DL, Dalton JP. Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. FASEB J. 2008; 22: 4022-4032.
11. Dowling DJ, Hamilton CM, Donnelly S, La Course J, Brophy PM, Dalton J, et al. Major secretory antigens of the helminth Fasciola hepatica activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses. Inf. Immu. 2010, 78(2):793-801.
12. Espino AM, Dumenigo BE, Fernandez R, Finlay CM. Immunodiagnosis of human fascioliasis by enzyme-linked immunosorbent assay using excretory-secretory products. Am J Trop Med. Hyg. 1987; 37: 605-608.
13. Figueroa-Santiago O, Espino AM. Fasciola hepatica Fatty Acid Binding Protein Induces the Alternative Activation of Human Macrophages. Inf Immu. 2014; 82: 5005-5012.
14. Espino AM, Rivera F. Quantitation of cytokine mRNA by real-time RT-PCR during a vaccination trial in a rabbit model of fascioliasis. Vet Parasitol. 2010; 169: 82-92.
15. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Anal. Biochem. 1985; 150: 76-85.

16. Morales A, Espino AM. Evaluation and characterization of Fasciola hepatica tegument protein extract for serodiagnosis of human fascioliasis. Clin. Vaccine Immunol. 2012; 19: 1870-1878.

17. Sotillo J, Valero ML, Sanchez Del Pino MM, Fried B, Esteban JG, Marcilla A, et al. Excretory/secretory proteome of the adult stage of Echinostoma caproni. Parasitol. Res. 2010; 107: 691-697.

18. Martin I, Caban-Hernandez K, Figueroa-Santiago O, Espino AM. Fasciola hepatica fatty acid binding protein inhibits TLR4 activation and suppresses the inflammatory cytokines induced by lipopolysaccharide in vitro and in vivo. J Immunol. 2015; 194: 3924-3936.

19. Kendall S, Parfitt J. The chemotherapy of fascioliasis. British Vet J. 1962; 118: 1-10.

20. Zafra R, Perez-Ecija RA, Buffoni L, Moreno P, Bautista MJ, Martinez-Moreno A, et al. Early and late peritoneal and hepatic changes in goats immunized with recombinant cathepsin L1 and infected with Fasciola hepatica. J Comp Patho. 2013; 148: 373-384.

21. Zafra R, Perez-Ecija RA, Buffoni L, Pacheco IL, Martinez-Moreno A, LaCourse EJ, et al. Early hepatic and peritoneal changes and immune response in goats vaccinated with a recombinant glutathione transferase sigma class and challenged with Fasciola hepatica. Res Vet Sci. 2013; 94: 602-609.

22. Adams PN, Aldridge A, Vukman KV, Donnelly S, O'Neill SM. Fasciola hepatica tegumental antigens indirectly induce an M2 macrophage-like phenotype in vivo. Parasite Immunol. 2014; 36: 531-539.

23. Donnelly S, O'Neill SM, Sekiya M, Mulcahy G, Dalton JP. Thioredoxin peroxidase secreted by Fasciola hepatica induces the alternative activation of macrophages. Inf. Immu. 2005; 73: 166-173.

24. Guasconi L, Serradell MC, Masih DT. Fasciola hepatica products induce apoptosis of peritoneal macrophages. Vet. Immunol. Immunopathol. 2012; 148: 359-363.

25. Serradell MC, Guasconi L, Cervi L, Chiapello LS, Masih DT. Excretory-secretory products from Fasciola hepatica induce eosinophil apoptosis by a caspase-dependent mechanism. Vet Immunol Immunopathol. 2007; 117: 197-208.

26. Rushton B, Murray M. Hepatic pathology of a primary experimental infection of Fasciola hepatica in sheep. J.Comp. Pathol. 1977; 87: 459-470.

27. Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen Fasciola hepatica: proteins associated with invasion and infection of the mammalian host. Mol Cell Proteomics. 2009; 8: 1891-1907.

28. Carnevale S, Rodriguez MI, Guarnera EA, Carmona C, Tanos T, Angel SO. Immunodiagnosis of fasciolosis using recombinant procathepsin L cystein proteinase. Diag Microbiol Inf Dis. 2001; 41: 43-49.

29. Carnevale S, Rodriguez MI, Santillan G, Labbe JH, Cabrera MG, Bellegarde EJ, et al. Immunodiagnosis of human fascioliasis by an enzyme-linked immunosorbent assay (ELISA) and a micro-ELISA. Clin Diag Lab Immunol. 2001; 8: 174-177.

30. Espino AM, Diaz A, Perez A, Finlay CM. Dynamics of antigenemia and coproantigens during a human Fasciola hepatica outbreak. J. Clin. Microbiol. 1998; 36: 2723-2726.

31. Mozer TJ, Tiemeier DC, Jaworski EG. Purification and characterization of corn glutathione S-transferase. Biochem. 1983; 22: 1068-1072.

32. van Riet E, Everts B, Retra K, Phylipsen M, van Hellemond JJ, Tielens AG, et al. Combined TLR2 and TLR4 ligation in the context of bacterial or helminth extracts in human monocyte derived dendritic cells: molecular correlates for Th1/Th2 polarization. BMC Immunol. 2009; 10: 9.

33. Marcilla A, Trelis M, Cortes A, Sotillo J, Cantalapiedra F, Minguez MT, et al. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS One. 2012; 7: 45974.

34. Aksoy E, Zouain CS, Vanhoutte F, Fontaine J, Pavelka N, Thieblemont N, et al. Double-stranded RNAs from the helminth parasite Schistosoma activate TLR3 in dendritic cells. J Biol Chem. 2005, 280: 277-283.

35. Goodridge HS, Marshall FA, Else KJ, Houston KM, Egan C, Al-Riyami L, et al. Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted products ES-62. J. Immunol. 2005; 174: 284-293.

36. Shen J, Xu L, Liu Z, Li N, Wang L, Lv Z, et al. Gene expression profile of LPS-stimulated dendritic cells induced by a recombinant Sj16 (rSj16) derived from Schistosoma japonicum. Parasitol Res. 2014; 113: 3073-3083.

37. Weatherill AR, Lee JY, Zhao L, Lemay DG, Youn HS, Hwang DH. Saturated and polyunsaturated fatty acids reciprocally modulate dendritic cell functions mediated through TLR4. J Immunol. 2005; 174: 5390-5397.

38. Mas-Coma MS, Esteban JG, Bargues MD. Epidemiology of human fascioliasis: a review and proposed new classification. Bull World Health Organ. 1999; 77: 340-346.

39. Ghendler Y, Arnon R, Fishelson Z. Schistosoma mansoni: isolation and characterization of Smpi56, a novel serine protease inhibitor. Exp Parasitol. 1994; 78: 121-131.

40. Hook VY, Hwang SR. Novel secretory vesicle serpins, endopin 1 and endopin 2: endogenous protease inhibitors with distinct target protease specificities. Biol Chem. 2002; 383: 1067-1074.

41. Hook VY, Yasothornsrikul S, Hwang SR. Novel chromaffin granule serpins, endopin 1 and endopin 2: endogenous protease inhibitors with distinct target protease specificities. Ann NY Acad Sci. 2002; 971: 426-444.

42. Hwang SR, Steineckert B, Toneff T, Bundey R, Logvinova AV, Goldsmith P, et al. The novel serpin endopin 2 demonstrates cross-class inhibition of papain and elastase: localization of endopin 2 to regulated secretory vesicles of neuroendocrine chromaffin cells. Biochemistry. 2002; 41: 10397-10405.

43. Zang X, Yazdanbakhsh M, Jiang H, Kanost MR, Maizels RM. A novel serpin expressed by blood-borne microfilariae of the parasitic nematode Brugia malayi inhibits human neutrophil serine proteinases. Blood. 1999; 94: 1418-1428.

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