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Fasciola hepatica – the pilot study of *in vitro* assessing immune response against native and recombinant antigens of the fluke

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Abstract

Fasciola hepatica is a liver fluke that infects 2.4 million of people and causes great economical loss in animal production. To date a 100% effective vaccine has not been developed and the disease is controlled by drug therapy. Great efforts are put into development of effective vaccine against parasite what is difficult since *Fasciola* spp. (like other helmints) during evolutionary process has developed sophisticated and efficient methods to evade immune response. During preliminary experiments it is convenient to use cell lines which are relatively cheap and allow for reproducible comparison of results between laboratories. We stimulated BOMA (bovine monocyte/macrophage cell line) and BOMAC (bovine macrophage cell line) with native or recombinant antigens of *Fasciola hepatica* and assessed IFN-γ, IL-4 and TNF-α level upon stimulation. We observed diminished secretion of proinflammatory TNF-α in LPS activated BOMA cells stimulated with Excretory/Secretory products of adult fluke (Fh-ES). We also observed greater changes in gene expression in LPS activated BOMA cells than in non activated BOMA cells upon stimulation using Fh-ES. The results show possibility of using cell lines for *in vitro* research of bovine immune response against liver fluke, although this model still requires validation and further characterization.

Keywords

Fasciola hepatica, antigens, immune response

Introduction

Fasciola hepatica is a liver fluke capable of infecting a wide range of mammals, including humans (2.4 million people infected and 180 million at risk) (McManus and Dalton 2006). It is also described as a problem in veterinary medicine, primarily due to infection of cattle, sheep and goats. The infection occurs after ingestion of metacercaria-contaminated food or water. The parasite migrates through the intestinal wall and abdominal cavity to the liver, where it grows and matures, causing inflammation, hemorrhages and fibrosis of liver parenchyma, thickening and dilatation (and sometimes calcification in cattle) of bile ducts. The economic losses in animal production, caused by reduction in milk yield, diminished fertility and weight gain, that are estimated at US$ 2 billion per year (McManus and Dalton 2006; Schweizer *et al*. 2005). To date a 100% effective vaccine has not been developed and control of the disease is achieved by drug use, which has led to resistance (Overend and Bowen 1995, Moll *et al*. 2000; Lane 1998) and a rise in concerns about drug residues in animal food products. Much of the research towards developing an efficient vaccine against liver fluke has focused on the cathepsin proteases, but there are also other antigens of interest. This has been reviewed previously by Wędrychowicz and Klockiewicz (1994) or Norbury *et al*. (2012).

Laboratory animal models do not always emulate the immune responses of target species. When working on vaccines

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for humans, scientists may process preliminary experiments using cell lines to determine the types of immune responses generated by the investigated antigens; however, for many other animals this tool is still to be developed due to a paucity of suitable cell lines.

Macrophages are one of the initial components of an immune response and can have an influence on shifting the type and magnitude of the subsequent immune response. In this study we estimated the suitability of two cell lines of bovine monocytes/macrophages for preliminary testing of the response against antigens. The cells were stimulated using *F. hepatica* Excretory/Secretory products (Fh-ES) known to interfere with host immune system causing e.g. induction of apoptosis in macrophages (Guasconi *et al*. 2012) and eosinophils (Serradell *et al*. 2009) and increase of macrophage arginase activity (Guasconi *et al*. 2011). *Fasciola hepatica* ES is also connected with high levels of immunosuppressive IL-10 and TGF-β (Guasconi *et al*. 2011). We also tested other important antigens, Fh-PGK, Fh-TPX and Fh-APR, for their influence on both bovine macrophage cell lines. Fh-PGK was tested in a previous study as a vaccine antigen (Jaros *et al*. 2010). Fh-TPX induces alternatively activated macrophages (Donnelly *et al*. 2005). *F. hepatica* aspartic proteases (like metalloproteases) have not been as deeply investigated as *F. hepatica* cysteine proteases (Norbury *et al*. 2011; McVeigh *et al*. 2012; Zawistowska-Deniziak *et al*. 2013), but apparent orthologs of *F. hepatica* aspartic protease (Fh-APR), a cathepsin D, have been widely investigated among other helminths – hookworms for their expression, biochemical and physiological function (Ranjit *et al*. 2009; Wasyl *et al*. 2013, Williamson *et al*. 2003, 2004) and vaccine potential (Pearson *et al*. 2009; Pearson *et al*. 2010; Loukas *et al*. 2005).

Materials and Methods

Obtaining newly excysted juvenile (NEJ) stage of *F. hepatica*

Metacercariae were obtained from the culture of a bovine strain of *F. hepatica* cultivated at the Witold Stefański Institute of Parasitology (Warsaw, Poland). For obtaining NEJ stadium slightly modified procedure described by Wilson *et al*. (1998) was used. Metacercariae were incubated at room temperature for 20 h, centrifuged (5 min, 1000 × g) and incubated for 60 min in 1% (w/v) pepsin and 0.2% HCl. The metacercariae were centrifuged rinsed with water, centrifuged again and incubated in CO_2 saturated 0.2 M Na_2SO_4 (37°C 1 h). After that the metacercariae were centrifuged and put in RPMI1640 supplemented with amphotericin B (2 µg/ml), gentamycin (10 µg/ml) and 2% taurocholic acid. After hatching NEJs were collected and transferred to fresh medium. The medium was changed every 2 hours to collect ES products which was used in other experiments. After the incubation NEJs were centrifuged and stored at –70°C.

Cloning of cDNA fragments encoding *F. hepatica* aspartic protease (Fh-APR) and *F. hepatica* tioperrodoxin (Fh-TPX)

RNA was isolated from NEJ using RNA Isolation Kit (A&A Biotechnology). First strand cDNA was generated by reverse transcription reaction using the pTXho primer (AGA GAA CTA GTC TCG AGT TTT TTTTTTTTTTT TTT) and Reverse Transcriptase (MBI Fermentas) according to manufacturer's protocol. For cloning of cDNA fragments, pairs of primers were used: Fh-aprL (GGATCCGCATGATTCAAGCATAAG) and Fh-aprP (TCTAGAGTATGACTTCAGTGGTCGGATTC) for cloning of *Fh-apr*; Fh-tpxL (CTGTCCTCT GGGTACGGCTGAT) and Fh-tpxP (TAGTCTCGAGTTTTTT TTTTTTTTTTT) for cloning of *Fh-tpx*. The achieved cDNA was used as a template in PCR for amplification of DNA fragments encoding *Fh-apr* and *Fh-tpx* with the primers mentioned above. The PCR products were separated in agarose gel, extracted and cloned into the pGEM-T EASY (Promega) vector and sequenced. The cloning procedure of another cDNA investigated in this paper – *Fh-pgk* (GenBank: FJ666097.1) was described elsewhere (Bąska *et al*. 2013a).

Expression of Fh-APR, Fh-TPX and Fh-PGK in *E. coli* and purification of the recombinant proteins

Primers Fh-apr-pETleft_*Bam*HI (GCGGATCCGACGTTTTAAGGACTAAA) and Fh-apr-pETright_*Xho*I (TGCTCGAGTTAAACCAACAAATTCCAAAAGC), or Fh-tpx-pETleft_*Eco*RI (CGGAATTCATGTGTGATCGCGATCAG) and Fh-tpx-pETright_*Xho*I (CCGCTCGAGCTAGTTGGCTGAGGAGAAA) were designed to create constructs allowing expression of respectively Fh-APR and Fh-TPX in *E. coli*. PCR products were separated in agarose gel, extracted from the gel and cloned into the pGEM-T EASY vector. Recombined vectors pGEM-T EASY/*Fh-apr* and pGEM-T EASY/*Fh-tpx* were digested with *Bam*HI and *Xho*I, or *Xho*I and *Eco*RI respectively. The digestion mixtures were separated in agarose gel, followed by extraction of the DNA fragments. The extracted fragments were ligated to pET28a+ vector digested with the appropriate pair of restriction enzymes. The vectors pET28a+/*Fh-apr* and pET28a+/*Fh-tpx* were maintained in *E. coli* DH5α, prior to transformation into expression strains: *E. coli* BL21 and *E. coli* ROSETTA. The detailed procedure for cloning of *Fh-pgk* into pET28a+ was described elsewhere (Bąska *et al*. 2013a).

Single colonies of *E. coli* BL21, and *E. coli* ROSETTA, carrying pET28a+/*Fh-apr*, pET28a+/*Fh-tpx*, or pET28a+/*Fh-pgk*, were used to inoculate overnight cultures in LB medium supplemented with kanamycin (60 µg/ml) (for *E. coli* BL21) or LB medium supplemented with kanamycin (60 µg/ml) and glucose (0.5%) (for *E. coli* ROSETTA). The overnight cultures were used to inoculate 500 ml of media, and were incubated (shaking) at 37°C to OD_{600} = 0.6. Protein expression was induced by adding 500 µl of 1 M IPTG. One milliliter samples were collected after 1, 2 and 3 hours and

the bacterial pellets after centrifugation were stored at –20°C until used.

The presence of the recombinant proteins was assessed using Western blot. The bacterial pellets were dissolved in SDS Sample Buffer and separated by SDS-PAGE followed by transfer to a nitrocellulose membrane, then the recombinant proteins were detected using anti-His antibody conjugated with horseradish peroxidase (Sigma-Aldrich).

To assess if the proteins were soluble in non-denaturing conditions, the bacterial pellets were dissolved in Equilibration buffer (10 mM imidazol, 0.3 M NaCl, 50 mM Na_3PO_4, pH 8), sonicated, then centrifuged. The supernatants were retained, the pellets were again dissolved in Equilibration buffer, sonicated and centrifuged. The supernatants and the pellets were retained. The Western blot analysis for the presence of the recombinant proteins in both supernatants and bacterial pellets was performed as described above.

E. coli cultures (300 ml) producing Fh-TPX, Fh-PGK or Fh-APR were spun and pellets were frozen (–20°C). The pellets (containing expressed Fh-TPX and Fh-PGK) were suspended in 30 ml of Equilibration buffer (50 mM Na_3PO_4, 0.3 M NaCl 10 mM imidazole, pH 8), sonicated and centrifuged (30 min, 4°C, 20 000 × g). Supernatants were retained and passed through a 0.22 µM filter. Pellet containing Fh-APR was suspended in Equilibration buffer for denaturing purification (0.1 M Na_3PO_4, 8 M Urea), sonicated and incubated for 16 hours (4°C) gently shaking. The mixture was centrifuged, supernatant was retained and passed through a 0.22 µM filter. Recombinant proteins were purified using His-affinity Gel (Sigma Aldrich) according to manufacturer`s protocol. Fh-TPX and Fh-PGK were purified in non-denaturing buffers whereas Fh-APR was purified using denaturing buffers (supplemented with 8 M urea).

To purify recombinant proteins from endotoxins the slightly modified procedure of Aida and Pabst (1990) was applied. The recombinant proteins were dialyzed against PBS using an AMICON filter (Millipore). 900 µl of the solutions of the recombinant proteins were combined with 100 µl of 10% TRITON X-114 followed by shaking (4°C, 20 min), incubation (37°C, 20 min) and centrifugation (30°C, 16 000 × g, 20 min). The aqueous phases were retained and the procedure was repeated 4 times. To eliminate TRITON X-114 residues incubation (30 min, 37°C) and centrifugation (30°C, 16 000 × g, 30 min) were repeated.

Expression of Fh-APR, Fh-TPX and Fh-PGK in *Pichia pastoris*

In order to clone cDNA encoding Fh-APR, Fh-TPX and Fh-PGK into the yeast expression vector pPICZαA, the mentioned cDNA fragments were amplified using primer pairs: Fh-apr_PichL (AAGAATTCGACGTTTTAAGGACTAAACTAC) and Fh-apr_PichR1 (ATGATGATGATGATGAACCAGATGAACCAACAAATTCCAAAAGC); Fh-tpx_PichiaL (ATGAATTCATGTGTGATCGCGATCAGTGCT) and Fh-tpx_PichiaR1 (ATGATGATGATGGTTGGCTGAGGAGGA); or Fh-pgk_PichiaL (ATGAATTCATGGGTCTGAAAAAGTTG) and Fh-pgk_PichiaR1 (ATTCTAGATTAATGATGATGATGATGATGGGCGT). The PCR products were separated in agarose gel, extracted, and used as a template for a second round of PCR. Briefly, first round PCR products for *Fh-apr*, *Fh-tpx* and *Fh-pgk* were used as a template and amplified using primer pairs: Fh-apr_PichL and Fh-apr_PichR2 (TTTCTAGATTAATGATGATGATGATGATGAACCAAC); Fh-tpx_PichiaL and Fh-tpx_PichiaR2 (GCTCTAGACTAATGATGATGATGATGATGGTTG); or Fh-pgk_PichiaL and Fh-pgk_PichiaR2 (ATTCTAGATTAATGATGATGATGATGATGGGCGT). The products were separated in agarose gel, extracted, and cloned into the pGEM-T EASY vector. The recombinant vectors pGEM-T EASY/*Fh-apr*, pGEM-T EASY/*F-tpx*, pGEM-T EASY/*Fh-pgk* were digested with *Eco*RI and *Xba*I enzymes. The inserts were ligated to the previously *Eco*RI and *Xba*I digested pPICZαA vector and cloned. The mentioned procedure with two rounds of PCR was applied since it allowed creation of a 6 x His sequence at the C terminus of the recombinant protein. The pPICZαA vector is designed to add a *c-myc* epitope and 6 x His sequence at the C-terminus of the protein. Due to concerns that the *c- myc* epitope might interfere with immune assays we used this detailed procedure to allow construction of the vector expressing proteins with a 6 x His sequence and without the c-*myc* epitope.

Each of the constructed vectors (pPICZαA/*Fh-apr*, pPICZαA/*Fh-tpx*, and pPICZαA/*Fh-pgk*) was linearized and introduced into cells of *Pichia pastoris* X-33, GS115 and KM71H using electroporation. The cells were seeded on YPD agar plates with zeocin (100 ug/ml). Single colonies were picked and 25 ml of BMG and BMGY were inoculated and grown for 48 h, vigorously shaking (28°C). The cultures were centrifuged (3 min, 3 000 × g) and supernatants were discarded. The yeast pellets from centrifuged BMG and BMGY medium were suspended in 200 ml of BMM or BMMY respectively. One milliliter of each sample was taken at 12, 24, 48, 72, 96 hours after inoculation. Each sample was centrifuged (1 min, 10 000 × g). Supernatants and pellets were frozen in separate tubes. The presence of recombinant proteins in yeast cells and in the medium was determined using SDS-PAGE and Western blot as previously described for determination of expression in *E. coli.*

Expression of Fh-APR, Fh-TPX and Fh-PGK in S2 cells

To clone cDNA encoding *Fh-apr*, *Fh-tpx* or *Fh-pgk* into the yeast expression vector pMT/Bip/V5-His the mentioned cDNAs were amplified with primer pairs: Fh-apr_S2L (TTAGATCTGACGTTTTAAGGACTAAACTAC) and Fh-apr_S2R (TTACCGGTAAACCAACAAATTCCAAAAGC); Fh-tpx_S2L (AAAGATCTATGTGTGATCGCGATCAGTG) and Fh-tpx_S2R (ACCGGTGTTGGCTGAGGAGAAATA); Fh-pgk_S2L (AAAGATCTATGGGTCTGAAAAAGTTGAGTATT) and Fh-pgk_S2R (TTACCGGTGGCGTTAGTGAG

TGCA). The products were cloned into pGEM-T EASY as outlined previously. The recombinant vectors pGEM-T EASY/ *Fh-apr*, pGEM-T EASY /*Th-tpx*, pGEM-T EASY/*Fh-pgk* were digested with *Bgl*II and *Age*I enzymes. The inserts were ligated to previously *Bgl*II and *Age*I digested pMT/Bip/V5-His vector and cloned. The vectors: pMT/Bip/V5-His/*Fh-apr*, pMT/Bip/V5-His/*Fh-tpx*, and pMT/Bip/V5-His/*Fh-pgk* were introduced to S2 cells using a Calcium Phosphate Transfection Kit (Invitrogen). The transfected cells were cultivated in 6 deep well plates (Nunc) in 2 ml of Schneider`s medium supplemented with 10% Fetal Bovine Serum (FBS) to a concentration of 3 x 10^6 cells/ml. The expression of the recombinant proteins was induced by adding $CuSO_4$ to a final concentration of 500 µM. Samples were taken daily for 4 days after induction. Each sample was centrifuged, and the supernatants and pellets were frozen in separate tubes. The presence of the recombinant proteins in insect cells and in the medium was determined using SDS-PAGE and Western blot as previously described for determination of expression in *E. coli.*

S2 cells were also transfected with each constructed vector (pMT/Bip/V5-His/*Fh-apr*, pMT/Bip/V5-His/*Fh-tpx*, and pMT/Bip/V5-His/*Fh-pgk*) and pCoBLAST (a vector giving resistance for blasticidin) and grown in Schneider's medium supplemented with 10% FBS and blasticidin. Protein expression was induced by adding $CuSO_4$ (500 µM) in 2 kinds of media: Schneider`s medium and Schneider`s medium supplemented with 10% FBS. Both cultures, after 4 days of incubation, were centrifuged, supernatants were dialyzed against Equilibration buffer and the proteins were purified using Nickel His affinity gel (Sigma Aldrich). The presence of recombinant proteins in elution fractions was determined using Western blot as previously outlined for determination of the expression in *E. coli.*

Collection of ES from adult *F. hepatica*

The detailed procedure is outlined elsewhere (Bąska *et al*. 2013a). Briefly, adult flukes were isolated from the livers of experimentally infected rats and incubated in PBS (with buffer changes) at 37°C for 2 h, then in RPMI 1640 medium at 37°C, 5% CO_2 for 2.5 h. The medium was discarded and flukes were incubated in RPMI 1640 medium replaced at 60 – 80 min intervals, with a total of 7 media changes. Each RPMI 1640 medium fraction containing Fh-ES was stored at –20°C. The obtained Fh-ES fractions were concentrated using an AMICON filter (Millipore) with a 3 kDa cut-off.

Bovine monocytes/macrophages cultivation and stimulation

We used the bovine monocyte/macrophage cell lines: BOMA (BioNutriTech) and macrophage cell line – BOMAC (Stabel and Stabel 1995) – a kind gift of Dr Judith Stabel (USDA-ARS-NADC, USA). BOMA cells were cultivated in DMEM (Gibco) supplemented with 5% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin. Trypsynization was used to detach cells from the plastic because of the adherent character of the cells. BOMAC cells were cultivated in RPMI1640 (Sigma-Aldrich) with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin. The cells were detached from the plastic with a cell scraper. For cultivation of both cell lines all purchased media and plastics were certified as endotoxin free. BOMAC cells were seeded at a concentration of 2 x 10^5 cells/ml in 1 ml of medium in 24 deep well plates (Nunclon). The BOMAC cells were stimulated (in quadruplicate) with Fh-TPX, Fh-PGK, or adult fluke ES (Fh-ES) at concentrations of 1 µg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml and 0.1 ng/ml for 40 hours. BOMAC cells were also activated with LPS (1 µg/ml) incubated for 2 hours and stimulated (without removal of LPS) with Fh-TPX, Fh-PGK (expressed in *E. coli*) for additional 40 hours, or BOMAC cells were activated with LPS (1 µg/ml) for 5 hours and stimulated (without removal of LPS) with Fh-ES for additional 40 hours at concentrations of 1 µg/ml, 100 ng/ml, 10 mg/ml, 1 ng/ml 0,1 ng/ml. Cells were scratched and centrifuged. Supernatants and pellets were stored at –20° C for further analysis. BOMA cells were seeded 5 x 10^5 cells/ml in 1 ml of medium in 24-deep well plates (Nunclon) and stimulated (in triplicate) with Fh-ES (5 µg/ml) for 48 hours. BOMA cells were also activated with LPS (1 µg/ml) for 60 min and stimulated with 5 µg/ml of Fh-ES (without removal of LPS) for 48 hours.

Determination of IFN-γ, TNF-α, IL-4 using ELISA

The concentration of IFN-γ, TNF-α, and IL-4 in the medium from stimulated cells was determined using IFN-γ Screening Set (Thermo Scientific), Bovine IL-4 Screening Set (Thermo Scientific), and Bovine TNF-alpha DuoSet (R&D Systems). The analyses were performed according to manufacturers` instructions.

Microarray analysis

Total RNA was isolated from BOMA cells stimulated with Fh-ES (5 µg/ml) and control cells using Total RNA Isolation Kit (A&A Biotechnology). Contamination with genomic DNA was eliminated using DNAseI (MBI Fermentas) according to the manufacturer`s protocol. The efficiency of the reaction was confirmed by PCR. RNA was purified using phenol-chloroform extraction. The concentration of the RNA was determined spectrophotometrically, followed by labeling with Cy3 and Cy5 using Quick Amp Labeling (Agilent Technologies). The further steps of the microarray experiment were performed according to the manufacturer`s instructions (Agilent Technologies).

Another microarray experiment was also undertaken. RNA was isolated from BOMA cells activated using LPS and stimulated with Fh-ES (5 µg/ml) and from control (LPS activated BOMA cells). Further steps of the experiment were done as described above, except of the labeling which was performed using Low Input Quick Amp Labeling (Agilent Technologies). The statistical analysis was performed in GeneSpring program

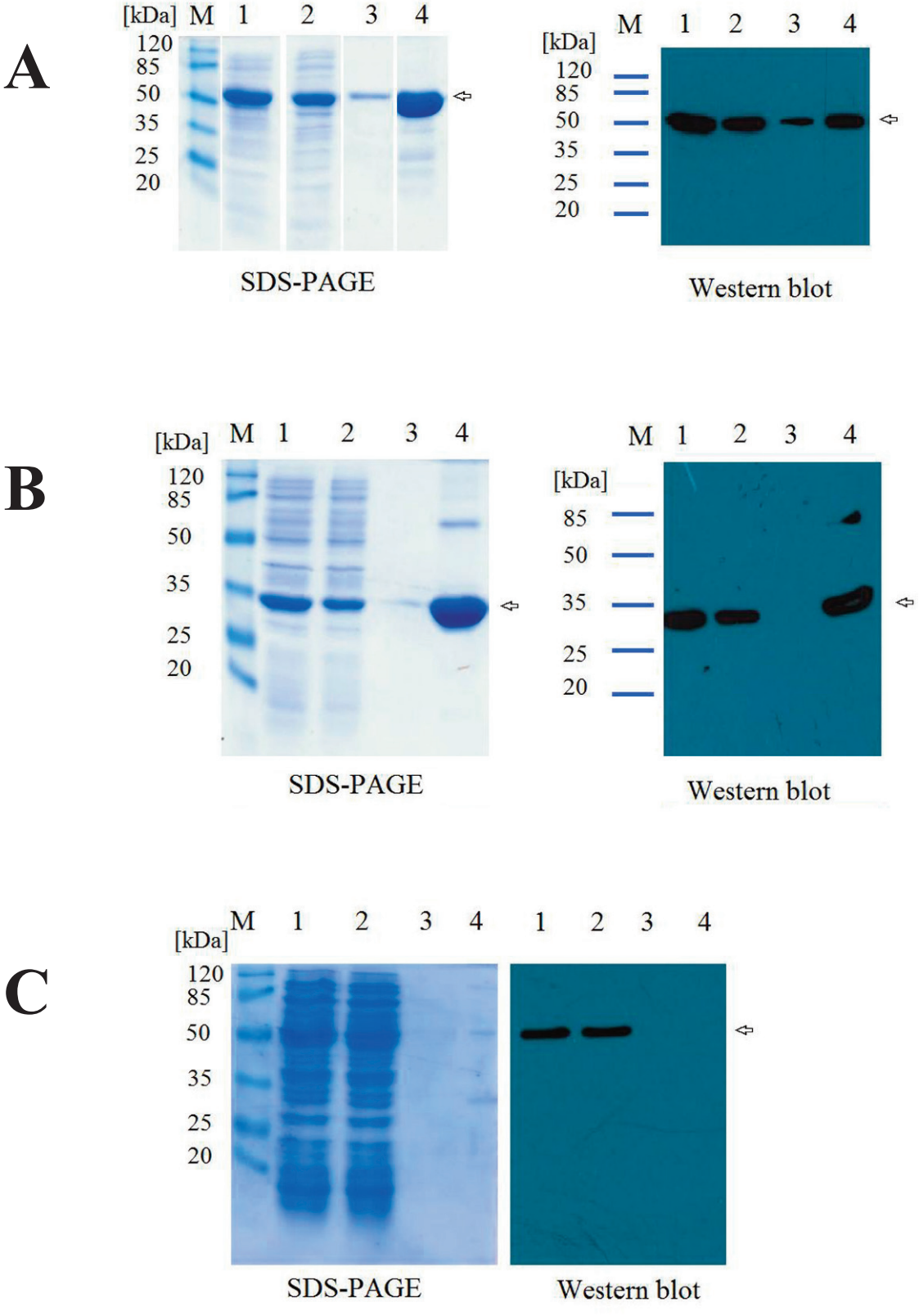

Fig. 1. SDS – PAGE and Western blot analysis of Fh-PGK **(A)**, Fh-TPX **(B)** and Fh-APR **(C)** purification. Lane 1 – bacteria lysate before incubation with his affinity gel. Lane 2 – bacteria lysate after incubation with His affinity gel. Lane – 3 proteins that detached from the gel during rinsing with Equilibration buffer. Lane 4 – elution fraction

using t-test. The genes with over 2-fold change of expression ($p < 0.05$) were considered as significant. The involvement of the identified genes in various biological process was determined using PANTHER Classification System.

Results

Cloning of cDNA fragments encoding Fh-APR and Fh-TPX

Cloned cDNAs coding regions of *Fh-apr* and *Fh-tpx* were deposited in GenBank and were given accession numbers: FJ168036.1 and FJ168037.1, respectively.

Expression of Fh-APR, Fh-PGK and Fh-TPX in *E. coli* and purification of the recombinant proteins

The expression of Fh-APR, Fh-PGK and Fh-TPX was induced using 1 M IPTG. The expression lasted up to 3 hours. Using Western blot we determined the expression of Fh-APR, Fh-PGK and Fh-TPX 1, 2 and 3 hours after induction. We did not determine significant changes during the course of expression for the investigated proteins. For use in immune assays, the proteins need to be soluble. We determined if the proteins were soluble in Equilibration buffer, which allows purification of the protein in non-denaturing conditions. Fh-APR was insoluble in non-denaturing buffers although it was soluble in the buffer containing 8 M Urea. Fh-PGK and Fh-TPX were soluble in non-denaturing Equilibration buffer. After determining the solubility we purified Fh-PGK and Fh-TPX in non-denaturing conditions (Fig 1) and Fh-APR in denaturing conditions although Fh-APR (Fig 1) did not attach to the His affinity gel.

Expression of Fh-APR, Fh-TPX and Fh-PGK in *Pichia pastoris*

Pichia pastoris strains X-33, GS115 and KM71H were transfected with vectors (pPICZαA/*Fh-apr*, pPICZαA/*Fh-tpx*, pPICZαA/*Fh-pgk*) allowing expression of the investigated antigens. The expression was performed in BMMY and BMM medium. The analysis of expression was performed using Western blot although no expression was determined.

Expression of Fh-APR, Fh-TPX and Fh-PGK in S2 cells

S2 cells were cotransfected with pCoBLAST and expression vectors (pMT/Bip/V5-His/*Fh-apr*, pMT/Bip/V5-His/*Fh-tpx*,

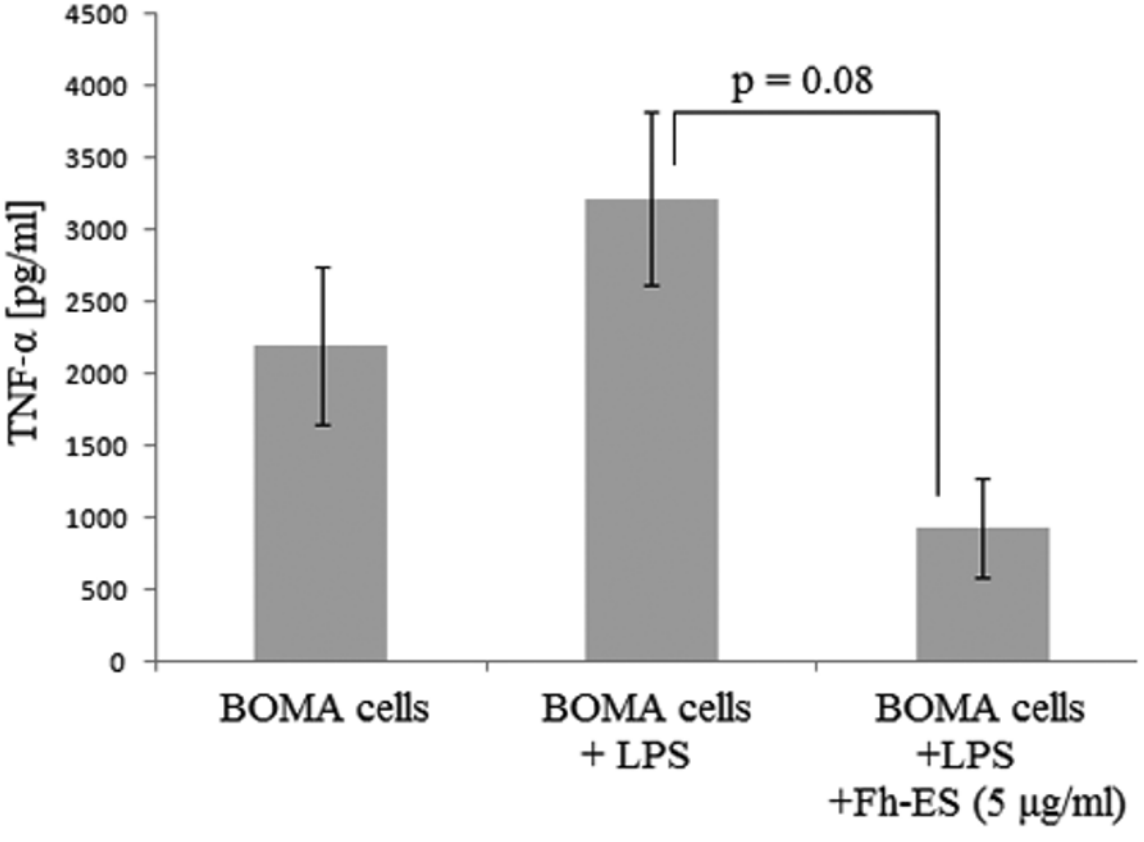

Fig. 2. Level of TNF-α secreted by BOMA cells. BOMA cells were incubated alone, stimulated with LPS for 48 hours or incubated with LPS for 60 min and then stimulated (without removal of LPS) with Fh-ES for additional 48 hours. There was statistically significant ($p = 0.08$) difference in TNF-α concentration between LPS activated cells and LPS activated cells stimulated with Fh-ES

pMT/Bip/V5-His/*Fh-pgk*) allowing expression of the investigated antigens. Stable lines were selected due to the resistance for blasticidin given by transfection using pCOBLAST. Expression was performed in Schneider`s medium with or without supplementation with 10% FBS. The analysis of expression was performed using Western blot although no expression was determined.

Determination of IFN-γ, TNF-α, IL-4 using ELISA

BOMAC cells and LPS activated BOMAC cells were stimulated with Fh-TPX, Fh-PGK or Fh-ES for 40 hours and the level of IL-4 and IFN-γ in surrounding medium was measured. None of the measurements was higher than background (data not shown). We checked a difference in TNF-α level secretion between BOMAC cells non-activated and activated (for 40 hours) with LPS. In both cases we observed no cytokine level in the medium (data not shown). We also investigated influence of LPS and Fh-ES on TNF-α secretion by BOMA cells. LPS did not affected TNF-α secretion, but we observed decreased level ($p = 0.08$) of TNF-α in medium of LPS activated BOMA cells stimulated with Fh-ES when compared to medium of LPS activated BOMA cells (Fig. 2).

Microarray analysis

To determine the influence of Fh-ES on BOMA cells and LPS activated BOMA cells we performed the microarray analysis. 23 genes showed change of expression level after stimulation BOMA cells with Fh-ES (12 genes were up regulated and 11 were down regulated). When LPS activated BOMA cells were stimulated with Fh-ES we noted change in expression of 319 genes (100 genes were up regulated, 219 genes were down regulated). To assess which process were affected by Fh-ES we analyzed genes showing changed expression level using PANTHER Classification System what showed influence of Fh-ES on various biological processes. The detailed analysis and number of genes that changed expression upon stimulation is shown in Fig. 3.

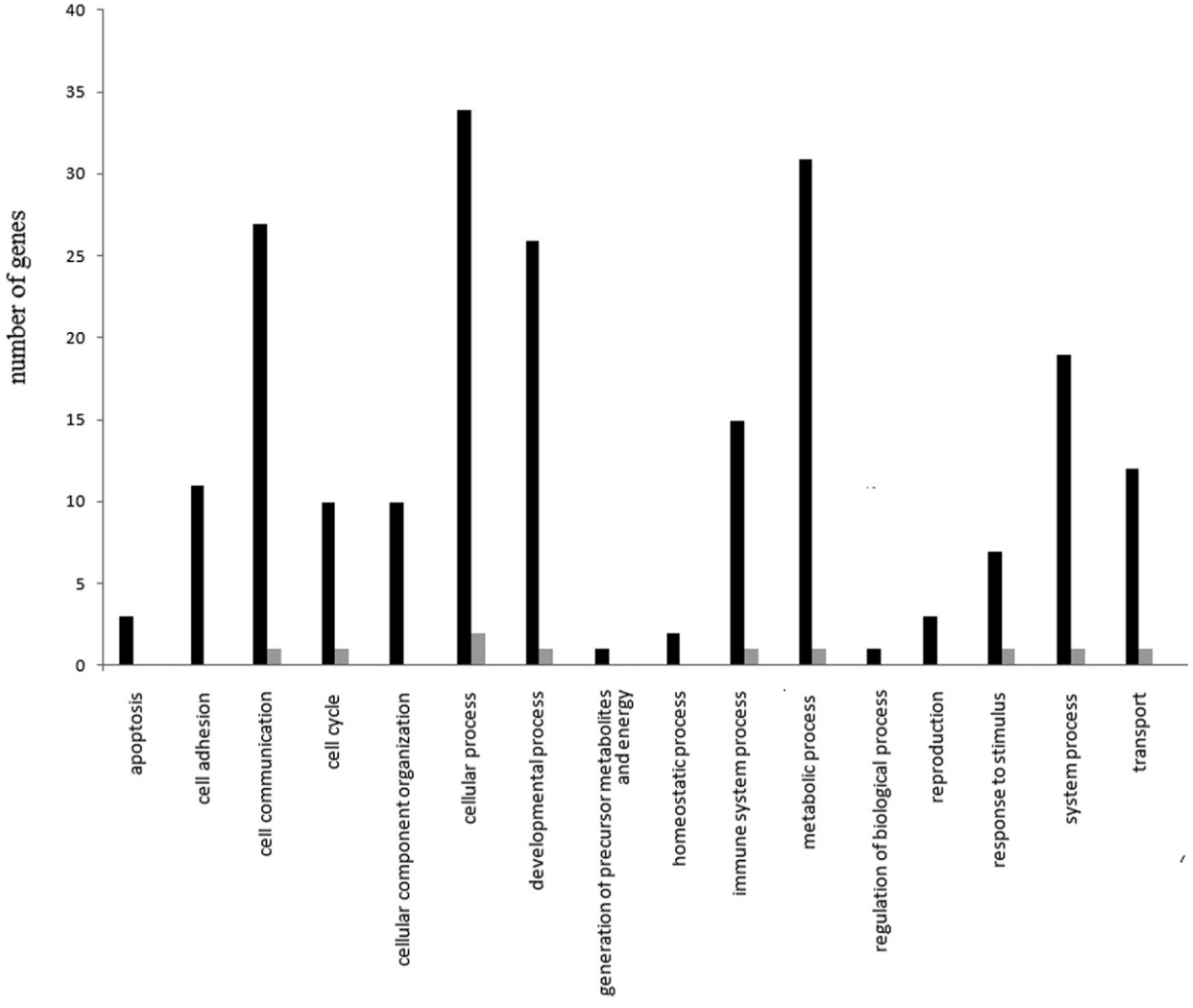

Fig. 3. Number of genes engaged in particular biological process that changed expression level after stimulation BOMA cells (grey bars) and LPS activated BOMA cells (black bars) with Fh-ES

Discussion

In this paper we described cloning of two and expression of three antigens form NEJ stage of *F. hepatica*: Fh-APR, Fh-TPX and Fh-PGK. Fh-TPX was soluble when expressed in *E. coli*. Salazar-Calderón *et al*. (2000) also managed to purify *F. hepatica* thioredoxin peroxidase. Although in that paper the authors used a different tag: GST, and during the purification procedure Triton X-100 detergent was used. Fh-PGK was also easily purified in non-denaturing conditions, although during purification of recombinant Fh-PGK from adult fluke, denaturing buffer (containing 4 M Urea) was applied during disrupting of bacterial cells (Jaros *et al*. 2010). We encountered problems when expressing Fh-APR in *E. coli.* Other literature data show that hookworm aspartic protease is insoluble when expressed in *E. coli* (Pearson *et al*. 2009) which is consistent with our results. Fh-APR shares only 55.1 % (data not shown) of amino acid similarity to *Ancylostoma caninum* Aspartic Protease 1 what may explain the differences in expression between these two aspartic proteases in yeast cells. We also tried to express the Fh-APR, Fh-TPX and Fh-PGK in S2 cells, but no expression was detected. Essentially, it is not possible to predict for any expression system if the protein will be expressed, in what yield and if it will be soluble or not. The protein might be expressed as truncated (De Maere *et al*. 2005) and slight variation in amino acid sequence may change heterologous protein solubility (Sreekrishna *et al*. 1998).

The assays with BOMAC cells showed no response to stimulation with either recombinant or native antigens of *F. hepatica*. BOMAC cells are suitable hosts for bovine virus cultivation (Donofrio *et al*. 2005), and were used for investigating interactions with intracellular parasite (*Theileria annulata*) gene (Shiels *et al*. 2004) or for analysis of gene expression after virus infection (Rola *et al*. 2011). We stimulated BOMA monocytes/macrophages with Fh-ES what resulted in no TNF-α change, but LPS activated BOMA cells diminished release of TNF-α after Fh-ES stimulation. Flynn and Mulcahy (2008) proved that blood mononuclear cells stimulated with Fh-ES release IL-4 in a time related manner after infection. In the beginning of the infection no IL-4 was observed, the level of IL-4 reached its highest point in 2nd or 6th week and was decreasing until the 12th week, when the infection became chronic. Although the variation between individual animals was observed. Other results shows that IFN-γ secretion by whole blood cultures from trickle infected calves, stimulated with adult and immature *F. hepatica* antigens, reaches a maximum 2 to 5 weeks post infection (Clery and Mulcahy 1998). Those results, supported by ours (BOMAC cells secreted neither IL-4, nor IFN-γ upon stimulation with Fh-ES), would suggest that host immune cells need to be previously stimulated by other parasite antigens during course of infection. LPS induces marked TNF-α release *in vitro* (Bąska *et al*. 2013b) in human THP-1 macrophages, so we wanted to asses if triggering a proinflammatory response might change the response to Fh-ES. We noted that LPS activated BOMA cells release lower amounts of TNF-α after Fh-ES stimulation. Fh-ES impairs the activation of dendritic cells (DC) induced by TLR ligands (Falcón *et al*. 2010), but the lack of a change of IL-4 and IFN-γ or TNF-α during our experiments might be caused by a non-physiological behavior of BOMAC cells, due to an absence of response observed to LPS. However, this question is still to be solved since BOMAC cells retain properties of ingestion of opsonised bacteria (Jolly *et al*. 2011) which indicates their phagocytic character. Bone marrow derived mouse dendritic cells do not change secretion of IL-6, 10, 12p70, TNF-α upon stimulation with Fh-ES, but they release significant lower amounts of those cytokines upon stimulation with LPS and Fh-ES than stimulated with LPS only (Falcón *et al*. 2010). This was also confirmed in this paper by BOMA cell line for TNF-α secretion. In general helminths bias immune response towards Th_2 phenotype as well as induce overall down-regulation of immune system (van Riet *et al*. 2007). TNF-α is rather associated with proinflammatory Th_{17} response (Weaver *et al*. 2006). The diminished TNF-α secretion in LPS activated BOMA cells stimulated by Fh-ES may be one of the parasite mechanism to down regulate proinflamatory response. Decrease in TNF-α concentration may also result in lower concentration of nitric oxide (NO), toxic to the worm, released by macrophages classically activated by IFN-γ and TNF-α.

The microarray experiments showed changes in a low number of genes in Fh-ES stimulated cells, while LPS activation of BOMA cells together with Fh-ES stimulation resulted in changes of expression to a dramatically higher number of genes. Nevertheless, to determine even small changes (in this preliminary screening microarrays analysis) in gene expression we used moderate criteria. Stringent analysis showed no changes in expression pattern. This might suggest that LPS activated monocytes/macrophages are more sensitive to Fh-ES than unactivated cells, but this is to be determined in further research by determining changes in protein level instead of mRNA. In conclusion, *in vitro* research with bovine monocyte/macrophage cell lines is promising and appears worth exploring, although for now further validation is required to provide more insight.

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