Possible Role for Toll-Like Receptors in Interaction of *Fasciola hepatica* Excretory/Secretory Products with Bovine Macrophages

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Alternative activation of macrophages (Mφ) during helminth infection is a characteristic feature of the host immune response. Alternatively activated macrophages (AAMφ) are distinguished from others by high arginase 1 (Arg-1) activity, low nitric oxide (NO), and high interleukin 10 (IL-10) production. In murine models, these cells have been shown to possess anti-inflammatory properties. They have also been implicated in exacerbating a subsequent infection with a secondary pathogen. In this study we used cattle experimentally infected with *Fasciola hepatica* to monitor the kinetics of IL-4 and IL-10 over the course of infection. Using naive Mφ in vitro, we examined the effects of exposure to *F. hepatica* excretory/secretory products (FhepES) alone or in combination with IL-4. Our results suggest that FhepES may work in combination with IL-4 to produce AAMφ. The effects of FhepES on the subsequent responses to lipopolysaccharide (LPS) and purified protein derivative from *Mycobacterium bovis* (PPD-B), which are bovine Toll-like receptor 4 (TLR4) and TLR2 antagonists, respectively, were also examined. We found that Mφ stimulated with FhepES together with LPS or PPD-B have reduced NO or gamma interferon production, respectively. The ability of FhepES to produce AAMφ was found to be heat labile and partially dependent on glycan residues. A possible role for TLR recognition is discussed.

Alternative activation of macrophages (Mφ) is a common feature of the immune response to helminth parasites, and their presence has been shown in a number of model infections, including those caused by *Schistosoma mansoni* (14), *Taenia crassiceps* (25), *Brugia malayi* (21), and *Fasciola hepatica* (9, 11). These alternatively activated macrophages (AAMφ) are distinguished from classically activated macrophages by preferential use of arginase 1 (Arg-1), instead of inducible nitric oxide (13), to metabolize L-arginine. A number of other markers of AAMφ have also been identified; these include increased expression of interleukin 10 (IL-10) and the mannose receptor (13), as well as production of interleukin, chitinases, and chitini-

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and oxidation of ES suggests that glycan residues have some role to play in parasite-host interactions.

**Materials and Methods**

Experimental infection and cytokine kinetics. Four castrated Friesian cattle were housed indoors and fed high-quality silage ad libitum at University College Dublin’s Lyons Research Estate, County Kildare, Ireland. Animals were experimentally infected with metacercariae as previously described (12). These infected animals were used to measure the kinetics of IL-4 and IL-10 production.

At the indicated time points, peripheral blood mononuclear cells (PBMCs) from *F. hepatica*-infected animals were isolated from Ficol-Histopaque (Sigma-Aldrich, United Kingdom) and cultured in complete RPMI 1640 (Invitrogen) containing 10% heat-inactivated fetal calf serum (Sigma-Aldrich), 200 U/ml penicillin (Sigma-Aldrich), and 200 μg/ml streptomycin (Sigma-Aldrich). Cells were plated, in triplicate, at a density of 1 × 10⁶ cells per well in 100-μl volumes. For cytokine production, *F. hepatica* excretory/secretory products (FhepES) were added at a concentration of 10 μg/ml with phosphate-buffered saline (PBS) as a control. Following 72 h of incubation, supernatants were harvested and stored at −20°C until analysis. All animal work was conducted under license from the Department of Children and Health following approval by the University College Dublin’s Ethics Committee.

Macrophage isolation, culture, and activation. Blood collected from naive healthy animals was used for the generation of mo nococyte-derived macrophages (MDM). Blood was collected into lithium heparin-coated Vacutainers, and isolation of Møs was performed as described elsewhere (12). Briefly, PBMCs were isolated from blood by centrifugation over Ficol-Histopaque (Sigma-Aldrich). Cells were washed in warmed complete medium (RPMI 1640 containing 10% fetal calf serum [Sigma-Aldrich]) and plated at 1 × 10⁶ cells/ml in 200-μl volumes. To generate MDM, cells were cultured for 7 days, with the medium changed every second day. Activating molecules were added to mature Møs in a final volume of 20 μl of medium at the indicated concentrations, and cells were incubated for a further 48 h. LPS derived from *Escherichia coli* 111:B4 (1 μg/ml) was used as a TLR4 antagonist (8). M. bovis purified protein derivative, PPD-B, used at indicated concentrations, was used as a stimulus for TLR2 activity (30).

Antigen preparation. FhepES were prepared as previously described and purified to remove contaminating endotoxin residues (12). To heat inactivate FhepES prior to use, samples were heated to 95°C for 15 min. Optical densities at 570 nm were measured and compared to a urea standard curve, where one unit of enzyme activity is equal to the amount of urea produced in the reaction. Results were expressed as milliunits of activity per 10⁶ cells.

(i) Arginase activity. Cell lysates (50 μl) were heated to 55°C for 10 min with 50 μl Tris buffer (25 mM Tris-HCl buffer with 10 mM MgCl2 [pH 7.5]). An equal volume (25 μl) of this mixture was added to 0.5 M arginine (pH 9.7) and incubated for 1 h at 37°C. The reaction was stopped by the addition of 400 μl of acid stop solution (96% H2SO4, 85% H3PO4, and H2O at a ratio of 1:3:7). 25 μl of 9% isonitrosopropionohexane was added and heated to 100°C for 45 min. Optical densities at 570 nm were measured and compared to a urea standard curve, where one unit of enzyme activity is equal to the amount of urea produced in the reaction. Results were expressed as millimoles of activity per 10⁶ cells (mM/10⁶ cells).

(ii) Nitrite oxide. Nitrite oxide levels were quantified using the Griess reaction. Briefly, 50 μl of supernatant was added to 100 μl of sulphanilamide solution and incubated in the dark at room temperature for 10 min. One hundred microliters of N1-naphthylenediamine dihydrochloride solution was then added and incubated as described above. Optical densities at 570 nm were measured, and nitrate was quantified by comparison with a standard curve.

(iii) Acidic chitinase activity. Chitinase activity was determined using a standard assay (3). Briefly, 10 μl of supernatant was added to 40 μl of 0.1 M sodium citrate buffer containing 0.025 mM of substrate, 4-methylumbelliferyl-β-D-N,N′,N′′-diacyctethylchitosamine. The assay was buffered to pH 2.5 specifically to measure acidic chitinase (AMCase) activity only. The reaction mixture was incubated at 37°C for 3 h and stopped by the addition of 200 μl of 0.25 M glycine-NaOH (pH 10.5). Fluorescence was measured by excitation at 365 nm and emission at 460 nm. Chitinase activity was quantified by comparison with a standard curve of 4-methylumbelliferyl (Sigma), and activity was expressed as nanomoles/hour/milliliter.

**Results**

The 2 cytokine production elicited by FhepES stimulation of lymphocytes from *F. hepatica*-infected animals. IL-4 and IL-10 production by PBMCs restimulated in vitro with FhepES was examined from four *F. hepatica*-infected animals over the course of a 12-week infection. Postinfection, IL-4 production peaked between weeks 2 and 6, with levels at week 6 ranging from 135.5 pg/ml to 423 pg/ml. Subsequently, decreasing levels of IL-4 were observed as the infection reaches chronic stages (Fig. 1a). IL-10 production in the same cultures was initially low with only one of four infected animals producing detectable IL-10 at week 2. By week 6, three of four infected animals were producing detectable levels, and these continued to rise steadily, reaching a peak at week 12 (Fig. 1b).

Alternative activation of macrophages by a *F. hepatica*-derived molecule. We generated AAMøs by exposing MDM from healthy uninfected cattle to FhepES concentration of 20 μg/ml (calculated from dose-response curves [data not shown]). High levels of Arg-1 (60.9 μM/l0⁶ cells), AMCase (30.3 nmol/h/ml), and IL-10 (ODI of 29.3) were produced by FhepES-stimulated cells (Fig. 2). The levels of Arg-1, AMCase, and IL-10 produced by FhepES-stimulated cells were significantly higher than those in LPS-stimulated cells. IL-10 was also detected in LPS-stimulated cultures. However, the levels of IL-10 produced by FhepES-stimulated Møs were significantly higher than those produced by LPS stimulation (ODI of 29.3 ± 2.8 versus 12.3 ± 1.01 [P < 0.005]). LPS incubation resulted in levels of NO significantly higher than those from FhepES-stimulated cells (P < 0.005).

Synergy between FhepES and IL-4 in macrophage activation. The optimal concentration of IL-4 for use in in vitro assays was determined by a dose-response curve. Using arginase and AMCase activity as markers of activation, we found...
20 ng/ml to be the optimal dose (Fig. 3a and b). We exposed MDM to IL-4 and found upregulation of Arg-1 and AMCase. When cells were treated with IL-4 and FhepES together, we found increased levels of Arg-1 and AMCase (Fig. 4a and b). The levels of Arg-1 were found to be significantly greater in cultures exposed to both ES and IL-4, with costimulation producing 62.43 ± 12.94 mU/10⁶ cells versus 40.01 ± 8.98 mU/10⁶ cells for cells treated with IL-4 only (P < 0.02). The same pattern was found for AMCase (P < 0.01). However, we found no evidence for IL-10 production in cultures stimulated with IL-4. IL-10 release was detected only after the addition of FhepES to IL-4-stimulated cultures. Stimulation with IL-4 alone or with IL-4 plus FhepES did not result in NO production (data not shown).

Effects of FhepES on responses of macrophages to PPD-B and LPS. To examine the plasticity of Mφ, cells were exposed to increasing concentrations of PPD-B and simultaneously incubated with FhepES, IFN-γ, and IL-10 levels were measured in the culture supernatants. IFN-γ levels were found to increase as the dose of PPD-B was increased (Fig. 5a). However, when FhepES was added to parallel cultures, IFN-γ production was reduced. No significant difference between the low-dose PPD-B culture and the parallel PPD-B–FhepES culture was observed in terms of IFN-γ production, suggesting that a high dose of PPD-B is required to stimulate IFN-γ production. IL-10 was produced following PPD-B stimulation alone. Again, the lowest dose of PPD-B alone was not sufficient to induce IL-10 levels above background. The addition of FhepES to parallel cultures resulted in massive release of IL-

FIG. 1. Stimulation of PBMCs from F. hepatica-infected animals with FhepES elicits Th2 cytokines. Lymphocytes isolated, at the indicated time point after infection from four animals (Fh 1, Fh 2, Fh 3, and Fh 4), were stimulated with FhepES (20 μg/ml), and after 72 h, supernatants were collected, and the relevant cytokines were measured. (a) IL-4 was measured by an ELISA, and the amounts are shown in picograms/milliliter. (b) IL-10 was measured by an ELISA, and results are shown as optical density index (ODI) calculated by subtraction of control (PBS reading) from experimental (ES-stimulated) reading, and then the optical density is multiplied by 1,000. All cells were cultured in triplicate, and results are average readings ± standard errors of the means (error bars). Wk, week.

FIG. 2. Matured Mφ, at a density of 2 × 10⁵ cells, were stimulated with PBS, FhepES (20 μg/ml), or LPS (1 μg/ml) for 48 h. (a) Arg-1 activity (in milliunits of activity per 10⁶ cells) in lysates. (b) AMCase activity (in nanomoles/hour/milliliter) from supernatants. (c) NO levels (in micromolar) in supernatants. (d) IL-10 (measured as ODI) was detected by an ELISA. The values for groups given experimental treatments were significantly different (P < 0.05 by the Student t test) as indicated by the asterisk. All cells were cultured in triplicate, and values are shown as averages ± standard errors of the means (error bars). Results shown are representative of three independent experiments.
10, with a four- to fivefold increase. The effect of FhepES on the ability of LPS to classically activate macrophages was evaluated. LPS alone induces high levels of NO (Fig. 2c and 5c). The addition of FhepES, however, inhibits this effect (Fig. 5c). Similarly, FhepES modulates the LPS-stimulated M/H9278 away from the classical phenotype, instead favoring production of Arg-1 (Fig. 5d). These results suggest that FhepES has the ability to modulate the phenotype of LPS- and PPD-B-stimulated M/H9278.

The effects of FhepES are heat labile and partially dependent on glycan residues. To examine the effects of heat inactivation on the capacity of FhepES (heat-inactivated FhepES [Hi-ES]) to activate Mφ, FhepES were heated to 95°C. When Hi-ES were used to stimulate cells, no response could be seen, suggesting that heat inactivation had denatured the components of FhepES responsible for Arg-1, AMCase, and IL-10 production (Fig. 6). FhepES were oxidized to destroy glycan residues, Gly-ES, within the parasite material. Stimulation of cells with this revealed a decrease in all parameters measured. While the effects of glycans removal were not as dramatic as heat inactivation, they resulted in a reduction of approximately 50% in IL-10 and AMCase production ($P < 0.05$). The reduction in Arg-1 production was the most pronounced with activity falling from 85.01 mU/10⁶ cells for FhepES to 18.51 mU/10⁶ cells for Gly-ES ($P < 0.005$) (Fig. 6a).

**FIG. 3.** Mφ were cultured at a density of $2 \times 10^6$ in the presence of increasing doses (10 to 40 ng/ml) of recombinant bovine IL-4 as indicated. (a) Arg-1 activity (in milliunits of activity per 10⁶ cells) was seen to peak at 20 ng/ml, while (b) AMCase had maximal activity at 20 ng/ml. The value for the group treated with 20 ng/ml was significantly different ($P < 0.05$) from the values for the other groups (indicated by the asterisk). The value for the group treated with 20 ng/ml was significantly different ($P < 0.05$ by ANOVA) from the values for the groups treated with 30 and 40 ng/ml (indicated by the # symbol). Values are shown as averages plus standard errors of the means (error bars).

**FIG. 4.** Mφ were cultured as described in the legend to Fig. 3 in the presence or absence of IL-4 (20 ng/ml), either alone or with FhepES (20 µg/ml). (a) Arg-1 activity (in milliunits of activity per 10⁶ cells). Cultures treated with the combination of FhepES and IL-4 had greater enzyme activation than cultures treated with either alone. (b) Effect of FhepES on IL-4-driven AMCase activity (in nanomoles/hour/milliliter). A similar upregulation was seen as with Arg-1. (c) IL-10 (measured as ODI) measured by an ELISA. All FhepES-stimulated cultures had significantly more IL-10 than those stimulated with IL-4 only. All cells were cultured in triplicate, and values are shown as means plus standard errors of the means (error bars). The values of cultures treated with IL-4 plus FhepES were significantly different ($P < 0.05$) from the values of cultures treated with either IL-4 or FhepES alone (indicated by the asterisk). The values of cultures treated with IL-4 plus FhepES were significantly different ($P < 0.005$ by ANOVA) from the values for cultures treated with IL-4 only (indicated by the ¶ symbol). Values are shown as averages plus standard errors of the means (error bars). Results shown are representative of three independent experiments.

**DISCUSSION**

Here we demonstrate the production of IL-4, a key Th2 cytokine, by PBMCs of *F. hepatica*-infected cattle following restimulation in vitro. We also describe the shift from IL-4 production to IL-10 production as infection progresses. This is similar to other helminth infections where chronic infections occur. Previous work detailing the immune response of
F. hepatica-infected animals demonstrated a strong Th2 bias, characterized by high immunoglobulin G1 production coupled with poor IFN-γ release and cellular proliferation by 4 weeks postinfection (5, 6). The results presented here, describing the kinetics of IL-4 and IL-10 production from animals infected with F. hepatica, add to the knowledge of immune responses to F. hepatica. The presence of IL-10 also explains previous findings, documenting a lack of cellular responsiveness and IFN-γ production in chronically infected animals (5). Previously published work has demonstrated that Th2 cytokine environments favor the development of AAM/H9278 (9, 21, 25), and the presence of IL-4 is thought to play a role in their development. F. hepatica infection in the murine model also favors this, and we have previously shown the presence of AAM/H9278 in F. hepatica-infected cattle (12). Over the course of infection, resting MΦ are exposed to parasite-driven IL-4, which favors alternative activation. Our results support this idea, and the data dealing with IL-4 in combination with FhepES suggest there may be synergy between the two.

The function of AAM/H9278 in helminth infections is not fully clear; they have been implicated in a number of roles, including protection from immunopathology (14), parasite expulsion (1), and direct modulation of lymphocyte responses (19, 26, 28, 29). A role for AAM/H9278 in increased susceptibility to coinfection was also demonstrated in T. crassiceps-infected mice (24). In this model, increased replication of Leishmania major and L. mexicana was due to the presence of AAM/H9278 in cestode-infected mice and not the absence of Th1 responses. Our results suggest that AAM/H9278 generated by F. hepatica infection or in vitro exposure to FhepES results in a phenotype that may modulate or simply be nonresponsive to another activating molecule. Incubation with PPD-B, a TLR2 antagonist, together with FhepES resulted in poor IFN-γ release yet high IL-10 production. These results suggest that IL-10 induced by FhepES is downregulating IFN-γ. This could have implications for coinfections with F. hepatica. We have previously demonstrated that F. hepatica alters the diagnostic capacity of assays designed to detect bovine tuberculosis in coinfected animals. Here we show that the high levels of IL-10 produced by MΦ stimulated by FhepES in the presence of PPD-B could be responsible for the poor skin test responses previously seen in coinfected animals (12). Modulation of the MΦ response to LPS was also another feature of cells incubated with FhepES. LPS is a potent MΦ activator and signals through TLR4 to produce NO (8). Here we found a reduction in NO levels in MΦ stimulated with both LPS and FhepES. We also found that Arg-1 production was upregulated even in the presence of LPS. This suggests that the mechanism by which FhepES signals is capable of interfering with LPS signaling. Our results also add to other evidence that MΦ possess a degree of plas-

**FIG. 5.** Effects of FhepES on TLR2 and TLR4 stimulation. (a and b) Effect of FhepES on PPD-B stimulation. The values for cells stimulated with FhepES and PPD-B were significantly different (P < 0.005 by ANOVA) from the values for cells stimulated with PPD-B only (indicated by asterisks). (a) Simultaneous incubation of MΦ with FhepES and increasing concentrations of PPD-B (in micrograms/milliliter) result in reduced IFN-γ production (optical density at 450 nm) as measured by an ELISA. (b) IL-10 (measured as ODI) is secreted following PPD-B stimulation but massively upregulated by the addition of FhepES. (c and d) Effect of FhepES (20 μg/ml) on LPS stimulation (1 μg/ml). Values for cells stimulated with FhepES plus PPD-B were significantly different (P < 0.005) from the values for cells stimulated with LPS plus FhepES and cells stimulated with LPS only (indicated by asterisks). (c) Nitric oxide level (in micromolar) in supernatant of stimulated cells. (d) Arg-1 activity (in milliunits of activity per 10⁶ cells) in cell lysates. All cells were cultured in triplicate, and values are means plus standard errors of the means (error bars). Results shown are representative of three independent experiments.
tivity in their response to signals promoting classical or alternative activation (27). However, the various conditions under which this may occur have yet to be explored. These include sequential stimulation, involving removal of the initial stimulus, washing steps of the cells, and finally the addition of subsequent stimuli (27).

Our results suggest that FhepES may be capable of overriding the effects of TLR2 and TLR4 antagonists on Mφ phenotype. This may occur in one of two ways; alternative activation by FhepES may alter the expression levels of TLR2 or TLR4, or conversely, FhepES may be interacting with one or both of these receptors, leaving them unavailable for additional signaling. The exact method by which helminths interact with the innate immune system is not fully understood, while their ability to modulate many facets of innate immunity is not in question. However, there is growing proof that helminths modulate the host’s response though TLR interaction. There is evidence that products derived from early infective S. mansoni larvae can signal through TLR4. In particular, the glycan residues within these extracts were found to be responsible for increased IL-6 and IL-12p40 production (16). Lewis sugars and other glycans from T. crassiceps have been shown to induce IFN-γ via TLR signaling from peritoneal murine Mφ (7). Elsewhere a role for TLR2 has also been implicated in immunopathology in S. mansoni infection (18). Indeed the role of TLR2 was demonstrated to be necessary only in the priming stage of the immune response, suggesting a role for TLR in early recognition of the worm. Our results also point toward a possible interaction with the TLR system during F. hepatica infection. The blockade of other TLR stimuli by FhepES and reduction of effectiveness of Gly-ES point toward this. The majority of interactions between parasite products and the TLR system that have been documented seem to stem from parasite glycoproteins (15). The overriding Th2 nature of helminth immune responses might be explained by two possible suggestions. The possible presence of molecules other than glycans in FhepES having stronger interactions with the innate immune system or the polarization of the immune response may depend on the strength of the binding of the sugars to TLRs. This phenomenon has been demonstrated recently where antibodies directed against T-cell immunoglobinulin mucin TIM-1 had differing effects on the response, with binding by one resulting in increased IFN-γ and IL-17 in experimental autoimmune encephalomyelitis, while a second antibody resulted in a Th2 response in the same model. These effects were seen to be related to the avidity with which each antibody bound their epitope (31).

The results presented here suggest that F. hepatica has a strong interaction with the host immune system that may benefit parasite survival. Evidence to suggest a parasite interaction with the TLR system and interference with subsequent signaling is also presented. This has implications for coinfections involving helminths and other pathogens and helps to outline the mechanism behind some of the effects seen in coinfected animals (12). One important unanswered question is the exact nature of the interaction between F. hepatica or its ES with the innate immune system, although the involvement of glycans is highly likely. Studies involving TLR blockade need to be undertaken to test directly the involvement of these receptors. Ultimately, the identification of these molecules and an understanding of how they direct the immune response will aid the quest for effective antiparasite vaccination strategies.

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