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Fasciola hepatica Fatty Acid Binding Protein Inhibits TLR4 Activation and Suppresses the Inflammatory Cytokines Induced by Lipopolysaccharide In Vitro and In Vivo

Ivelisse Martin, Kimberly Cabán-Hernández, Olgary Figueroa-Santiago, and Ana M. Espino

TLR4, the innate immunity receptor for bacterial endotoxins, plays a pivotal role in the induction of inflammatory responses. There is a need to develop molecules that block either activation through TLR4 or the downstream signaling pathways to inhibit the storm of inflammation typically elicited by bacterial LPS, which is a major cause of the high mortality associated with bacterial sepsis. We report in this article that a single i.p. injection of 15 μ g fatty acid binding protein from *Fasciola hepatica* (Fh12) 1 h before exposure to LPS suppressed significantly the expression of serum inflammatory cytokines in a model of septic shock using C57BL/6 mice. Because macrophages are a good source of IL-12p70 and TNF- α , and are critical in driving adaptive immunity, we investigated the effect of Fh12 on the function of mouse bone marrow–derived macrophages (bmM Φ s). Although Fh12 alone did not induce cytokine expression, it significantly suppressed the expression of IL-12, TNF- α , IL-6, and IL-1 β cytokines, as well as inducible NO synthase-2 in bmM Φ s, and also impaired the phagocytic capacity of bmM Φ s. Fh12 had a limited effect on the expression of inflammatory cytokines induced in response to other TLR ligands. One mechanism used by Fh12 to exert its anti-inflammatory effect is binding to the CD14 coreceptor. Moreover, it suppresses phosphorylation of ERK, p38, and JNK. The potent anti-inflammatory properties of Fh12 demonstrated in this study open doors to further studies directed at exploring the potential of this molecule as a new class of drug against septic shock or other inflammatory diseases. *The Journal of Immunology*, 2015, 194: 3924–3936.

Helminths, known as masters of immunomodulation, use several immunomodulatory strategies to evade and/or modify immune responses to survive into the mammalian host for long periods (1). The parasitic helminth *Fasciola hepatica* causes fascioliasis, an emerging important human disease that affects around 17 million persons worldwide (2, 3). Fascioliasis also infects livestock, causing economic losses estimated at more than \$3 billion annually (4). As with many other helminths, *F. hepatica* polarizes the immune system of the host to a dominant Th2/T regulatory status with suppression of inflammatory responses (5–8). As a result, hosts infected with *F. hepatica* are rendered more susceptible to secondary bystander

infections, such as with *Bordetella pertussis* and *Mycobacterium tuberculosis*, which require Th1 immunity for protection (7–9).

The potent immune suppression exerted by *F. hepatica* is mediated by the copious amounts of excretory-secretory products (ESPs) released by the parasite, particularly the cathepsin-L peptidases, which represent ~80% of ESPs. Studies have demonstrated that ESPs of *F. hepatica* can mimic the immunomodulatory effect that is observed during active infection, without the tissue pathology, and also suppress the development of the Th1 response (5, 6). For example, administration of *F. hepatica* Cathepsin-L1 cysteine protease suppressed the onset of protective Th1 immune responses to bacterial infections in mice (6) and prevented the development of a Th1 response in mice inoculated with *B. pertussis* vaccine (8, 10). GST, another major Ag comprising 4% of ESPs, inhibited the proliferation of rat spleen cells in response to Con A stimulation in vitro (11). Both Cathepsin-L1 and GST were shown to partially activate dendritic cells (DCs) via TLR4, a pattern recognition receptor, using different intracellular signaling pathways (12). Other *F. hepatica* polypeptides that also play a role in host immunomodulation are the tegument Ags. The tegument constitutes the parasite–host interface and is the place where much of the immune interplay between the fluke and the host occur (13–17). *F. hepatica* tegumental Ags (FhTeg) have been shown to significantly suppress the serum levels of IFN- γ and IL-12p70 and to suppress expression of the cell-surface markers CD80, CD86, and CD40 by targeting multiple TLRs of DCs (18). Moreover, FhTeg have also been shown to impair DC function in a mouse model of septic shock by inhibiting their phagocytic capacity and ability to prime T cells (18).

Proteomic studies have demonstrated that both ESPs and tegument Ags consist of highly complex mixtures of polypeptides that include proteolytic enzymes, transporters, membrane-associated

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Abbreviations used in this article: A₆₅₅, absorbance read at 655 nm; bmM Φ , bone marrow–derived macrophage; DC, dendritic cell; ESP, excretory-secretory product; FABP, fatty acid binding protein; Fh12, FABP from *F. hepatica*; FhTeg, *F. hepatica* tegumental Ag; HEK293, human embryonic kidney 293 cell; HKLM, heat-killed *Listeria monocytogenes*; IEF, isoelectric focusing; iNOS2, inducible NO synthase-2; LBP, LPS binding protein; MD2, myeloid differentiation protein-2; PLA, proximity ligation assay; PMB, polymyxin B; poly(I:C), polyinosinic-polycytidylic acid; qPCR, quantitative RT-PCR; RT, room temperature; SEAP, secreted embryonic alkaline phosphatase; XTT, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate.

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proteins, antioxidants, and many other trematode-specific proteins (19–22). Members of the 12-kDa fatty acid binding protein (FABP) family have been identified in most of these studies. FABPs play an essential role in parasite nutrition and have been recently categorized as antioxidant molecules (23). These proteins can potentially prevent oxidative damage to trematode cellular components by binding fatty acids and ions involved in oxidative stress (23, 24). Previous studies have shown that vaccines containing FABPs induce partial immune protection in experimentally infected mice and sheep (25, 26). Moreover, *F. hepatica* FABPs also appear to be important molecules for inducing cross-immunity against *Schistosoma* species (27). Although numerous published articles have explored the vaccine potential of *F. hepatica* FABPs, no studies have investigated whether FABPs have anti-inflammatory effects or whether they may interact with cells of the host immune system.

In this study, we purified native forms of FABP (named Fh12 [FABP from *F. hepatica*]) from adult fluke extract and investigated the anti-inflammatory properties of the purified protein *in vitro* and *in vivo*. To our knowledge, this study is the first to report the anti-inflammatory properties of *F. hepatica* FABP, providing evidence of its mechanism of action.

Materials and Methods

Animals

C57BL/6 female mice, 6–8 wk old, were purchased from Charles River Laboratory (Wilmington, MA). B6.129S4 CD14 knockout (CD14^{-/-}) female mice (on C57BL/6 background), 6–8 wk old, were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were kept under conventional germ-free conditions in the animal care facility of the University of Puerto Rico School of Medicine, and treated according to Institutional Animal Care and Use Committee Standards (protocol 7870215).

Preparation of soluble whole-worm extract of adult *F. hepatica*

F. hepatica adult worms were homogenized with a Teflon homogenizer in 0.1 M PBS, pH 7.4, in the presence of a protease inhibitor mixture and then centrifuged at 30,000 × *g* for 30 min at 4°C as previously described (28). The supernatant was used immediately or stored at –20°C until use for the purification of FABPs.

Purification of 12-kDa native Fh12

Native Fh12 was purified from whole-worm extract of adult *F. hepatica* using a previously optimized protocol (29) that involves an initial ultracentrifugation step at 30,000 × *g*, followed by gel filtration chromatography with Sephadex G-50 (XK 26/100 column) and two consecutive preparative isoelectric focusing (IEF). Each aliquot from the last IEF separation was subjected to 12.5% SDS-PAGE, and the proteins were visualized by silver staining or Coomassie blue staining. Fractions that exhibited a single polypeptide band of around 12 kDa were manually excised from the gel and their identity as FABP confirmed by MALDI and tandem mass spectrometry as previously described (29, 30). Fractions were pooled and termed Fh12.

Production of anti-Fh12 polyclonal Ab

A New Zealand White rabbit was immunized by s.c. injection with 200 µg purified Fh12 protein mixed with an equal amount of CFA. The rabbit was boosted twice with equal amounts of protein mixed with IFA at 2-wk intervals. The rabbit IgG fraction was purified using protein A affinity chromatography (GE Healthcare).

Endotoxin removal

Endotoxins were removed from the Fh12 by using polymyxin B (PMB) columns (31) according to the manufacturer's instructions. The presence of endotoxins was assessed before and after removing endotoxins using the Chromogenic *Limulus* Amebocyte Lysate QCL-1000 Assay (Lonza, Walkersville, MD) following the manufacturer's instructions. Endotoxin levels were quantified using a standard curve and reported as endotoxin units per milliliter. Protein concentration was adjusted to 1 mg/ml as determined by the bicinchoninic acid method using a Pierce protein assay kit

(Pierce, Cambridge, NJ). Purified endotoxin-free Fh12 was stored in aliquots at –20°C until use.

Septic shock

Groups of 5 animals each were injected i.p. with Fh12 (15 µg for each mouse), 1 h before i.p. injection with LPS (*E. coli* 0111:B4, 1 µg per each mouse). Control mice received PBS, Fh12, or LPS only (i.p.). Mice were sacrificed by cervical dislocation 12 h later, and blood samples were collected from orbital vein or by cardiac puncture. Serum concentration of IFN-γ, IL-12p70, TNF-α, GM-CSF, IL-3, IL-9, IL-10, IL-15, and IL-10 were measured by cytokine microarrays (RayBiotech, Norcross, GA).

Isolation and treatment of bone marrow-derived macrophages

Cells were collected from femoral shafts of mice by flushing with 3 ml cold sterile PBS. The cell suspensions were passed through a sieve to remove large clumps, washed three times with sterile complete RPMI 1640 medium (supplemented with 20 mM L-glutamine, 1 ml penicillin and streptomycin/100 ml medium, and 10% heat-inactivated FCS; Sigma-Aldrich). Cells were adjusted to 0.5 × 10⁶ cells/well with differentiation medium (complete RPMI 1640 supplemented with 20 ng/ml M-CSF; R&D Systems) and cultured in 24-well plates (Nunc) at 37°C, 5% CO₂. On day 3 of culture, nonadherent cells were removed and the adherent cells were placed in fresh differentiation medium, and the incubation was prolonged for 7 d to cause full maturation of macrophages, which was assessed by FACS analysis and 4/80 surface Ag expression.

Bone marrow-derived macrophages (bmMΦs) were seeded into 24-well plates (Nunc) at 10⁶/ml in complete RPMI 1640 medium and then treated with a predetermined concentration of Fh12 (5 µg/ml) (29) for 30 min before being exposed to heat-killed *Listeria monocytogenes* (HKLM; 10⁸ cells/ml), polyinosinic-polycytidylic acid [poly(I:C)] (100 µg/ml), LPS (100 ng/ml), flagellin (1 µg/ml), imiquimod (10 µg/ml), or thiazoloquinolone (CL075, 10 µg/ml). Control cells were treated with PBS, Fh12, or TLR ligands alone.

TLR screening

Fh12-induced stimulation of TLRs was assayed in THP1-Blue-CD14 cells expressing all TLRs, as well as a reporter gene (secreted embryonic alkaline phosphatase [SEAP]) driven by the NF-κB promoter (Invivogen, San Diego, CA). Each TLR was induced with a known specific ligand as a positive control. Fh12 was assayed in concentrations between 1 and 20 µg/ml. Upon TLR stimulation, cells activate transcription factors and subsequently the secretion of SEAP, which is detected by using QUANTI-Blue medium that turns purple/blue in the presence of SEAP. Absorbance was read at 655 nm (A₆₅₅). Cells stimulated with PBS-endotoxin free were used as negative controls.

Phagocytosis assay

The phagocytic ability of bmMΦs was measured using a CytoSelect 96-well phagocytosis assay (Cell Biolabs) that uses enzyme-labeled *Escherichia coli* particles as a phagocytosis pathogen. In brief, bmMΦs were plated at 0.5 × 10⁶ cell/ml and treated with Fh12 (5 µg/ml), LPS (100 ng/ml) alone, or Fh12 and LPS at the same time 2.5 h before addition of 10 µl/well of *E. coli* suspension. After 6 h of undisturbed incubation, the supernatants were gently aspirated and adherent cells were fixed with 3.2% buffered formaldehyde solution, and blocked for 30 min at room temperature (RT) in an orbital shaker. Cells were washed three times with PBS and permeabilized by incubation with 100 µg/well of 1% Triton-X100 in PBS for 5 min. After another washing step, the substrate solution was added and the mixture was incubated for 30 min at RT. The reaction was stopped and absorbance was read at 450 nm. Cells treated with 2 µM cytochalasin D were used as control for phagocytosis inhibition as per the manufacturer's instructions.

NF-κB activation in TLR4-transfected HEK cells

To investigate the mechanism of interaction between Fh12 and TLR4, we used Human Embryonic Kidney 293 cells (HEK293; Invivogen). These cells (HEK293-TLR4) are cotransfected with genes encoding cluster differentiation Ag-14 (CD14), myeloid differentiation protein-2 (MD2), TLR4 coreceptor, and a SEAP reporter gene (HEK293-TLR4). Cells were maintained in DMEM supplemented with 4.5 g/l glucose, 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml Normocin, and 2 mM L-glutamine. For all experiments, a cell suspension was prepared using HEK-Blue Detection medium at 14 × 10⁴ cells/ml. For stimulation experiments, cells were seeded at 2.52 × 10⁴ cells/well in 96-well flat-bottom plates and treated with Fh12 (from 0.62 to 10 µg/ml) or LPS (5 µg/ml), and incubated

at 37°C, 5% CO₂ for 24 h. In the inhibition experiments, cells were cultured with Fh12 (0.6–10 µg/ml) 30 min before LPS (5 µg/ml) stimulation. Cells cultured with PMB (100 µM; Invivogen) were used as an antagonist control. In other experiments, cells were stimulated with LPS (5 µg/ml) and then cultured with Fh12 (5 µg/ml) 30 min, 1, 2, and 4 h after LPS stimulation. All readings were done at 655 nm after 24 h of LPS stimulation. Cells treated with PBS were used as a negative control. Cells treated with LPS were used as a positive control. The percent of reduction (R%) of the absorbance values was calculated by the formula $R(\%) = [1 - (A - C/B - C)] \times 100$, where A is the mean A₆₅₅ of three replicates obtained when cells were stimulated with Fh12, B is the mean A₆₅₅ value obtained when cells were exposed to LPS, and C is the mean A₆₅₅ of three replicates obtained when cells were stimulated with PBS.

Cell viability

To determine whether our experimental conditions affect cell viability of HEK293-TLR4 cells or macrophages, we seeded cells at 2.52×10^4 cells/well in 96-well flat-bottom plates or at 0.5×10^6 cells/well and cultured in 24-well plates, respectively. Cells were treated with LPS (100 ng/ml) and/or Fh12 (5 µg/ml) for 24 or 48 h. After incubation, cell viability was assessed by adding 50 µl sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) labeling reagent (Roche Life Science) to each well. After an additional incubation of 24 h at 37°C, the absorbance of each well was read at 480 nm.

Quantitative real-time PCR analysis

Total RNA was extracted using a PureLink RNA Mini kit (Invitrogen) followed by treatment with Turbo DNase free endonuclease (Ambion, Grand Island, NY) to remove contaminating genomic DNA. RNA was quantified using a Nanodrop-1000 spectrophotometer (Thermo Scientific) and reverse transcribed to cDNA by a high-capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA). cDNA was amplified using a StepOne Plus Real-Time PCR system (Applied Biosystems) with cDNA equivalent to 5 ng total RNA and SYBR green PCR master Mix (Applied Biosystems). The cycling conditions were as follows: 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 55°C for 30s, and 72°C for 30s. The primers used for each gene are listed in Table I. Primer concentration was optimized and dissociation curves were generated for each primer set to verify the amplification of a single PCR product. Quantitative RT-PCR (qPCR) experiments were conducted in triplicate using a StepOne Plus real-time PCR system (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method (32) was used to quantify relative gene expression using β-actin as an internal control and expressed as fold change relative to expression in the control (cells stimulated with PBS). The values reported are the mean of three replicates. The SD of the mean is shown as error bars in each group.

Protein extraction and Western blot

Total protein was extracted from cell lysates using radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% NaDodSO₄, and protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Cells were incubated in the extraction buffer on ice using a rocking platform for 30 min before being centrifuged at $20,000 \times g$ for 10 min at 4°C. Supernatants were transferred to clean tubes, and protein concentrations were determined using a bicinchoninic acid protein assay kit (31).

Protein samples (20 µg) and prestained protein markers (Precision Plus protein standards; Bio-Rad) were separated by 10% SDS-PAGE and blotted onto 0.45-µm nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 h at RT in 3% albumin in PBS and incubated overnight at 4°C with the primary Ab. The Abs used in this study were mouse mAb to TLR4 (2 µg/ml), goat polyclonal Ab to CD14 (1 µg/ml), rabbit polyclonal Ab to MD2 (2 µg/ml; Imgenex, San Diego, CA), mouse anti-β-actin (0.37 µg/ml; Sigma-Aldrich), or rabbit anti-GAPDH (1:10,000; Abcam, Cambridge, MA). In the MAPK pathway studies, we used rabbit mAbs anti-phospho-p44/42 MAPK (Erk1/2) (Thr²⁰²/Tyr²⁰⁴) and anti-p44/42 MAPK (Erk1/2) (Thr²⁰²/Tyr²⁰⁴) diluted 1:2000 (Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), anti-p38 MAPK (D13E1), anti-phospho-SAPK/JNK (Thr¹⁸³/Tyr¹⁸⁵), anti-SAPK/JANK (56G8) Abs, and rabbit anti-GAPDH Ab diluted 1:10,000. Membranes were washed in 50 mM Tris-HCl-buffered saline pH 7.5 (TBS) with 0.1% Tween 20 and incubated for 1 h at RT with the secondary Ab (anti-mouse IgG, anti-goat IgG, or anti-rabbit IgG Ab) labeled with biotin. After additional washing steps, membranes were incubated 30 min at RT with a solution of avidin-peroxidase (Sigma-Aldrich) diluted 1:10,000 in PBS. Proteins were detected by addition of ECL substrate (Thermo Scientific). Densitometry analysis was performed on all immuno-

blots. Values were normalized to the PBS-treated control group, and all values are expressed in arbitrary units as a percentage increase over the PBS control group.

LPS binding protein/LPS interaction assay

The Endoblock LPS binding protein (LBP) ELISA test kit (Hycult Biotech, Plymouth Meeting, PA) was used according to the manufacturer's instructions to investigate whether Fh12 disrupts the interaction between LPS and LBP. In brief, binding of biotinylated LPS to precaptured human LBP was assessed using streptavidin peroxidase, followed by addition of tetramethylbenzidine. Fh12 (0.05–10 µg/ml) was preincubated for 30 min with biotinylated LPS and added to wells precoated with anti-LBP + LBP. PMB (0.2–100 µM), which competes with LBP for binding to LPS, was used as a control for inhibition by preincubation with biotinylated LPS.

Immunofluorescence staining

HEK293-TLR4 cells and bmMΦs from naive and CD14 knockout mice were grown for 48 h to 50% confluence on microscope coverslips and then treated for 4 h with LPS (1 µg/ml), Fh12 (5 µg/ml), or PBS. After the incubations, the cells were fixed for 10 min at 4°C in cold acetone/methanol (1:1). After several washes with cold PBS, the cells were incubated for 30 min at RT with blocking solution (0.2% w/v gelatin, 0.5% w/v BSA in cold PBS). After removing the blocking solution, we placed the slides in a humidity chamber and incubated them overnight at 4°C on a rocking platform with primary Abs (rabbit anti-Fh12 IgG [1:50], mouse anti-lipid A [1:10]; Abcam ab8467) or goat anti-CD14 (15 µg; Imgenex IMG 3991) diluted in blocking solution. The cells were then washed six times 5 min each with cold blocking solution and incubated for 1 h with the corresponding secondary Ab (anti-rabbit IgG, anti-goat IgG, or anti-mouse IgG) labeled with FITC diluted 1:2000 in blocking solution. Nuclear chromatin was stained by incubation for 5 min in 0.5 mg/ml DAPI (Bio-Rad). Preparations were mounted using ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). Images were observed with a Zeiss Observer Z1 confocal laser-scanning microscope coupled to a Zeiss LSM 510 Meta EC. The system was controlled using Zeiss ZEN 2009 software.

Immunostaining by in situ proximity ligation assay

Proximity ligation assay (PLA) was performed in HEK293-TLR4 cells to determine whether Fh12 could be interacting with CD14 coreceptor. Cells were grown to 50% confluence on BD BioCoat poly-D-lysine eight-well culture slides (BD Biosciences, Franklin Lakes, NJ), and after 4 h of treatment with Fh12, LPS, or PBS, were fixed as described earlier. After three washes with cold PBS, the cells were incubated with blocking buffer for 30 min. After removing the blocking solution, slides were placed in a humidity chamber and incubated overnight at 4°C on an orbital platform with the pair of primary Abs rabbit anti-Fh12 IgG and mouse anti-lipid A IgG or anti-Fh12 IgG and goat anti-CD14 IgG at the concentrations described earlier. Protein-protein interactions were detected using a Duolink II PLA orange kit composed of anti-rabbit PLA probe minus, anti-mouse PLA probe minus, and anti-goat probe plus (OLINK Bioscience, Uppsala, Sweden) following the manufacturer's instructions. Images were observed with a Zeiss Observer Z1 confocal laser-scanning microscope coupled to a Zeiss LSM 510 Meta EC. The system was controlled using Zeiss ZEN 2009 software.

Docking studies

The protein sequence of *F. hepatica* FABP1 (Fh15, Q7M4G0.3) was obtained from the UniProt database. No structures of these parasite proteins are known; thus, models were prepared using the Protein Homology/AnalogY Recognition Engine (PHYRE) server (33), which predicts protein structure based on homology modeling. Human CD14 coreceptor structure (4GLP) was obtained from the PDB database (34). Proteins were docked using the ClusPro server (35, 36). The top 10 balanced models were manually examined using PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.4; Schrödinger), and the distances between the LPS binding site and FABP1 residues were examined and evaluated for relevance.

Statistical analysis

All data were analyzed for normality before statistical testing. When comparisons of the values for multiple groups were made, data were analyzed using one-way ANOVA. For comparison of values for two groups, the Student *t* test was used using GraphPad Prism software (Prism-6). For all tests, a *p* value <0.05 was deemed significant.

Results

Purification of native 12-kDa Fh12

Purification of native Fh12 was previously optimized and published in detail (37). After the purification process, fractions containing a polypeptide band of around 12 kDa with isoelectric points of 5.1 to 7.25 were confirmed as FABP1 by mass spectrometry and its purity verified by 12.5% SDS-PAGE as recently reported (29). Fh12 gave endotoxin levels <0.1 endotoxin units/ml, which is similar to background levels and to complete RPMI 1640 medium, so the purified Fh12 protein was considered to be endotoxin free.

Fh12 suppresses proinflammatory cytokines in vivo in a model of septic shock

Because it has been reported that helminthic infections, including inoculation with *F. hepatica* and other parasite Ags, prevent the symptoms of inflammatory diseases (38, 39), we assessed whether Fh12 could suppress proinflammatory and inflammatory cytokines in vivo using a mouse model of septic shock. As expected, i.p. injection of 1 μ g LPS alone induced significantly higher levels of serum IFN- γ ($p < 0.0001$), IL-12p70 ($p < 0.0002$), IL-3 ($p < 0.0005$), IL-9 ($p < 0.045$), IL-10 ($p < 0.0001$), IL-15 ($p < 0.0001$), and TNF- α ($p < 0.0021$) compared with injection of PBS. Injections of Fh12 (15 μ g) alone induced cytokine responses similar to those induced by PBS (data not shown). However, in-

jection of mice with 15 μ g Fh12 30 min before LPS injection resulted in significantly reduced levels of IFN- γ ($p < 0.0001$), GM-CSF ($p < 0.006$), IL-12p70 ($p < 0.0002$), IL-3 ($p < 0.0003$), IL-9 ($p < 0.0029$), IL-10 ($p < 0.0001$), IL-15 ($p < 0.0001$), and TNF- α ($p < 0.0006$) cytokines compared with LPS alone (Fig. 1).

Fh12 modulates the production of inflammatory markers from *bmMΦs* in response to LPS

Macrophages are very specialized APCs, and when stimulated in vitro, they are excellent producers of proinflammatory cytokines. We previously demonstrated that Fh12 exerts a strong immunomodulatory effect on human macrophage-derived monocytes (29). Our results in this study demonstrate that in the presence of Fh12 alone, *bmMΦs* do not express proinflammatory or inflammatory cytokines, which suggests that Fh12 does not activate any TLR on macrophages. However, treatment of *bmMΦs* with Fh12 before LPS stimulation resulted in a significant suppression of TNF- α ($p < 0.0029$), IL-1 β ($p < 0.0081$), IL-12p35 ($p < 0.0006$), and IL-6 ($p < 0.0152$) cytokines and inducible NO synthase-2 (iNOS2) ($p < 0.0104$) (Fig. 2) (Table I). Concurrently to the suppression of IL-12p35, we also found the subunit IL-12p40 to be significantly overexpressed ($p < 0.0298$) in cells treated with Fh12 + LPS in comparison with cells that were treated with LPS only. An excess of the IL-12p40 subunit may exert a profound inhibitory effect on IL-12p70 functions, as has been reported in other studies (40, 41);

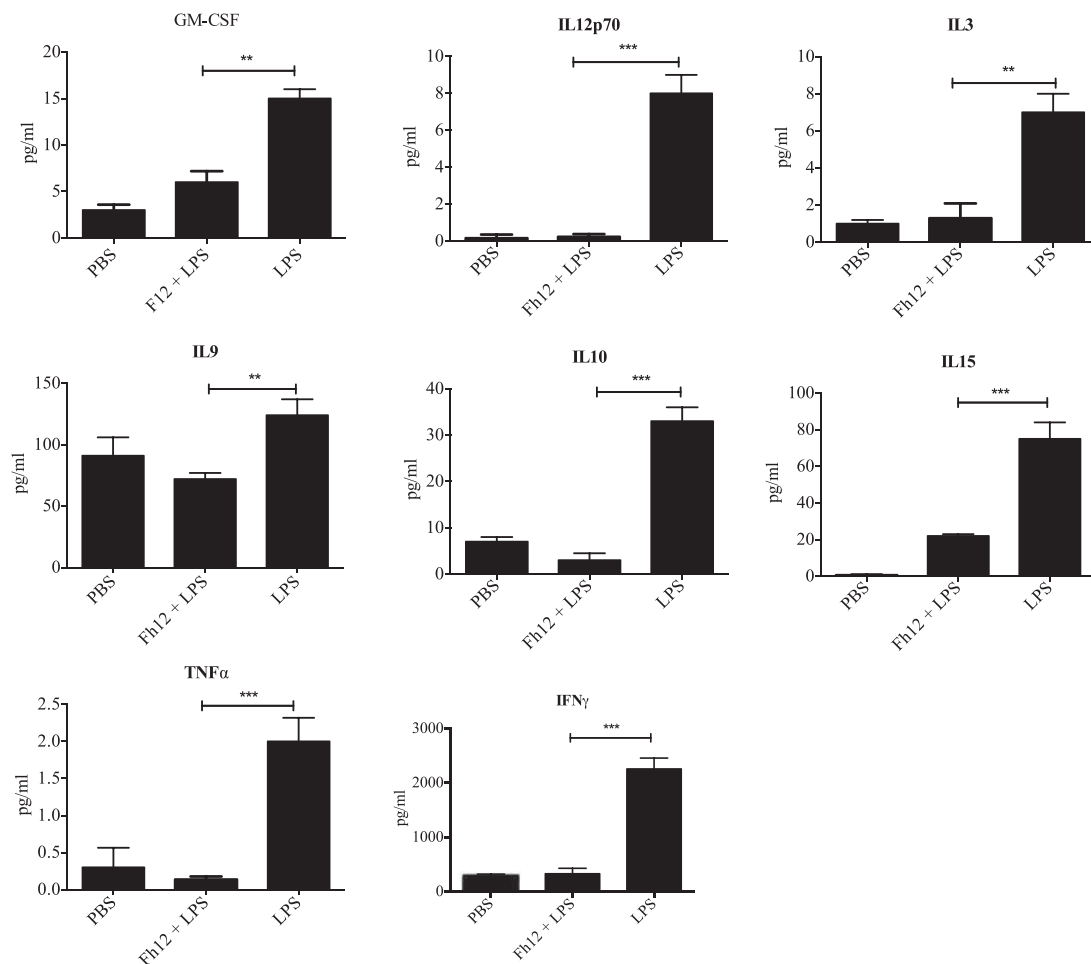


FIGURE 1. Fh12 suppresses inflammatory cytokines in vivo in a murine model of septic shock. C57BL/6 mice, 6–8 wk old, were used. Groups of five animals each were injected i.p. with a volume of 15 μ l Fh12 (15 μ g for each mouse), 1 h before i.p. injection with LPS (*E. coli* 0111:B4, 1 μ g per each mouse). Control mice received PBS, Fh12, or LPS only (i.p.). Mice were sacrificed by cervical dislocation 12 h after LPS exposure, and blood samples were taken by cardiac puncture. Concentrations of serum IFN- γ , IL-12p70, TNF- α , GM-CSF, IL-3, IL-9, IL-10, and IL-15 cytokines were measured by cytokine microarrays. ** $p < 0.0085$, *** $p < 0.0006$.

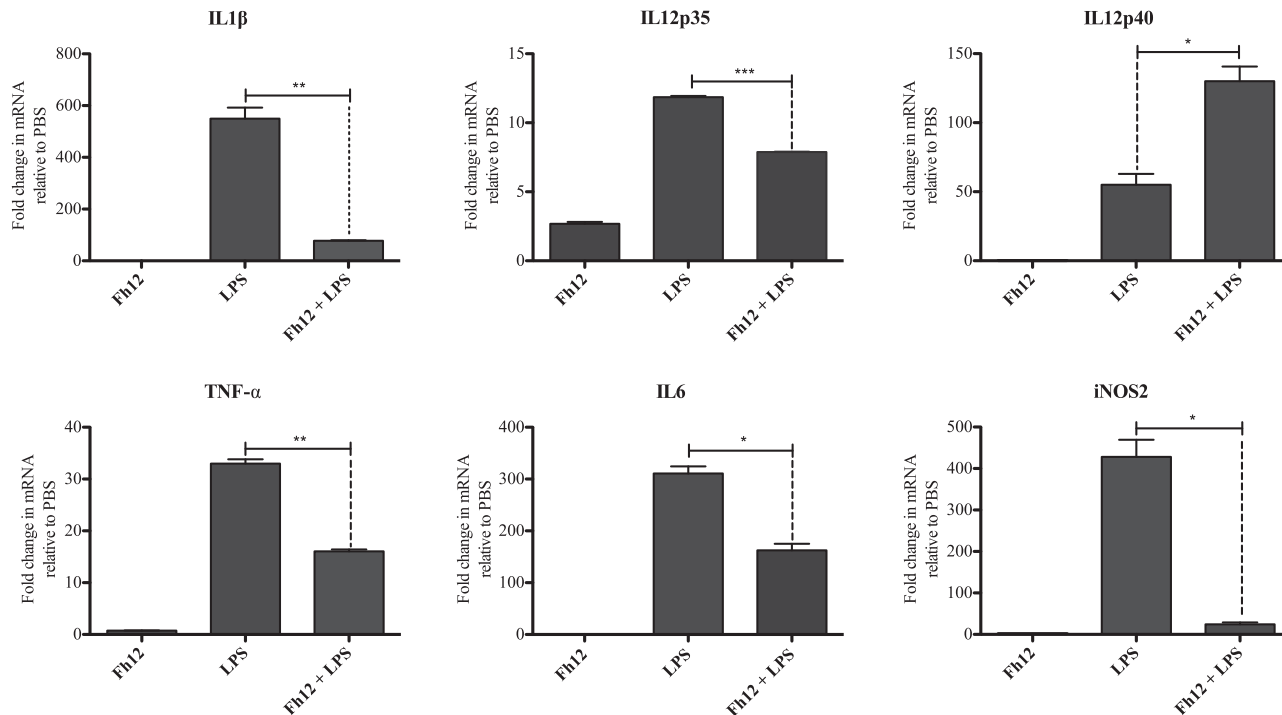


FIGURE 2. Fh12 suppresses the expression of inflammatory markers from mouse bmMΦs in response to LPS. Macrophages from naive C57BL/6 mice were treated with Fh12 (5 μg/ml) or PBS 30 min before stimulation with LPS (100 ng/ml) for 24 h. Control cells were treated with Fh12 or PBS alone. Expression of IL-1β, IL-12p35, IL-12p40, TNF-α, IL-6, and iNOS2 was measured in treated cells by qPCR. Results are shown as the fold change in expression relative to cells stimulated with PBS and represent the mean ± SD of a minimum of three experiments, each in triplicate. Values that were significantly different from the value for the group stimulated with LPS and Fh12 + LPS are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

this is in agreement with the observed suppression of serum IL-12p70 induced by Fh12 in the septic shock experiment.

To determine whether Fh12 has a broad suppressive effect on macrophages rather than targeting a single TLR pathway, bmMΦs were cultured with Fh12 1 h before TLR-ligand stimulation with HKLM (10⁸ cells/ml), poly(I:C) (100 μg/ml), flagellin (1 μg/ml), imiquimod (10 μg/ml), and orthiazoloquinoline (CL075, 10 μg/ml). Fh12 had a limited effect on the cytokine expression profile of MΦs induced by ligands other than TLR4 because it only suppressed the expression of IL-1β ($p < 0.0084$) and IL-12p35 ($p < 0.089$) in response to HKLM (TLR2 ligand), and the expression of IL-12p35 ($p < 0.0499$) in response to imiquimod (TLR7 ligand) but had no effect on other inflammatory markers induced by TLR2 or TLR7 nor on the expression of iNOS2 in response to other TLRs (Fig. 3A). The lack of involvement of TLR activation in Fh12-mediated suppression was further demonstrated by the absence of stimulation of THP1-CD14 or HEK293 cells, which functionally express TLR proteins (Fig. 3B).

Fh12 suppresses phagocytosis by mouse bmMΦs

Phagocytosis is a major function of macrophages, allowing these key cells of the innate immune system to engulf and destroy foreign pathogens. To determine whether Fh12 was interfering with the phagocytic ability of bmMΦs, we cultured cells with PBS or LPS in the presence or absence of Fh12 before exposure to *E. coli* particles. The results demonstrated that incubation with Fh12 alone did not induce phagocytosis of bacteria by macrophages. Incubation with Fh12 significantly suppressed the phagocytic ability of macrophages in response to LPS ($p < 0.01$; Fig. 4A). With such a potent inhibitory effect on macrophage function, we measured the influence of Fh12 on cell viability using an XTT assay. The results demonstrated that treatment of HEK293-TLR4 cells or macrophages with LPS, Fh12, or Fh12 + LPS for 24 or 48 h did not compromise the cell viability (Fig. 4B, 4C).

Fh12 blocks LPS-induced NF-κB activation in HEK293-TLR4 cells

Based on the observed suppression of phagocytic activity and cytokine expression induced by LPS in the mouse model of septic shock, and the immunomodulation of helminth molecules via targeting TLR4 (12, 42, 43), we focused on the interaction between Fh12 and TLR4 by using HEK293-TLR4 cells. By dose-response analyses, optimal concentrations of LPS and the antagonist PMB were determined for subsequent analysis. A concentration of 5 μg/ml LPS induced maximum activation of the NF-κB transcription factor, and concentrations of 100 μM PMB completely suppressed NF-κB expression. When Fh12 was added to cells, NF-κB was not activated at any of the Fh12 concentrations used. To investigate whether the suppressive effect of Fh12 is

Table I. Primers used in the qPCR experiments

Primer	Sequence (5' to 3')
CD14	Sense: AGCCACAGGACTTGCCTTT Antisense: TGGGCAATGCTCAGTACCTT
MD2	Sense: CCGAGGATCTGATGACGATT Antisense: TGGGCTCCCAGAAATAGCTT
TLR4	Sense: CGGAGGCCATATATGCTATGT Antisense: TCCCTTCTCCTTTTTCCTA
IL12p70	Sense: TCCAGAAGGCCAGACAACTC Antisense: AATGGTAAACAGGCCCTCCACT
TNFα	Sense: TGGGATCATTGCCCTGTTGAG Antisense: TCTAAGCTTGGGTTCCGACC
IL1β	Sense: GCTCGCCAGTGAAATGATGG Antisense: GTCCCTGGAAGGAGCACCTTCAT
iNOS2	Sense: TCACCTACCACACCCGAGAT Antisense: TTCAGGCTGTTGAGCCATGT
ACTB	Sense: AGAAAATCTGGCACACACCC Antisense: GGGGTGTTGAAGGTCTCAA

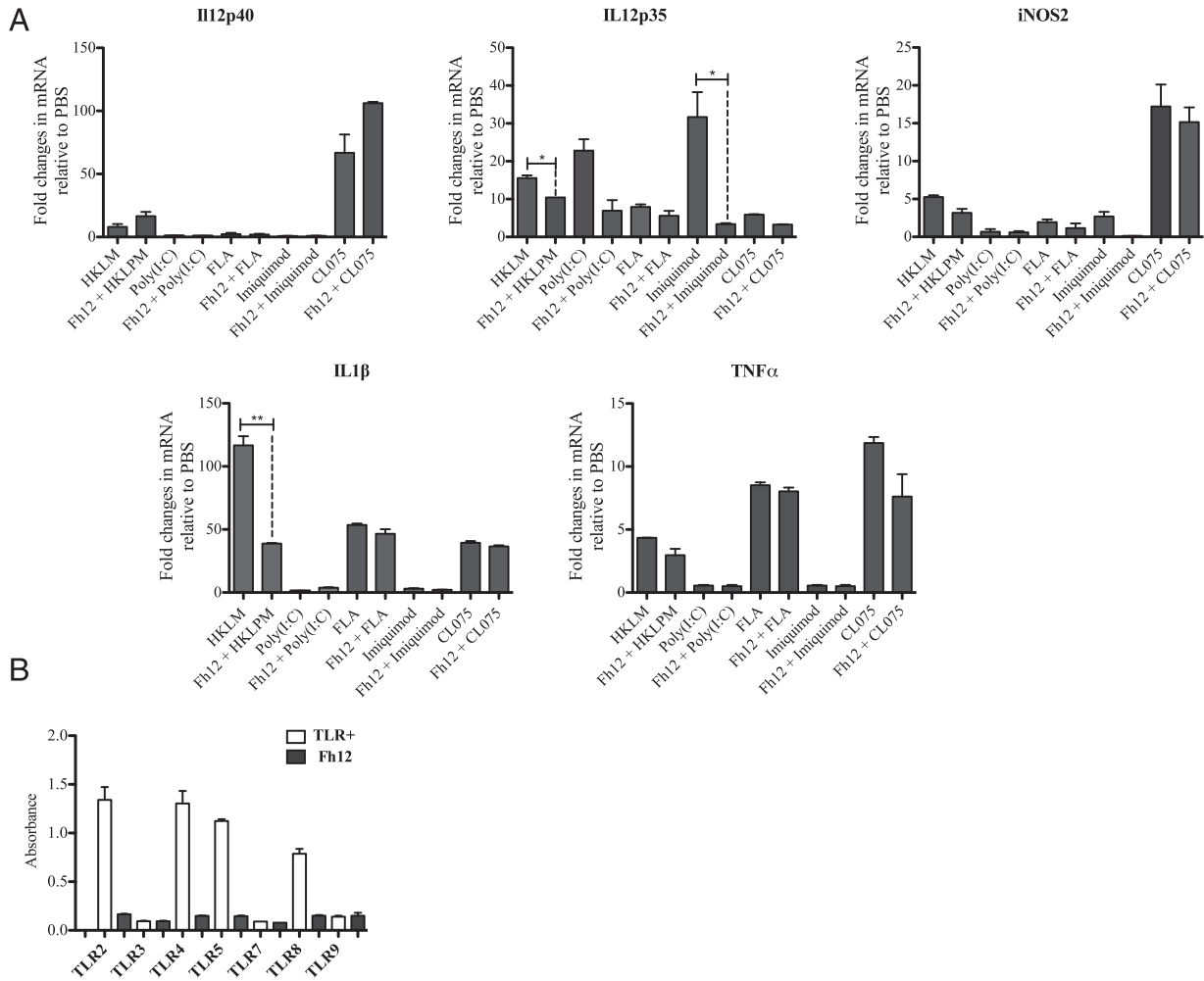


FIGURE 3. Fh12 has a limited effect on the cytokine expression in macrophages in response to TLR ligands other than TLR4. **(A)** Macrophages from naive mice were stimulated with Fh12 (15 $\mu\text{g/ml}$) or PBS 30 min before stimulation with HKLM (10^8 cells/ml); poly(I:C) (100 $\mu\text{g/ml}$), flagellin (FLA; 1 $\mu\text{g/ml}$), imiquimod (10 $\mu\text{g/ml}$), or orthiazoloquinoline (CL075; 10 $\mu\text{g/ml}$). Control cells were stimulated with PBS or a single TLR protein. Expression of IL-1 β , IL-12p35, IL-12p40, TNF- α , and iNOS2 was measured in treated cells by qPCR. Results are shown as the fold change in expression relative to cells stimulated with PBS and represent the mean \pm SD of a minimum of three experiments, each in triplicate. **(B)** THP1-CD14-Blue cells, functionally expressing TLR proteins as well as a reported gene, were stimulated with a specific agonist (TLR $^+$) or Fh12 (5 $\mu\text{g/ml}$). Values that were significantly different from the value for the group stimulated with each TLR ligand (TLR $^+$) and Fh12 are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$.

dependent on the dose and the time of exposure, cells were cultured with different concentrations of Fh12 30 min before LPS stimulation. The results demonstrated that, starting at 0.625 $\mu\text{g/ml}$, Fh12 suppressed NF- κB activation by 55%, and at concentrations $\geq 2.5 \mu\text{g/ml}$, NF- κB activation was 100% suppressed, which is similar to the effect seen in the PMB control (Fig. 5A). To investigate whether Fh12 could suppress the activation of NF- κB after the cells have been stimulated with LPS, we added 5 $\mu\text{g/ml}$ Fh12 at different time points (30 min, 1, 2, and 4 h) after LPS exposure. The results demonstrate that NF- κB activation is fully suppressed when Fh12 is added to cells between 30 min and 4 h after LPS stimulation (Fig. 5B). Taken together, these results indicate that Fh12 block the entire TLR4 pathway in a manner that is dose dependent, and that the timing of exposure to Fh12 makes no difference.

Fh12 suppresses the phosphorylation of ERK, p38, and JNK in response to LPS

Having demonstrated that Fh12 suppresses the expression of inflammatory cytokines of macrophages in response to LPS and suppresses the NF- κB activation within HEK293-TLR4 cells incubated with Fh12 either before or after LPS stimulation, we

then investigated whether Fh12 might target common components of TLR pathways. Such targeting has been reported for MAPKs by other helminth products, such as FhTeg (12, 44, 45). To address this question, we cultured bmM Φ s with Fh12 before or after LPS stimulation, and phosphorylation of ERK, JNK, and p38 was measured. Initially, we measured the phosphorylation levels at different time points (15, 30, and 60 min) after LPS stimulation and determined that the maximal phosphorylation levels of p38 and ERK are reached at 30 min, which is consistent with the findings of other authors in RAW267.7 cells (46), whereas the maximal levels of JNK phosphorylation are reached at 60 min (Supplemental Fig. 1). Fh12 alone did not induce phosphorylation of these components at any time point but was able to suppress significantly LPS-induced phosphorylation of ERK (p-ERK; $p < 0.0035$), p-JNK ($p < 0.001$), and p-p38 ($p < 0.01$) at every time point studied (Fig. 6), which could explain the suppression of inflammatory markers induced by Fh12.

Fh12 alters the expression of various TLR4 pathway components

To investigate the mechanism through which Fh12 specifically blocks the interaction between LPS and TLR4, we first assessed

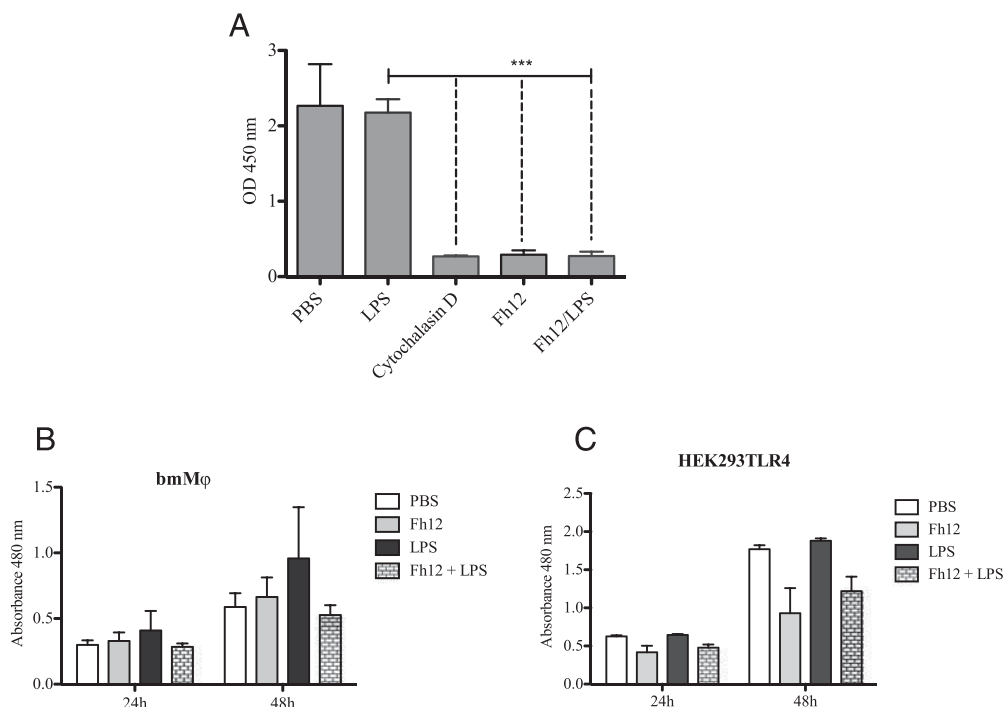


FIGURE 4. Fh12 suppresses phagocytosis by macrophages. **(A)** bmMΦs from naive mice were cultured with PBS or LPS (100 ng/ml) in the presence and absence of Fh12 (5 μg/ml) before the addition of enzyme-labeled *E. coli* particles. Negative controls were treated with Fh12 alone or with 2 μM cytochalasin D to block phagocytosis. Data are the mean (plus SD [error bars]) for three wells and are representative of three experiments. Values were found to be significantly different (***) $p < 0.001$. **(B)** Macrophages or **(C)** HEK293 TLR4 cells were exposed from naive mice were exposed to Fh12 (5 μg/ml), LPS (100 ng/ml), or Fh12 (5 μg/ml) + LPS (100 ng/ml) during 24 or 48 h. Cell viability was determined by adding 50 μl XTT to each well. After an additional incubation of 24 h at 37°C, the absorbance of each well was read at 480 nm.

whether Fh12 exposure alters either the interaction of LPS with LBP or the expression of any TLR4 complex component. To assess whether Fh12 prevents binding of LPS to LBP, we used an LBP-ELISA kit, which detects binding of biotinylated LPS to LBP. PMB, which competes with LBP for binding to LPS, was used as a positive control. At concentrations ≤ 10 μg/ml, Fh12 did not prevent LPS binding to LBP after preincubation with either LPS or LBP (Fig. 7), indicating that Fh12 does not bind to LPS or LBP.

We next investigated whether Fh12 achieves its effect by regulating various components of the TLR4 complex and signaling cascades. The results show that Fh12 significantly reduced the expression of CD14 by >5-fold compared with cells stimulated with LPS alone, and this difference was found to be highly significant ($p < 0.001$). Fh12 also reduced by 1.6-fold the expression of MD2 and by 2.1-fold the expression of TLR4 mRNA, and these reductions were both significant ($p < 0.05$; Fig. 8A). Consistent

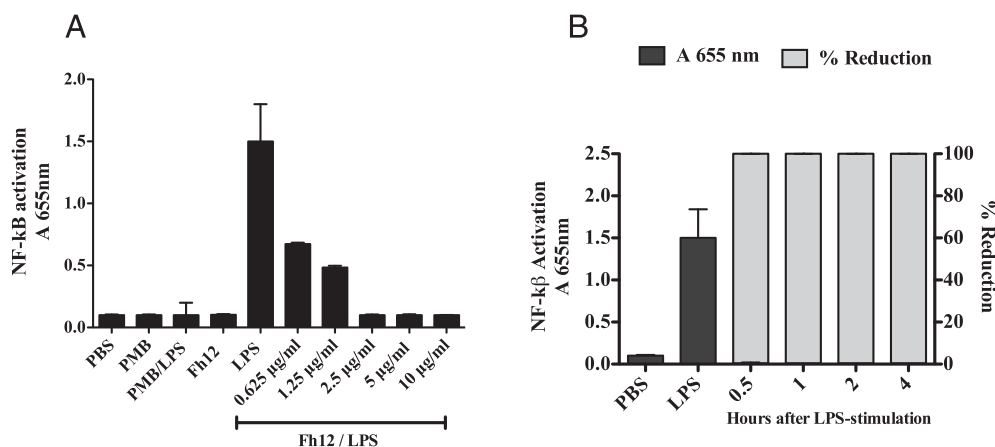


FIGURE 5. Fh12 suppresses LPS-induced NF-κB activation in vitro in a manner that is dose dependent but is independent of the duration of LPS exposure. HEK293 cells cotransfected with genes encoding cluster differentiation Ag-14 (CD14), MD2, TLR4 coreceptor, and an SEAP reporter gene (HEK293-TLR4) were maintained in DMEM and seeded at 2.52×10^4 cells/well in 96-well flat-bottom plates. The levels of NF-κB activation were estimated by readings at 655 nm, 24 h after LPS stimulation. **(A)** Cells were cultured with Fh12 (from 0.625 to 10 μg/ml) before stimulation with LPS. Cells cultured with PMB or PBS were used as the antagonist and negative controls, respectively. Cells stimulated with LPS were used as the activation control. **(B)** Cells were stimulated with LPS (5 μg/ml) and then exposed to Fh12 (5 μg/ml) at different time points (30 min, 1, 2, 4 h) after LPS stimulation. The reduction (R%) of NF-κB activation was calculated by the formula $R (\%) = [1 - (A - C/B - C)] \times 100$, where A represents the mean NF-κB activation measured at 655 nm of three replicates obtained when cells were cultured with Fh12, B is the mean NF-κB activation measured at 655 nm when cells were stimulated with LPS, and C is the mean NF-κB activation measured at 655 nm when cells were stimulated with PBS.

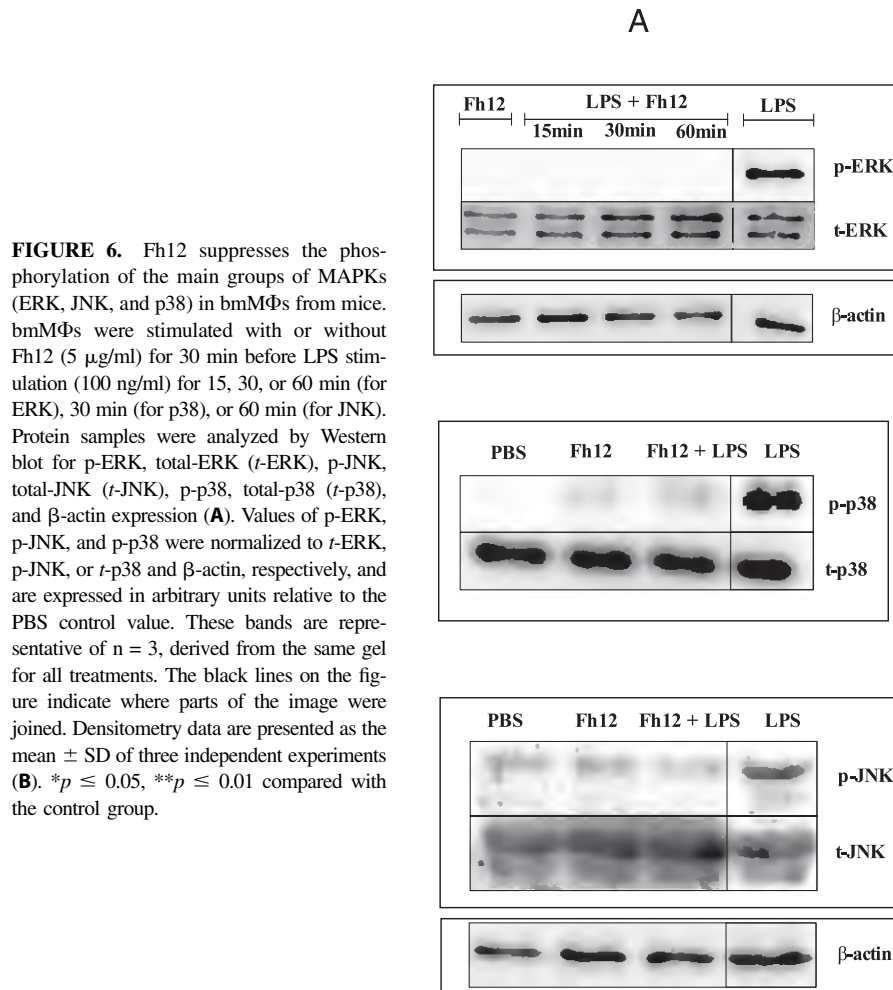
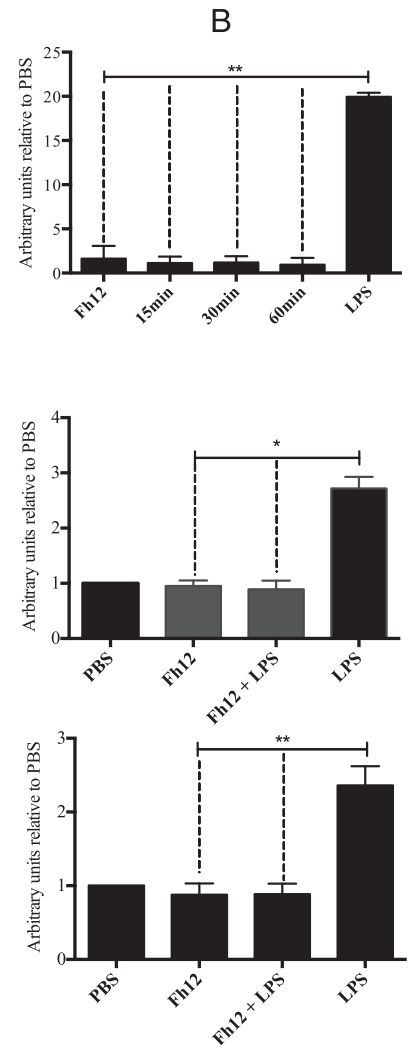


FIGURE 6. Fh12 suppresses the phosphorylation of the main groups of MAPKs (ERK, JNK, and p38) in bmMΦs from mice. bmMΦs were stimulated with or without Fh12 (5 μg/ml) for 30 min before LPS stimulation (100 ng/ml) for 15, 30, or 60 min (for ERK), 30 min (for p38), or 60 min (for JNK). Protein samples were analyzed by Western blot for p-ERK, total-ERK (*t*-ERK), p-JNK, total-JNK (*t*-JNK), p-p38, total-p38 (*t*-p38), and β-actin expression (**A**). Values of p-ERK, p-JNK, and p-p38 were normalized to *t*-ERK, *t*-JNK, or *t*-p38 and β-actin, respectively, and are expressed in arbitrary units relative to the PBS control value. These bands are representative of $n = 3$, derived from the same gel for all treatments. The black lines on the figure indicate where parts of the image were joined. Densitometry data are presented as the mean \pm SD of three independent experiments (**B**). * $p \leq 0.05$, ** $p \leq 0.01$ compared with the control group.



with these results, the expression of CD14 protein was also significantly reduced ($p < 0.001$), as detected by Western blot analysis of cells exposed to Fh12 before stimulation with LPS. However, expression of MD2 and TLR4 protein was not significantly reduced after Fh12 treatment (Fig. 8B, 8C). These results suggest that Fh12 might achieve its effect impacting the expression of CD14 coreceptor.

Fh12 colocalize on the cell surface and interact with CD14 coreceptor

To assess whether Fh12 could be in proximity to interact with the CD14 coreceptor, we used the in situ PLA, which was recently developed to detect and visualize protein–protein interactions (47, 48). Results demonstrate that Fh12 localizes on the surface of cells in HEK293-TLR4 cells, which was also observed for LPS and CD14 coreceptor separately. Fh12 was also localized on the cell surface of bmMΦs generated from wild-type mice, but not on the surface of macrophages from CD14 knockout mice, which confirms that Fh12 binds to the CD14 coreceptor (Supplemental Fig. 2). Next, we performed the PLA using HEK293-TLR4 cells. As expected, by using the control pair of Abs anti–lipid A and anti-CD14, we observed a large number of intense orange dots around cells (Fig. 9A), indicating colocalization and interaction of LPS and CD14 coreceptor. Similarly, numerous intense orange dots were also observed when the experiment was performed with the pair of Abs anti-Fh12 and anti-CD14 Ab, demonstrating close proximity and interaction of Fh12 and CD14 (Fig. 9B). No orange

dots were observed in cells treated with PBS incubated with the anti-Fh12 and anti-CD14 Abs simultaneously (Fig. 9C), demonstrating there were no nonspecific interactions in this assay. Furthermore, the modeled structure of human CD14 and the predicted tertiary structure of *F. hepatica* FABP were subjected to docking analysis to investigate potential molecular interactions between the two molecules. Amino acid residues K21, K22, K83,

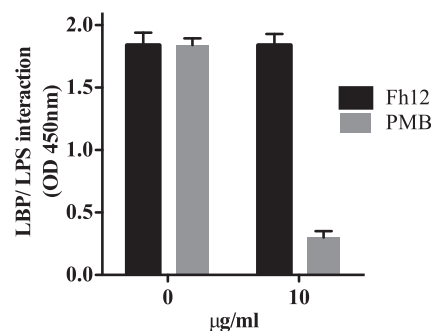


FIGURE 7. Fh12 does not disrupt lipid binding protein/LPS (LBP/LPS) binding. A lipid binding protein assay (Endoblock) was used to investigate whether Fh12 disrupts the interaction between LPS and LBP. PMB (100 μM), which is known to compete with LBP for binding to LPS, blocked LBP/LPS binding. Fh12 (10 μg) preincubated for 30 min with biotinylated LPS and added to anti-LBP + LBP was unable to block the (LBP/LPS) binding.

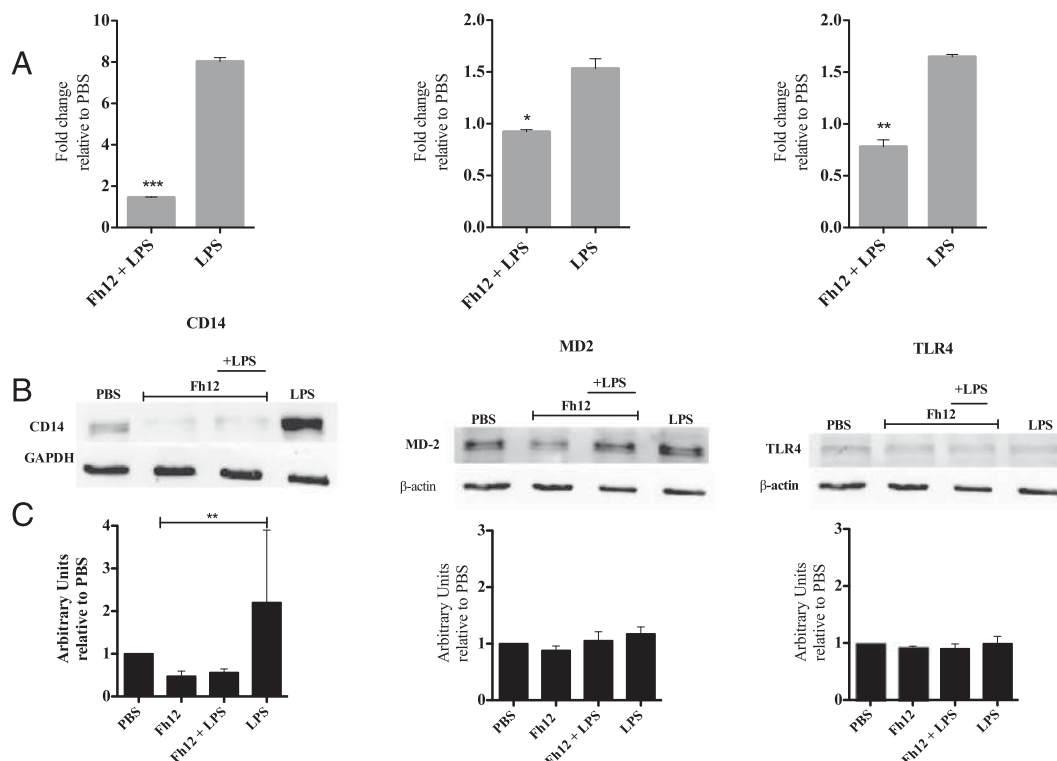


FIGURE 8. Fh12 inhibits TLR4 activation targeting the human CD14 coreceptor. HEK293 cells were treated with 5 $\mu\text{g/ml}$ Fh12 before stimulation with LPS (5 $\mu\text{g/ml}$). Cells stimulated with LPS were used as a positive control, and cells treated with PBS were used as a negative control. After 24 h of incubation at 37°C, 5% CO₂, the cells were lysed for RNA and protein extraction. **(A)** Expression of CD14, MD2, and TLR4 was determined by qPCR, and results were expressed as fold-changes relative to the PBS-control. **(B)** Protein extract (30 $\mu\text{g/sample}$ of protein) was separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and sequentially probed with anti-CD14, anti-MD2, or anti-TLR4 Ab. Immunoblots incubated with anti- β -actin or anti-GAPDH Abs were used as a control for protein concentration. Representative blots are shown from three experiments. **(C)** Densitometric analysis was performed on all immunoblots. Values were normalized to β -actin or GAPDH, and all values are expressed in arbitrary units as units of increase over the PBS control. * $p < 0.01$, ** $p < 0.005$, *** $p < 0.0001$ compared with the PBS control group.

K97, E100, E104, and D122 of the modeled structure of FABP1 were predicted to bind D44, S46, K71, N72, V73, Y82, Q81, and D84 of the LPS binding pocket of the CD14 coreceptor (Fig. 9D and 9E).

Although Fh12 binds to CD14, its anti-inflammatory effect could be independent of this coreceptor

Having demonstrated that Fh12 binds and interacts with the CD14 coreceptor, we proceeded to investigate whether CD14 is essential for the anti-inflammatory action of Fh12. We therefore exposed bmM Φ s generated from CD14 knockout mice to LPS and HKLM in the presence of Fh12 and measured the expression of IL-1 β and IL-12p35 cytokines, which were suppressed by Fh12 in the wild-type mice in response to TLR4 and TLR2 ligands. The expression of IL-1 β and IL-12p35 in response to LPS was found significantly lowered in CD14 knockout compared with wild type ($p < 0.0001$), which is an expected finding because it is well-known that CD14 is an essential coreceptor for the activation of the TLR4 cascade (49, 50). Similarly, the expression of both cytokines in response to HKLM was also found significantly reduced in knockout compared with wild type ($p < 0.0059$). We found that Fh12 did not affect the expression of IL-1 β or IL-12p35 of CD14 knockout mice in response to LPS, and neither affects the expression of IL-12p35 in response to HKLM. However, it suppressed the expression of IL-1 β in response to HKLM ($p < 0.013$). These results suggest that CD14 coreceptor could not be the only target used by Fh12 to exert its anti-inflammatory effect. (Fig. 10)

Discussion

F. hepatica FABPs are immunogenic proteins of 12–15 kDa that play important roles in nutrient acquisition and survival of the parasite within the mammalian host. Although FABPs are considered cytosolic proteins, in proteomic studies, two FABP isoforms (FABP1 and FABP2) have been identified among the ESPs of the liver fluke (23, 24). Also, FABP1, known as Fh15, has been located in abundance at the surface of *F. hepatica*, indicating a possible role in the uptake of fatty acids from the environment (19). Because the tegument is shed every 2–3 h during the course of infection, FABP1 may be released into the surrounding medium as the tegument is sloughed. Recently, by using proteomics, our research group identified FABP1 in *F. hepatica* tegument extracts, specifically in one fraction highly reactive with sera from patients with chronic fascioliasis (30). These results corroborate that *F. hepatica* FABP1 is exposed to the immune system and that specific Abs are elicited against this protein during the course of active infection. Thus, as for many other *F. hepatica* molecules, FABP could interact with APCs during the course of the innate immunity response. Recently, studies demonstrated that FhTeg target innate immune cells, inhibiting their ability to drive Th1 immune responses (51, 52). Based on the earlier discussion, we purified native *F. hepatica* Fh12 and assessed its interaction with innate immune responses.

The ability of helminth Ags to inhibit activation of DCs and macrophages in response to stimulation with bacterial ligands suggests they could be ideal therapeutic candidates for the treatment of chronic inflammatory conditions such as septicemia. Indeed, septicemia is not a common event during helminth infection

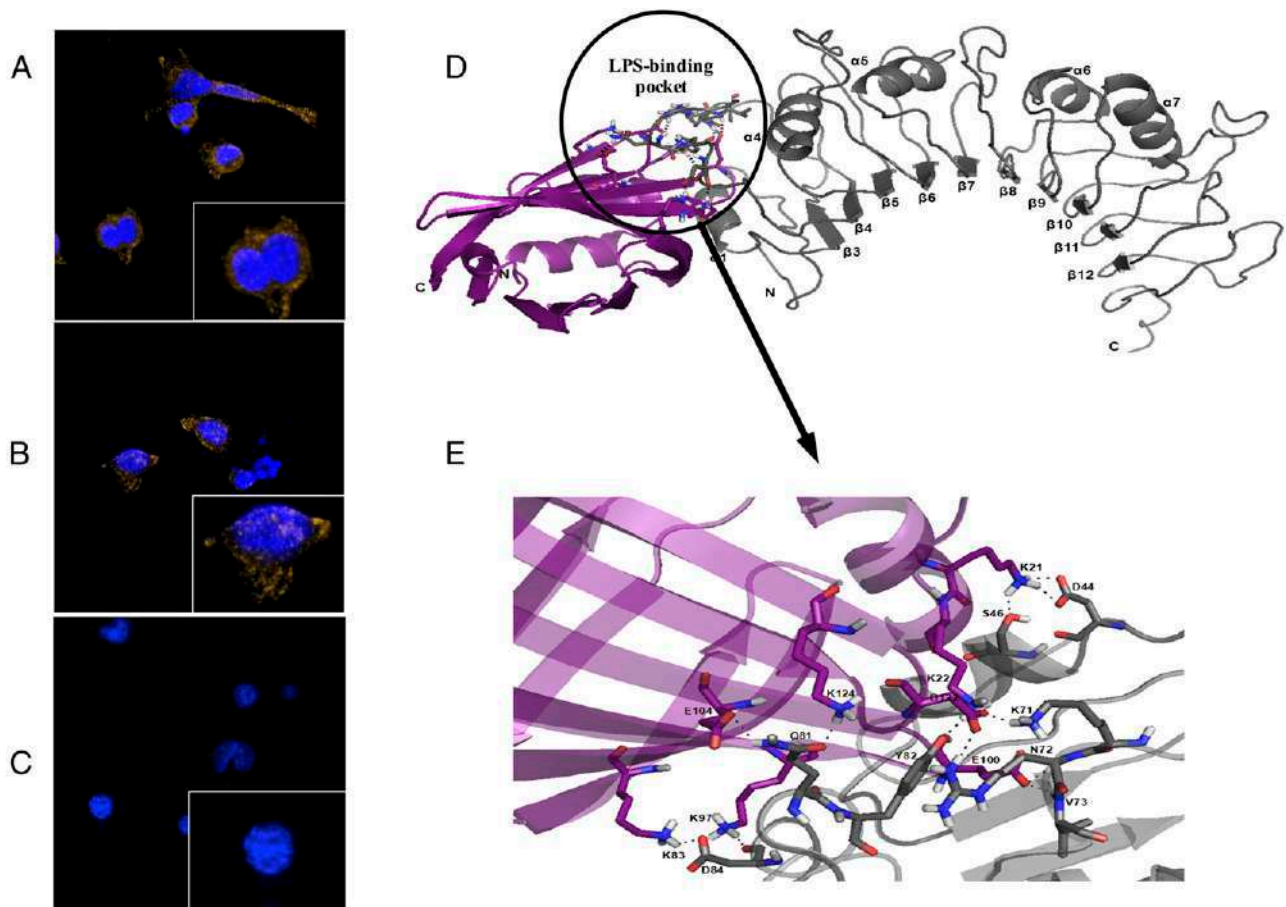


FIGURE 9. PLA demonstrates that Fh12 could interact with CD14 coreceptor. HEK293 cells were grown for 48 h to 50% confluence on microscope coverslips and then treated for 4 h with LPS (5 $\mu\text{g}/\text{ml}$), Fh12 (5 $\mu\text{g}/\text{ml}$; positive control), or PBS (negative control). **(A)** Cells treated with LPS and incubated with the pair of Abs mouse anti-lipid A IgG (diluted 1:10) and goat anti-CD14 IgG (15 μg), **(B)** cells treated with Fh12 incubated with the pair of Abs anti-Fh12 IgG (diluted 1:50) + goat anti-CD14 IgG (15 μg), and **(C)** control cells treated with PBS and incubated with the pair of Abs anti-lipid A IgG (diluted 1:10) or anti-Fh12 IgG (diluted 1:50) and anti-CD14 IgG (15 μg). The numerous orange dot signals around the cells show the typical interaction between LPS and CD14 coreceptor (A) and between Fh12 and CD14 (B). No orange dots were observed in cells stimulated with PBS where two Abs anti-Fh12 and anti-CD14 were present (C), which demonstrates that there are no nonspecific reactions in this assay. Images were observed with a Zeiss Observer Z1 confocal laser-scanning microscope coupled to a Zeiss LSM 510 Meta EC using a Plan-Apochromat 63 \times /1.40 oil differential interference contrast M27 objective. The system was controlled using Zeiss ZEN 2009 software. **(D)** Docking analysis between FABP1 and human CD14 coreceptor was performed using the ClusPro server, demonstrating the potential interaction of FABP1 with the LPS pocket localized on the structure of CD14. **(E)** The LPS-binding pocket region was zoomed to show interactions among residues K21, K22, K83, K97, E100, E104, and D122 of the model structure of FABP1 (purple), which are predicted to interact with the residues D44, S46, K71, N72, V73, Y82, Q81, and D84 of the LPS binding pocket of the CD14 coreceptor (gray).

(53), which supports the therapeutic potential of helminth Ags. Using specific helminth molecules to target innate immune cell signaling will circumvent the problem of global immune suppression associated with parasite infection and also with current immune therapies. Given that Fh12 is an Ag previously identified in ESPs and FhTeg, we investigated whether it could prevent chronic inflammation in a mouse model of sepsis. Our results showed that injection of Fh12 (15 μg) into mice before administration of a sublethal dose of LPS reduced significantly the serum expression of IFN- γ , TNF- α , GM-CSF, IL-12p70, IL-3, and IL-15, which are all cytokines associated with inflammatory responses (54–56). *F. hepatica* has been reported to suppress Th1 responses in concurrent bacterial infections, thus demonstrating its anti-inflammatory effect in vivo (8, 9). These findings support the therapeutic potential of Fh12 as an anti-inflammatory agent. It was unexpected to find that Fh12 also reduced the levels of IL-10, a cytokine generally associated with anti-inflammatory and regulatory responses, which was elevated in mice injected with LPS. At least one study has demonstrated that Th1 cells can produce IL-10 (57); thus, our results emphasize

that IL-10 is a versatile cytokine that could play different roles during infection (57).

To ascertain whether Fh12 could target TLR pathways, we optimized a screening system based on NF- κ B activation using THP1-Blue-CD14. Fh12 did not activate NF- κ B, indicating that it is not a ligand for any of the TLRs expressed on these cells. Because Fh12 is present in FhTeg, these results are consistent with those of previous studies, which reported failure of FhTeg to activate HEK293 cells expressing a range of TLR proteins (18). Although Fh12 alone failed to induce iNOS2 expression and cytokine production of macrophages from naive mice, treatment of macrophages with Fh12 rendered the cells hyporesponsive to LPS with significant reductions in iNOS2, TNF- α , IL-12p35, IL-6, and IL-1 β expression. iNOS2 is an enzyme that catalyzes the production of NO from L-arginine, and its expression is directly associated with the classical activation of macrophages (58, 59), as is expression of IL-12p70, TNF- α , and IL-1 β (60). These findings are consistent with recent data obtained by our research group using naive human macrophages, which showed an alternative activation pattern in the presence of Fh12 that was characterized

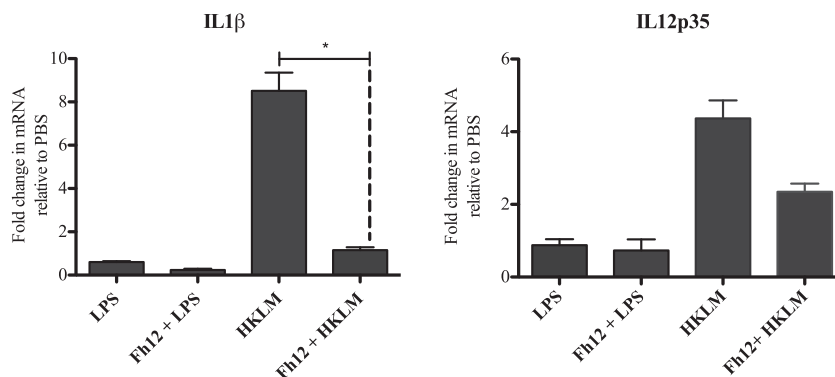


FIGURE 10. Fh12 had no effect on IL-12p35 or IL-1 β of macrophages from CD14 knockout mice in response to LPS but had a moderate effect on expression of cytokines in response to HKLM. bmM Φ s were isolated from CD14 knockout mice and stimulated with LPS (100 ng/ml) or HKLM (10^8 cells/ml) in the presence or absence of Fh12 (15 μ g/ml) for 24 h. Expression of IL-12p35 and IL-1 β was determined by qPCR. Results are shown as the fold changes in expression relative to cells stimulated with PBS and represent the mean \pm SD of a minimum of three experiments, each in triplicate. Values that were significantly different from the value for the group stimulated with TLR ligand and Fh12 + TLR ligand are indicated as $*p \leq 0.05$.

by low levels of iNOS2 and NO, and high levels of Arg-1, arginase activity, and chitinase activity (29).

It was interesting that in the presence of Fh12, macrophages overexpressed IL-12p40 and suppressed the expression of IL-12p35. The IL-12p40 subunit binds with either the p35 or p19 subunit to form the functionally active IL-12p70 (61) or IL-23 (62) cytokine, respectively, which are required for development of Th1 responses, and plays a central role in the autoimmune process. As a result of the IL-12p40 overregulation, homodimers are formed (IL-12p80) that competitively bind to the common receptor component IL-12R β 1 (63), which prevents IL-12-mediated shock in the murine model (64). This differential regulation of both IL-12 subunits induced by Fh12 could partially explain the low IL-12p70 levels observed in this study. It is therefore consistent with the significant downregulation of phagocytic capacity of macrophages and evidence of the suppressive impact of Fh12 on the maturation and function of these cells. Because *F. hepatica* needs to suppress inflammatory processes to survive in the host, reduction of IL-12p70, which is a Th1-driving cytokine (65), could influence the suppression observed during bacterial coinfection (7, 9, 10).

Fh12 significantly suppresses iNOS2, as well as a large number of inflammatory cytokines, in response to LPS but fails to suppress most of these markers in response to other ligands. This suggests that the anti-inflammatory effect of Fh12 mainly occurs through a single receptor, TLR4. Interestingly, FhTeg, of which Fh12 is a component (30), suppressed significantly a large number of inflammatory cytokines from DCs in response to several TLR ligands (18). This apparent contradiction could be explained by the complex composition of FhTeg, which contain a myriad of molecules as has been demonstrated in proteomic analysis (22), each of which has its own mechanism of immunomodulation. Thus, it is likely that molecules other than Fh12, with broader suppressive effects, on multiple TLRs might overlap the effect of Fh12.

To explore the mechanism by which Fh12 reduces the inflammatory response via TLR4, we tested Fh12 in HEK293 cells stably transfected with CD14, MD2, and TLR4 genes and a SEAP reporter gene, a system that permits direct and efficient detection of NF- κ B when it is activated by stimulation with TLR4 ligands. The results demonstrate that Fh12 completely inhibited the TLR4 activation induced by LPS whether it was added to the cell culture medium either before or after LPS stimulation, which is consistent with the results previously obtained with macrophages. Moreover, the fact that Fh12 was able to suppress NF- κ B activation when added to

the culture before or 4 h after LPS stimulation reinforces the prophylactic and therapeutic potential of this molecule for the prevention of bacteria-induced sepsis, because Fh12 blocks LPS-induced activation of innate immune responses. FhTeg (18) and parasites such as *Toxoplasma gondii* (66) and *Brugia malayi* (67) also inhibited the activation of NF- κ B.

LPS is the major lipid present in the outer membrane of Gram-negative bacteria (68, 69), and the induction of proinflammatory responses through TLR4 is achieved by the sequential and coordinate action of four principal proteins: LBP, CD14, MD2, and TLR4. This process is initiated when LBP disaggregates LPS from the bacterial membrane and catalytically transfers it to CD14 (70), which, in turn, shuttles LPS to MD2/TLR4 to form the activated (TLR4-MD2-LPS) $_2$ complex that has a pivotal role in initiating the inflammatory cascade. Our results demonstrate that Fh12 does not bind LBP or disrupt the LBP-LPS binding, and although Fh12 reduced the levels of expression of MD2 and TLR4 at the RNA level, these reductions did not significantly impact the expression of MD2 and TLR4 proteins. However, Fh12 caused a significant reduction in the expression of CD14 protein. This observation strongly suggests that CD14 coreceptor is likely the target that Fh12 uses to block the LPS-TLR4 interaction. Moreover, the PLA demonstrated that Fh12 colocalizes in close proximity to the CD14 coreceptor, and the docking analysis supports Fh12 binding to the LPS pocket, which is located within the 65 N-terminal residues and clusters around the hydrophobic pocket of the horseshoe-shaped structure of CD14 (71). This is further supported by the failing of Fh12 to localize on the surface of cells from CD14 knockout mice. Also, in CD14 knockout mice, Fh12 lost the capacity to suppress IL-12p35 and IL-1 β of macrophages that are released in response to LPS or HKLM, which are ligands that use CD14 in their respective activation cascades (72, 73). The finding that Fh12 did not suppress IL-1 β in response to HKLM in CD14 knockout mice suggests that Fh12 could impact molecules downstream the signaling cascade such as MAPKs that are common to several TLRs.

The MAPKs are a highly conserved family of serine/threonine protein kinases involved in a variety fundamental cellular processes such as proliferation and macrophage maturation through known ligands (74). Activation of the main mammalian groups, JNK, ERK, and p38, culminates in the release of cytokines from macrophages after downstream activation of a signaling cascade involving adaptor proteins such as MyD88 (75). In this study, we demonstrated that Fh12 suppresses the LPS-induced phosphorylation of ERK, JNK, and p38 in mouse macrophages. Our findings

correlate with studies performed by others in which FhTeg reduced the ERK-phosphorylation induced by LPS on DCs (18). A study using *Schistosoma mansoni* egg Ags reported a reduction in LPS-stimulated phosphorylation of ERK in murine DCs (43). Also, it was demonstrated that phosphorylation of NF- κ B and ERK is crucial in Th2 immune responses induced by *Trichuris trichiura* (76). The fact that Fh12 does not induce but suppresses cytokine production could suggest that Fh12 has a role in the suppression of Th1 responses. However, we have yet to determine whether Fh12 drives Th2 or T regulatory immunity. This hypothesis and the biological importance of these findings will be investigated in further experiments in which the therapeutic and prophylactic potential of Fh12 will be evaluated *in vivo*, including its effect on the function of APCs.

The selective targeting of CD14 coreceptor could be a way to inhibit the entire TLR4 pathway. Because CD14 is potentially a target of a new generation of antiseptis agents (77), the finding that Fh12 targets the CD14 coreceptor could have pharmacological applications. A glycoconjugate preparation from *Treponema spirochetes* (Tm-Gp) was also reported to inhibit the interaction of LPS with CD14, acting as an antagonist of TLR4 (78); however, this preparation is chemically heterogeneous, and it was not possible to determine the component responsible for the interaction. In other studies, synthetic lipids have been used to inhibit LPS-induced TLR4 activation in HEK293 cells by targeting the CD14 coreceptor (79). Benefits of suppressed TLR4 activation have been documented in several experimental models of lethal shock (44, 80), as have the benefits of using anti-CD14 (81) and anti-TLR4 (82) Abs in humans. Also, synthetic LPS antagonists such as Eritoran and Tak-242 have been tested in experimental models of endotoxic shock (83). In this context, this study offers a significant promising alternative, a well-characterized helminth protein (Fh12) obtained by a relatively inexpensive methodology that has anti-inflammatory properties mostly via TLR4.

In summary, to our knowledge, this is the first study to report the anti-inflammatory properties of *F. hepatica* FABP (Fh12) and its modulatory effect on macrophage function. Several novel findings stand out from the results being reported in this article: 1) that Fh12 blocks induction of inflammatory mediators *in vitro* and *in vivo*, and doing so completely inhibits activation of TLR4 by LPS in a dose-dependent manner; and 2) that this anti-inflammatory effect occurs through various concurrent mechanisms. First, Fh12 targets the CD14 coreceptor. Thus, *F. hepatica* Ags with Fh12 as a constituent could be saturating CD14 on circulating monocytes in infected subjects rendering them refractory to LPS-induced inflammatory activation. Second, Fh12 inhibits the activation of transcription factor NF- κ B, as well as ERK, JNK, and p38, which is a common molecule of multiple TLR pathways. Third, Fh12 overexpresses the IL-12p40 subunit, which may have a profound inhibitory effect on IL-12p70 function and consequently on macrophage maturation and function. Thus, this study represents a significant contribution to the development of drugs that block either activation through TLRs or their downstream signaling pathways, leading to inhibition of the storm of inflammatory molecules implicated in the pathology of many diseases.

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Disclosures

The authors have no financial conflicts of interest.

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