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Invited Review

Fasciola hepatica: the therapeutic potential of a worm secretome

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ABSTRACT

The success of helminth parasites is partly related to their ability to modulate host immune responses towards an anti-inflammatory/regulatory phenotype. This ability resides with the molecules contained in the secretome of various helminths that have been shown to interact with host immune cells and influence their function. Consequently, there exists a unique opportunity to exploit these molecules for the prophylactic and therapeutic treatment of human pro- and auto-inflammatory disorders (for example septic shock, transplant rejection and autoimmune disease). In this review, we describe the mechanisms used by the trematode parasite, *Fasciola hepatica*, to modulate the immune responses of its host and discuss the potent immune-modulatory effects of three individual molecules within the secretome; namely cathepsin L1, peroxiredoxin and helminth defence molecule. With a focus on the requirements from industry, we discuss the strategies by which these molecules may be clinically developed to control human immune responses in a way that is conducive to the prevention of immune-mediated diseases.

Keywords: *Fasciola hepatica*, Cysteine protease, Peroxiredoxin, Helminth defence molecule, Macrophage, Diabetes

1. Introduction

Over millennia of co-evolution (Jackson et al., 2009), helminth parasites have developed unique and effective mechanisms by which to regulate the immune responses of their mammalian hosts to create an environment favouring parasite survival and longevity (Wilson et al., 2007; Allen and Maizels, 2011). The requirement for the host to overcome this parasite-induced immune regulation has resulted in compensatory adjustments to interleukin genes, which have ultimately allowed the parasite to be tolerated while concomitantly minimising tissue damage for the host. One outcome of these immunological adaptations is that exposure to helminth parasites is a requirement to establish and maintain normal immunological balance in humans (Barnes et al., 2005; Moller et al., 2007; Fumagalli et al., 2009; Maizels, 2009). In populations where parasitic infections are no longer endemic this fine-tuning of immunological responses has likely become disrupted, leading to inappropriate immune responsiveness, and consequently the development of auto-inflammatory diseases such as Crohn's disease, multiple sclerosis (MS), rheumatoid arthritis, and type 1 diabetes (T1D) (Bilbo et al., 2011; Rook, 2012). This premise is corroborated by epidemiological evidence demonstrating an association between a decreased incidence of helminth infections and an increased incidence of auto-inflammatory diseases in developed countries (Correale and Farez, 2007; Gale, 2002; Fleming and Cook, 2006; Zaccone et al., 2006). Studies using murine models (Cooke et al., 1999; La Flamme et al., 2003; Walsh et al., 2009; Melon et al., 2010) and human clinical trials using helminth infections (Summers et al., 2005a,b) provide additional support for the therapeutic potential of helminth infection. Consequently, the investigation of helminth infection, or worm therapy, as a treatment for auto-inflammatory disorders is a rapidly expanding field of research.

The merits of using helminth infection as a means of controlling auto-inflammatory responses in patients has been extensively discussed (Bilbo et al., 2011; Rook, 2012; Pritchard, 2011, 2012). However, the use of live parasites as a therapy is problematic because infection is associated with detrimental physiological side effects, as some tissue damage is still incurred.

Additionally, there is a lack of immunological specificity associated with active infection, which induces a multitude of effects that compromise normal immunity (for example elicitation of the immunological responses required for effective vaccination) (McSorley and Maizels, 2012). Consequently, it is more judicious to identify the specific immune-modulatory molecules produced by helminth parasites and to characterise their precise mechanisms of action. The therapeutic use of the specific immune-modulatory molecules, either singly or in combination, would permit a more targeted and selective level of treatment control, thereby increasing therapeutic efficacy and safety.

Many studies have focused on characterising the molecules secreted by helminths since these are expected to interact locally and systemically with host immune cells. Advances in mass spectrometry-based proteomics have facilitated the detailed characterisation of helminth secretomes, and, when integrated with interrogation of transcriptome datasets, this approach has revealed that helminths secrete a wide array of proteins. Additionally, helminths produce non-protein molecules such as phosphorylcholine, glycans and lipids, which can also exert profound immune-modulatory effects. Given the diverse composition of secretomes between helminth species, the challenge now is to identify the specific molecules that possess immune-modulatory characteristics and hence offer therapeutic potential. This discovery process, and subsequent translation into a clinical therapy, requires a strategic multi-faceted approach that elucidates not only the molecular biology and biochemistry of the parasite-derived molecules but also their effects on the phenotype and function of specific immune cells, and on the collective immunological responses generated.

2. *Fasciola hepatica*: a judicious choice in the search for novel immune modulators

Our laboratory has studied the immunological interaction between the trematode parasite, *Fasciola hepatica*, and its mammalian hosts for over 25 years. This helminth parasite has an extensive host range, including humans, cattle, sheep, buffalo, goats, rats, mice, and rabbits (Garcia et al., 2007). Interestingly, *F. hepatica* can infect, and complete its life cycle, in mammalian hosts to which the helminth has only been exposed in the last few centuries due to the export of infected

cattle from Europe; for example, capybara, alpaca and llama in South America and kangaroos in Australia (Robinson and Dalton, 2009). It follows that the parasite has not only acquired efficient mechanisms of infection and tissue invasion, but has also developed effective immune-modulatory ability that allows long-term residency in varied mammalian hosts.

All mammalian hosts of *F. hepatica* become infected by ingestion of vegetation containing encysted dormant larvae. Infective juveniles emerge in the duodenum, rapidly traverse the intestinal wall, and enter the peritoneal cavity where they break through the liver capsule. Following a period of consistent burrowing, feeding and growth within the liver parenchyma, they move to their final destination within the bile ducts where they mature and produce eggs (Andrews, 1999). Thus, during their migration and development, parasites encounter different host tissues and macromolecules and are confronted with dynamic physiological microenvironments (differing in conditions such as pH and oxygen availability) and host immune responses (both humoral and cellular). Despite all of these obstacles, *F. hepatica* can live for extended periods in its host (for example, up to 20 years in sheep) (Andrews, 1999).

Fasciola hepatica induces Thelper (Th2, anti-inflammatory) immune responses in its host, as do many other helminths. In contrast to gastrointestinal nematodes, however, where immune expulsion is generally mediated by host Th2 responses, the Th2 responses induced by *F. hepatica* parasites are not protective. Expulsion can be induced by vaccination, but this is dependent upon the generation of strong Th1 responses (Mulcahy et al., 1998, 1999; Golden et al., 2010). We have shown that during the acute phase of infection in mice, *F. hepatica* polarizes immune responses by (i) suppressing the production of Th1-associated cytokines, and (ii) inducing a potent parasite-antigen specific Th2 immune response (Brady et al., 1999; Donnelly et al., 2005). This modulation of host immune responses occurs within just a few hours of the parasite penetrating the intestinal wall. Peritoneal macrophages display characteristic markers of regulatory/M2 macrophage phenotype (for example Arg-1 and PD-L1 expression and secretion of IL-10 and TGFβ) within 24 hours of infection and are hyporesponsive to Th1-associated activation (Donnelly et al., 2005, 2008

and unpublished). As infection enters the chronic phase in mice (21 days p.i.), the frequency of IL-10-secreting $CD4^+$ regulatory T-cells (Tregs) increases significantly and results in the suppression of parasite-specific Th2 responses (Walsh et al., 2009). Administration of adult *F. hepatica* excretory/secretory products (FhES) to mice, like natural infection, inhibits the development of host Th1 responses and induces parasite-specific Th2 type immune responses in vivo (O'Neill et al., 2001; Donnelly et al., 2005, 2008, 2010), but does not directly activate Tregs (S. Donnelly and B.A. O'Brien, unpublished data).

Although immunological studies performed using domesticated animals are fewer and less extensive, as compared with those using murine models, these studies show that infection with *F. hepatica* induces potent and highly polarized Th2 responses. Cattle infected with the parasite show an almost complete absence of the Th1-associated antibody isotype, IgG2, and high levels of the Th2-associated antibody, IgG1, (Clery et al., 1996). Peripheral blood mononuclear cells (PBMCs) isolated from cattle and sheep, 4 weeks after experimental infections, fail to secrete IFNγ in response to parasite antigens (Clery et al., 1996; Brown et al., 1994), but do secrete elevated levels of IL-4 in vitro in response to stimulation with parasite antigens (Flynn et al., 2008; Donnelly and Dalton unpublished). The magnitude of these Th2 responses correlates positively with parasite burden (Clery et al., 1996), suggesting that *F. hepatica* is not only actively suppressing the production of host protective Th1 immune responses, but is also inducing robust Th2 responses. As infection progresses towards chronicity (12 weeks p.i.), continued IgG1 production is one of the few remaining features indicating persistence of a Th2 response (Flynn et al., 2008), and regulatory responses begin to predominate. At this stage, lymphocytes isolated from cattle and sheep show reduced secretion of IL-4, but an increase in parasite driven IL-10 and TGFβ production (Flynn et al., 2008; Donnelly and Dalton unpublished).

3. Consequences of *F. hepatica* immune modulation

A bystander consequence of *F. hepatica* infection is the suppression of immune responses directed against concurrent or secondary bacterial infections. For example, mice co-infected with *F. hepatica* and *Bordetella pertussis* (causative agent of whooping cough) exhibited a significant reduction in bacterial-specific Th1 responses and a consequential inability to eliminate the microbe. In addition, *F. hepatica*-infected mice displayed a reduced Th1 response to immunization with a bacterial whole cell whooping cough vaccine (Brady et al., 1999).

In cattle, infection with *F. hepatica* confers susceptibility to infection with *Salmonella dublin* due to the inhibition of Th1 immune responses (Aitken at al., 1979). Experimentally, PBMCs isolated from animals co-infected with *F. hepatica* and *Mycobacteria bovis* secreted reduced levels of IFNγ in response to stimulation with mycobacterial antigens, compared with PBMCs from animals infected with *M. bovis* only (Flynn et al., 2009). Importantly, this suppression of Th1 immune responses by *F. hepatica* not only affects immune responses to other infections but also significantly compromises the predictive capacity of diagnostic tests for bovine tuberculosis (BTB; Flynn et al., 2007) which are reliant on the production of *M. bovis*-specific IFNγ from whole blood cells. A recent epidemiological study involving 3,026 herd of cattle in England and Wales confirmed that, in the field, a significant negative association exists between exposure to *F. hepatica* and diagnosis of BTB, with an approximate under-ascertainment of one-third (Claridge et al., 2012).

While *F. hepatica* infection exerts a negative impact upon the host's ability to mount effective Th1 immune responses, the potent immune-modulatory properties of *F. hepatica* may be exploited to suppress the detrimental Th1 immune responses that precipitate auto-inflammatory disease. Recent studies demonstrated that infection of mice with *F. hepatica* attenuated the clinical symptoms of murine experimental autoimmune encephalomyelitis (EAE), a model for human MS. Protection against neuronal tissue degradation was associated with TGFβ-mediated suppression of autoantigen-specific IFNγ and IL-17 production, and thus destructive pro-inflammatory responses were attenuated (Walsh et al., 2009).

Our laboratory has shown that systemic suppression of host Th1 responses in mice can also be induced by the administration of FhES. A single i.v. injection of FhES (100 µg) was sufficient to prevent the activation of antigen-specific Th1 cells in response to immunization with a whole cell *B. pertussis* vaccine (O'Neill et al., 2001). Most recently, we have shown that i.p. delivery of FhES over a short time course (6 x 10 µg injections on alternate days) was sufficient to prevent the development of T1D in non-obese diabetic (NOD) mice (Fig. 1). In these experiments, the incidence of disease in female NOD mice treated with FhES approximated only 15%, with an average of 85% (over three separate trials) of mice remaining normoglycaemic up to 30 weeks of age. By contrast, approximately 82% of mice treated with vehicle control (PBS) developed diabetes by 20 weeks of age. FhES-induced prevention of T1D was associated with a reduction in the level of autoantigen (insulin)-specific Th1 immune responses (unpublished data).

4. Identification of the specific immune modulators in the *F. hepatica* secretome

Given our observations that FhES exerted analogous immune-modulatory effects to those seen during infection with *Fasciola hepatica*, it represents a valuable source of parasite-secreted immune modulators that can be mined to isolate novel therapeutic agents. To this end, our approach was to first reduce the FhES into fractions that were then assessed for their ability to mimic the immune-modulatory activity of infection in vivo; namely the inhibition and promotion of Th1 and Th2 immune responses, respectively. Analysis of the FhES by gel permeation chromatography identified two distinct major fractions, termed PI (approximately 200 kDa) and PII (20-60 kDa) (Donnelly et al., 2008). Intraperitoneal delivery of PI to mice caused the induction of regulatory/M2 macrophages and the development of antigen-specific Th2 responses. While PII did not induce Th2 responses, T cells isolated from the spleen of mice injected with this fraction showed reduced secretion of Th1 cytokines in response to antigen stimulation, thereby indicating inhibition of Th1 immune responses (Donnelly et al., 2008). To identify the specific Th2-inducing proteins within PI, the fraction was separated by one-dimensional gel electrophoresis. Peptides were subjected to in-gel

digestion with trypsin and analysed by mass spectrometry (M.W. Robinson, unpublished data; Fig. 2). This analysis revealed that PI primarily contained the antioxidant, peroxiredoxin (FhPrx), and a second more abundant protein with a molecular mass of <10 kDa, which due to its structural homology with host defence peptides, notably human CAP18/LL-37, we termed *F. hepatica* helminth defence molecule-1 (FhHDM-1) (Robinson et al., 2011). Molecular and biochemical analysis revealed that the Th1-inhibiting PII fraction consisted of cathepsin L cysteine proteases (FhCL) (Smith et al., 1993). Subsequent proteomics studies showed that a family of cathepsin L cysteine proteases are highly represented in FhES, comprising 80% of the total protein secreted (Robinson et al., 2009).

5. Mechanisms of immune modulation by secreted *F. hepatica* proteins

On the basis of the data described above, we have selected FhPrx and FhHDM-1 (from the PI fraction) and FhCL1 (from the PII fraction) as candidate molecules possessing immune-therapeutic potential. Significantly, homologues of all three of these molecules exist in the secretions of related trematodes that are major pathogens of humans, including the liver flukes, *Clonorchis sinensis* and *Opisthorchis viverrini*, the lung fluke, *Paragonimus westermani*, and the blood flukes, *Schistosoma mansoni* and *Schistosoma japonicum* (Donnelly et al., 2008; Robinson et al., 2011). Elucidating the mechanisms of action of FhPrx, FhHDM-1 and FhCL1 will be an important step in the translation to therapeutic applications.

5.1. Peroxiredoxin (FhPrx)

Within their vertebrate hosts, helminth parasites are exposed to reactive oxygen species (ROS) that are released from immune effector cells such as eosinophils, macrophages and neutrophils. Accordingly, helminths utilise an array of antioxidants for protection against oxidative stress. *Fasciola hepatica* expresses high levels of superoxide dismutase, which reduces superoxide

to hydrogen peroxide (H_2O_2) and Prx, which prevents the accumulation of H_2O_2 (Barrett, 1980; Callahan et al., 1988; McGonigle et al., 1995, 1998).

Immunocytochemical analyses have revealed that FhPrx is located in the gut epithelium of *Fasciola* worms (J.P. Dalton, unpublished data) and proteomic studies have shown that, despite lacking a predicted N-terminal signal peptide, FhPrx is secreted by *F. hepatica* (Robinson et al., 2009). FhPrx is produced throughout the lifecycle of *F. hepatica*, with expression levels during the parasite's development being positively correlated with exposure to host generated ROS. For example, the highest level of FhPrx protein expression occurs during the infective stage of the parasite's life cycle as it traverses the intestine of the host (Robinson et al., 2009). Tissue invasion and penetration is a vulnerable time in the parasite's lifecycle during which the parasite must circumvent vigorous host immune responses that are mounted in response to the tissue damage incurred.

The function of FhPrx is not limited to antioxidant effects, as we have shown experimentally that this molecule skews the phenotype of macrophages towards a regulatory/M2 phenotype. Intraperitoneal delivery of a functional recombinant FhPrx to BALB/c mice (3 x 5 µg injections on alternate days) induced the activation of regulatory/M2 macrophages, as verified by the expression of the markers, Ym1 and Arg1 (Donnelly et al., 2005). A similar result was observed when FhPrx was administered to IL-4- or IL-13-deficient mice, suggesting that FhPrx modulated the macrophage phenotype independently of these Th2-signalling cytokines (Donnelly et al., 2008). Recombinant FhPrx induced the expression of Ym1 by peritoneal macrophages in vitro, which validated that FhPrx directly interacted with, and modulated the phenotype of, macrophage populations. This immune modulatory effect was not dependent upon the antioxidant activity of FhPrx since an inactive recombinant variant of FhPrx also induced the expression of Ym1 and Arg1 in macrophages, both in vivo and in vitro (Donnelly et al., 2008).

Collectively, this data suggests that FhPrx-mediated activation of macrophages likely involves direct interaction of a conserved FhPrx structural motif with a receptor that is yet to be

identified. A recent report showed that a malarial Prx (Furuta et al., 2008) and extracellular mammalian Prx molecules, originating from damaged tissues, interact with toll-like receptor (TLR) 4 (Riddell et al., 2010). Furthermore, it was reported that the binding of mammalian Prx was dependent upon a conserved region of the protein (located between amino acid residues 70 and 90) (Shichita et al., 2012). Therefore, it is likely possible to design peptide derivatives of FhPrx that exert potent immune-modulatory effects.

A deviation towards M2 macrophage polarisation modulates disease progression in auto-inflammatory diseases. For example, the adoptive transfer of M2 macrophages into pre-diabetic NOD mice, in which the initiation and perpetuation phases of auto-reactive T cell responses have occurred, significantly reduced the incidence of T1D (Parsa et al., 2012). Furthermore, in a model of human MS (rodent EAE), the administration of M2 macrophages to rats, after the induction of clinical symptoms, suppressed the progression of disease by preventing relapse of paralysis (Mikita et al., 2011). While the mechanisms of protection afforded by the regulatory/M2 macrophage populations in these studies were not fully elucidated, we have shown that FhPrx-induced regulatory/M2 macrophages promote Th2 cell differentiation and suppress Th1 cell development in vitro (Donnelly et al., 2008). Furthermore, the adoptive transfer of FhPrx-activated M2 macrophages to naive murine recipients results in the polarisation of T cells towards a Th2 phenotype in response to stimulation with anti-CD3 (S. Donnelly, unpublished data). Collectively, these observations illustrate that the generation of regulatory/M2 macrophage populations is both necessary and sufficient to suppress pathogenic Th1 immune responses and suggest that the delivery of FhPrx, or perhaps the adoptive transfer of FhPrx-treated macrophages, has the potential to deviate the Th1/Th17 immune responses that precipitate auto-inflammatory disease.

5.2. Cathelicidin-like helminth defence molecule

Cathelicidin peptides represent an evolutionarily conserved component of innate immunity (Boman, 1995). For many years it was believed that these molecules acted solely as antimicrobial

peptides, however studies have revealed that the cathelicidin peptides interact with host cells to induce a multitude of effects not directly related to microbial killing (Scott and Hancock, 2000; Yang et al., 2001a, 2001b, 2002). New functions attributed to these peptides include the modulation of physiological processes, such as the activation of wound healing, angiogenesis and cartilage remodelling (Frasca et al., 2012). Therefore, the cathelicidin peptides represent potent effector molecules, not only in the generation of innate defences against bacteria, but also in the regulation of immune cell activation and migration and, accordingly, play a putative role in the pathogenesis of auto-inflammatory disease.

Due to their overall lack of primary sequence homology, cathelicidins are broadly classified according to their secondary structure, namely a linear amphipathic α-helical peptide (Hazlett and Wu, 2010). Using this classification, we noted that the FhHDM-1, the 8 kDa protein secreted by *F. hepatica*, could be classified as a cathelicidin, as circular dichroism spectroscopy studies indicated that both native and recombinant FhHDM-1 have a high propensity to adopt an α-helical structure, in both the presence and absence of helix-stabilising agents, and under both neutral and acidic pH conditions (Robinson et al., 2011).

The most widely studied cathelicidin is the human peptide, LL-37. This peptide is secreted as an inactive precursor protein, known as CAP18, which undergoes cleavage by endogenous proteases to release the bioactive 37-residue peptide, LL-37 (Agerberth et al., 1995; Gudmundsson et al., 1996). Residues 13–34 of LL-37 form an amphipathic helix that anchors the peptide to phospholipid membranes, via interaction with its hydrophobic face (Agerberth et al., 1995; Porcelli et al., 2008), and this confers antimicrobial activity (Giuliani et al., 2010). In addition, the amphipathic helix of LL-37 facilitates binding to bacterial endotoxin, thereby blocking its interaction with TLR4 and preventing the induction of pro-inflammatory immune responses (Nagaoka et al., 2001). Similarly, following secretion, FhHDM-1 can be proteolytically cleaved by parasite cathepsin L protease to release a C-terminal peptide fragment (Robinson et al., 2011). This 34-residue peptide (FhHDM-1 p2) contains a 21-residue amphipathic helix, which structurally

resembles the bioactive LL-37 peptide. Furthermore and analogous to the actions of LL-37, FhHDM-1 p2 binds *Escherichia coli* endotoxin in a concentration-dependent manner to prevent the classical/M1 activation of macrophages (Robinson et al., 2011).

Examination of gene expression profiles in newly excysted juvenile worms, migratory immature worms and mature adult parasites revealed that FhHDM-1 was constitutively expressed during all three life-cycle stages of *F. hepatica* (Robinson et al., 2011). The migration of parasites across the intestinal wall disrupts the epithelial barrier, and consequently facilitates the movement of luminal antigens (bacteria and their toxins) into the circulation (McDermott et al., 2003; Farid et al., 2008). Despite this translocation of enteric microbes, fatal septicaemia, in the presence of helminth infection, is not a common occurrence (Onguru et al., 2011). We proposed that the active secretion of FhHDM-1 by the parasite throughout its existence in the mammalian host ensures that the presence of potentially lethal bacterial lipopolysaccharide (LPS), from either intestinal flora or microbial co-infections, is neutralised, such that LPS-mediated activation of macrophages is avoided. Consequently, excessive inflammatory responses, that would precipitate septic shock, are avoided and the survival of the host, and therefore the parasite, are ensured.

The amphipathic helix is a structural motif that commonly mediates binding to cell membrane surfaces (Cornell et al., 2006). Indeed, LL-37 has been shown to interact with lipid rafts on the cell surface of CHO-K1 cells (Sandgren et al., 2004). Our recent studies showed that FhHDM-1 binds to macrophage plasma membrane lipid rafts, via selective interaction with phospholipids and/or cholesterol, before being endocytosed and localising to endolysosomal structures (Robinson et al., 2012). Active lysosomal cathepsin L, but not cathepsin S, processed FhHDM-1 releasing a C-terminal peptide containing the conserved amphipathic helix. This peptide inhibited the activity of vacuolar ATPase, thus preventing the acidification of endolysosomes. The resultant alkaline environment impeded the functional activity of lysosomal proteases, which optimally operate at low pH, and therefore prevented the processing of endocytosed proteins (Robinson et al., 2012). Accordingly, macrophages exposed to FhHDM-1 are unable to produce

antigenic peptides for loading onto major histocompatibility complex (MHC)II molecules for presentation to T cells. By this mechanism, FhHDM-1 effectively modulates macrophage function to prevent antigen-specific adaptive immune responses. Elucidation of this mechanism of immune-modulation opens up significant avenues for the prevention of the priming events (i.e. MHC presentation of (auto)antigen) that would generate cytotoxic T cells to break tolerance and precipitate auto-inflammatory disease. FhHDM-1 could also be of therapeutic benefit in situations in which the inhibition of vATPase or lysosomal acidification would halt the progress of pathologies such as cancer and osteoarthritis (Fais et al., 2007; Kartner et al., 2010).

5.3. Cathepsin L cysteine proteases (FhCL)

Cathepsin L cysteine peptidases are major components of the FhES during all life cycle stages of *F. hepatica* in the mammalian host (Tort et al., 1999). These enzymes are stored as inactive zymogens (pro-enzymes) within secretory vesicles of the gastrodermal epithelial cells and are subsequently secreted into the lumen of the parasite gut in large quantities, before being released externally into the host tissues (Dalton and Heffernan, 1989; Collins et al., 2004). The secreted cysteine peptidases degrade host interstitial matrix proteins such as collagen, laminin and fibronectin, and primarily function to acquire nutrients for the parasite by degrading host proteins into peptides (Berasain et al., 1997; Robinson et al., 2008). Given that these biological activities are central to survival of the parasite, it is not surprising that enzyme activity, ascribable to the papain family of cysteine proteases, has been identified as a major component of the secretions of most helminth parasites of humans, livestock and companion animals (Tort et al., 1999; Sajiid and McKerrow, 2002).

Protease activity is also a central biochemical property of many allergenic molecules such as the cysteine protease of the house dust mite, Der p1, the food allergens, actinidin, bromelain and papain, and the major grass-derived allergens (Chua et al., 1998; Grobe et al., 1999; Mills et al., 2004). The induction of Th2 and IgE responses associated with allergic responses has been shown

to be dependent upon the enzymatic activity of these molecules (Matsumura et al., 2012). Accordingly, innate immune cells have evolved to respond directly to protease activity (Cocks et al., 2000; Gottar et al., 2006). For example, activation of the protease-activated receptor (PAR)-2 on airway epithelial cells, induced by environmental fungi proteases, results in the production and secretion of thymic stromal lymphopoietin (TSLP), which subsequently activates dendritic cells (DCs) to mediate Th2 immune responses (Kouzaki et al., 2009). Papain stimulates the production of ROS in DCs and epithelial cells, which orchestrate the development of Th2 immune responses by inducing the formation of oxidized lipids that trigger TLR4-TIR-domain-containing adapter-inducing interferon-β (TRIF)-mediated induction of TSLP by epithelial cells (Tang et al., 2010).

Despite the structural and biochemical similarities between the helminth proteases, and other members of the papain-like cysteine peptidase clan, delivery of native FhCL1, or a functional recombinant FhCL1 protein, to mice does not induce an antigen-specific Th2 response (O'Neill et al., 2001). Instead, mice receiving either of these proteases exhibit a reduced ability to mount Th1 or Th17 immune responses, following bacterial infection or exposure to a whole cell bacterial vaccine (Brady et al., 1999; our unpublished data). Our data indicate that, unlike the major papain-like allergens, the role of FhCL1 is not to activate Th2 immune responses, but rather to prevent the differentiation of host protective pro-inflammatory Th1 and Th17 cells.

In fact, FhCL1 modulates the function of both macrophages and DCs, and thus influences the nature of developing antigen-specific adaptive immune responses. The secretion of IL-12 and IL-23 from DCs is required to promote the differentiation of Th1 and Th17 cells, respectively. However, FhCL1-treated DCs are unable to secrete IL-23 and are therefore compromised in their ability to induce antigen-specific Th17 cells (Dowling et al., 2010). Similar to the effect on DCs, FhCL1 prevented the secretion of Th1-associated cytokines from macrophages in response to TLR ligands, via degradation of endosomal TLR-3 and thus inactivation of MyD88-independent TRIF-dependent TLR signalling pathway (Donnelly et al., 2010). The modulation of innate immune responses by FhCL1 in vivo was sufficient to protect mice from the lethal effects of bacterial

endotoxin by preventing the release of the inflammatory mediators, nitric oxide, IL-6, TNF and IL-12, from macrophages (Donnelly et al., 2010; Fig. 2). By inhibiting these activation pathways in innate immune cells, FhCL1 prevented the generation of host protective immune responses. An additional consequence of TLR-3 cleavage by FhCL1 might be the promotion of Th2 immune responses induced by other parasite molecules (for example FhPrx or FhHDM-1) or by components of host tissue. For example, TLR3-deficient mice have a propensity to develop IL-4 dominant Th2 immune responses accompanied by an increase in numbers of regulatory/M2 macrophages, in response to both viral and parasitic infection (Joshi et al., 2008; Abston et al., 2012).

Collectively, our data to date suggest that the mechanism of action of FhCL1 is to re-establish tissue homeostasis by dampening the production of pro-inflammatory mediators and facilitating the development of Th2 immune responses, which is strongly associated with wound healing and tissue repair. In the context of auto-inflammatory diseases this scenario is of significant benefit as such disorders are generally mediated by pro-inflammatory Th1/Th17 immune responses.

6. Translation of *F. hepatica*-derived immune therapies from bench to bedside

After identification of an immune modulating helminth molecule, the next step most commonly taken by academic researchers is to test it prophylactically and/or therapeutically in a range of murine models of disease (reviewed in Harnett and Harnett, 2010). However, with an interest in reducing the rate of attrition at the earliest possible stage of development, we have found that the pharmaceutical industry is equally, if not more, interested in testing potential helminth-derived therapeutic proteins on human cells. This type of analysis raises the preclinical value of a molecule by demonstrating the translatability of the immune-modulatory effect from murine to human cells and exposing potential adverse side effects (such as platelet aggregation and haemolysis), and issues of stability in physiological conditions (for example half-life in plasma). Thus we are now investigating the in vitro pharmacology of our *F. hepatica*-derived immune-modulatory molecules using primary human cells, as well as testing those in various available

397 animal models of disease. In addition, we are assessing the immunogenicity and global immune
398 suppressive effects of helminth-derived molecules to further de-risk the development of these
399 molecules for clinical use.

400

401 *6.1. Avoiding immunogenicity*

402 Quantifying antibody titres in response to exposure to putative immune-modulatory
403 molecules is now a regulatory requirement for the development of therapeutic proteins, and post-
404 marketing surveillance of immunogenicity is an industry requirement (Stas and Lasters, 2009).
405 Large molecules carry increased immunogenic potential and therefore carry a higher risk of
406 inducing unwanted immune responses during treatment. However, some highly immunogenic drugs
407 have proved to be commercially successful, including Humira and the existing versions of Factor
408 VIII (West et al., 2008; Pisal et al., 2012). In cases such as these, where a potential therapeutic drug
409 has a unique mode of action, or is more potent than existing therapies, it will likely progress to
410 further development. Considering the absence of effective treatments for chronic auto-inflammatory
411 disease that do not carry adverse side-effects (such as toxicity or global immune suppression),
412 helminth-derived therapeutic proteins offer an attractive therapeutic avenue for novel drug
413 development.

414 Pre-clinical immunogenicity studies can be facilitated using in vitro and in silico testing. In
415 silico molecular modelling can predict the binding potential of peptides to different MHC class II
416 molecules or T cell receptors (TcRs), allowing the determination of the contribution of individual
417 amino acids to peptide binding, which will inform the design of 'deimmunised' sequence variants in
418 which peptide epitopes are mutated to disrupt MHC and/or TcR binding. Combining this
419 technology with in vitro analysis of $CD4^+$ T cell activation will allow the prediction of clinical
420 potential of specific helminth-derived molecules (De Groot et al., 2008).

421 Of the immune-modulatory molecules derived from *F. hepatica*, FhCL1 may not represent a
422 favourable candidate because it is a highly immunogenic 25 kDa protein (O'Neill et al., 1998),
423 which cannot be reduced to a smaller moiety since its immune-modulatory effect on the function of

innate cells depends upon its proteolytic activity. By contrast, our studies indicate that employing only a portion of the protein that interacts with innate cell receptors can reduce the immunogenicity of FhPrx. FhHDM-1 is a small molecule (8 kDa) and active peptides derived from FhHDM-1 are only 29-37 residues in length and hence it is inherently low-risk according to its immunogenicity potential. We have found that the administration of FhHDM-1 to mice (on alternate days for a total of six i.p. injections) does not induce memory T cell responses or FhHDM-1-specific antibodies (S. Donnelly, unpublished data).

6.2. Avoiding global immune suppression

Infections with helminth parasites can induce systemic modulation of host immune responses. *Fasciola hepatica* compromises Th1 responses in the host and interferes with immunity to concurrent infections with pathogenic bacteria and vaccination (Aitken et al., 1979; Brady et al., 1999; O'Neill et al., 2001; Claridge et al., 2012). The suppression of host immune responses during helminth infection has been shown to depend upon the continuing presence of the parasites in vivo, with fully functional immune responses being restored following anti-helminthic chemotherapy and subsequent expulsion of the parasite (Sartono et al., 1995; Grogan et al., 1996). This would suggest that any immune suppression induced by treatment with a helminth-derived protein may only be for the duration of the treatment regime, however this may be sufficient to redress the balance of the patient's immune system, thus preventing auto-inflammatory responses in the long term. In support of this premise, we found that protection of autoimmune diabetes in NOD mice by administration of FhES was maintained up to 30 weeks of age even though the final treatment was delivered when mice were only 6 weeks old. Although peritoneal macrophages isolated from mice during the FhES treatment regime responded poorly to simulation with IFN-γ (i.e. reduced expression of iNOS) compared with non-treated mice, by 10 weeks of age their reactivity was fully restored to levels observed in control animals (S. Donnelly, unpublished data).

Even if, as our data suggests, a patient's immune system is compromised during the treatment regime, this strategy offers a better alternative to the currently available immune therapies, which are life-long prescriptions and are associated with global immune suppression, debilitating side effects and toxicity. However, the extent to which a parasite-derived molecule impacts upon the development of an effective immune response is yet to be fully elucidated. Recent studies indicate that even in the presence of a helminth infection, a significant degree of immune functionality remains. For example, macrophages isolated from mice harbouring a helminth infection retained some antimicrobial ability, despite lacking IL-12 production (Mylonas et al., 2009). In addition, helminth infection has been shown not to effect the establishment of bacterial-specific responses induced by immunisation with a DNA vaccine (Frantz et al., 2012), or to inhibit the development of Th1 responses induced by a concurrent infection with *Toxoplasma gondii* (Miller et al., 2009). Therefore, the immune-modulatory activity of any *F. hepatica*-derived therapeutic molecule will need to be assessed for its capacity to generate systemic suppressive effects on protective immune responses, vaccination and/or anti-tumour immunity.

7. Concluding remarks

There are over 100 different auto-inflammatory diseases affecting hundreds of millions of people worldwide, however few effective treatments have been developed. The majority of existing, and potential, therapies treat disease symptoms or block the inflammation triggered by the immune response, rather than prevent disease. Many such therapies fail to exhibit immunological selectivity and thus cause global immune suppression that leads to unwanted side effects such as susceptibility to infection, bone loss, neurodegenerative impacts and epithelial thinning. Since helminth parasites have evolved to produce molecules that selectively modulate immunological responses to promote their own survival, while concomitantly reducing excessive tissue damage, helminth-derived molecules offer a first in class mechanistic approach to address the underlying cause of auto-inflammatory disease.

Characterising the predominant proteins within the secretome of *F. hepatica* has allowed the identification of the specific modulatory pathways that are targeted by the parasite (Fig. 3), thereby revealing the strong therapeutic potential of these molecules. While individual proteins may not be sufficient to protect against disease, the identification of these immune-modulating proteins secreted by *F. hepatica* ideally positions us to create a defined recombinant (or synthetic) version of FhES. However, unlike the native FhES, the recombinant proteins can be modified during synthesis to enhance stability and to reduce immunogenicity and toxicity. Further, an optimal combination of proteins can be selected, based on their specific modulatory function; therefore a therapeutic cocktail can be custom-made for specific clinical requirements.

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768 **Figure legends**

769

770 **Fig. 1.** Treatment of NOD mice with the excretory/secretory products of *Fasciola hepatica* (FhES)
771 prevents the development of autoimmune diabetes. Four week old female NOD mice were injected
772 i.p. with FhES (10 µg in 100 µl of sterile PBS) or vehicle (PBS), on alternate days, for a total of six
773 injections. The data shown are the percentages of mice that were hyperglycaemic (as defined by two
774 consecutive blood glucose concentrations ≥ 14 mmol/L) or normoglycaemic, at the experimental
775 end point (22-30 weeks of age) from three independent experiments.

776

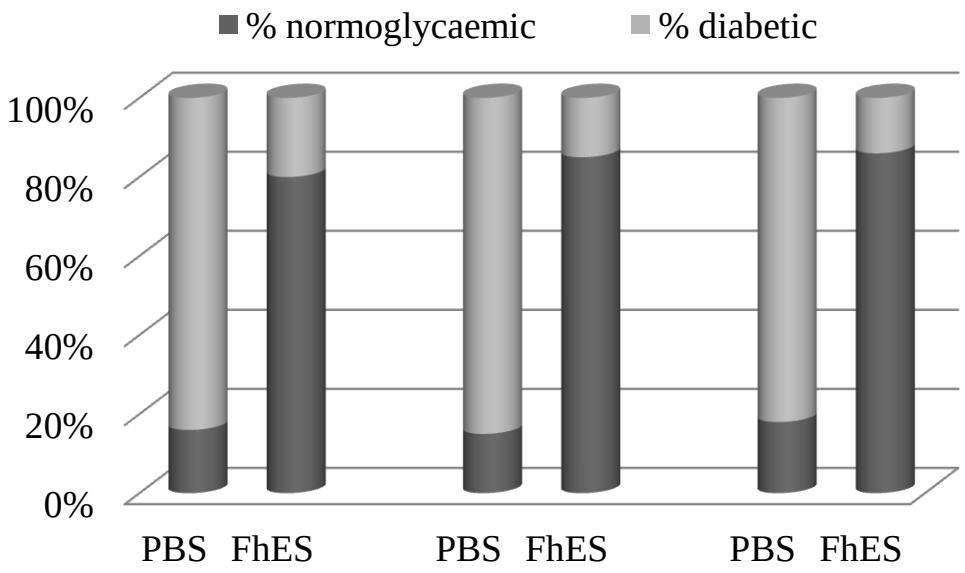
777 **Fig. 2.** Proteomics analysis of *Fasciola hepatica* secreted immune-modulatory fraction PI. **(**A)
778 Excretory/Secretory products of *F. hepatica* (FhES) were separated by gel filtration
779 chromatography and proteins in the resulting immune-modulatory fraction (PI) were
780 electrophoresed on a 4-12 % reducing gel. The most prominent protein bands were digested with
781 trypsin and identified by mass spectrometry (B). [a]Matched to *F. hepatica*; [b]identity confirmed as *F.*
782 *hepatica* helminth defence molecule (FhHDM) by N-terminal sequencing. In addition to its
783 monomeric form (band 3), a comparatively small amount of peroxiredoxin was present in dimeric
784 (band 2) and other oligomeric forms (band 1).

785

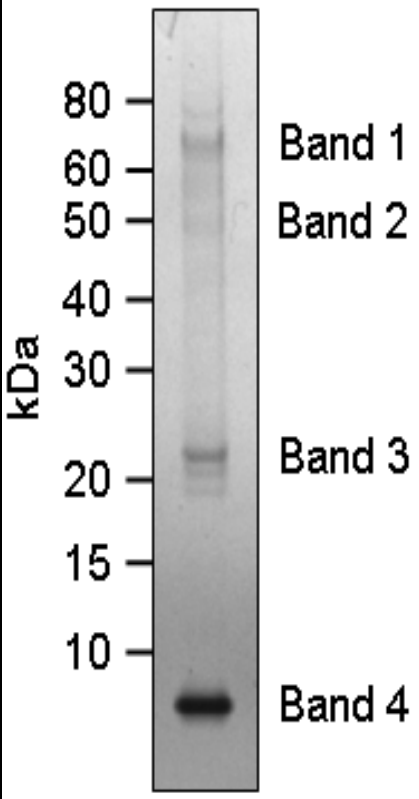
786 **Fig. 3.** Summary of putative mechanisms through which *Fasciola hepatica* may modulate auto-
787 inflammatory disease. (A) Antigen presenting cells (APCs) play an important role in the initiation
788 and perpetuation of auto-inflammatory disease. Activated dendritic cells (DCs) prime auto-antigen
789 specific T cells after migration to draining lymph nodes. This activation process is promoted by the
790 inflammatory cytokines, IL-12 and IL-23, secreted by the classical/M1 phenotype of macrophage.
791 APCs also play a significant role in the progression of disease, by facilitating the continued
792 activation and expansion of auto-reactive lymphocytes at the site of disease (for example the CNS
793 in multiple sclerosis (MS) and the pancreatic islet cells in type 1 diabetes (T1D)) and secreting

794 destructive pro-inflammatory mediators, such as TNF, IL-1β and nitric oxide (not shown). (B)
795 *Fasciola* secreted proteins influence the development of antigen-specific responses through contact
796 with APCs. Interaction of macrophages with *F. hepatica* peroxiredoxin (FhPrx) converts
797 macrophages to a M2/regulatory phenotype, which secrete the regulatory cytokines, IL-10 and
798 TGFβ (Donnelly et al., 2005), and promote the development of Th2 cells. *Fasciola hepatica*
799 cathepsin L1 (FhCL1) inhibits the ability of both macrophages and DCs to secrete the pro-
800 inflammatory cytokines, IL-12 and IL-23, which are necessary to promote the development of
801 antigen-specific Th1 and Th17 immune responses, respectively. *Fasciola hepatica* helminth defence
802 molecule (FhHDM-1) is internalised and cleaved in the lysosomes of APCs to release a peptide,
803 which reduces the capacity of APCs to process and present antigen, thus reducing the proliferation
804 of antigen-specific T cell responses. TcR, T cell receptor; Treg, regulatory T cell.

805

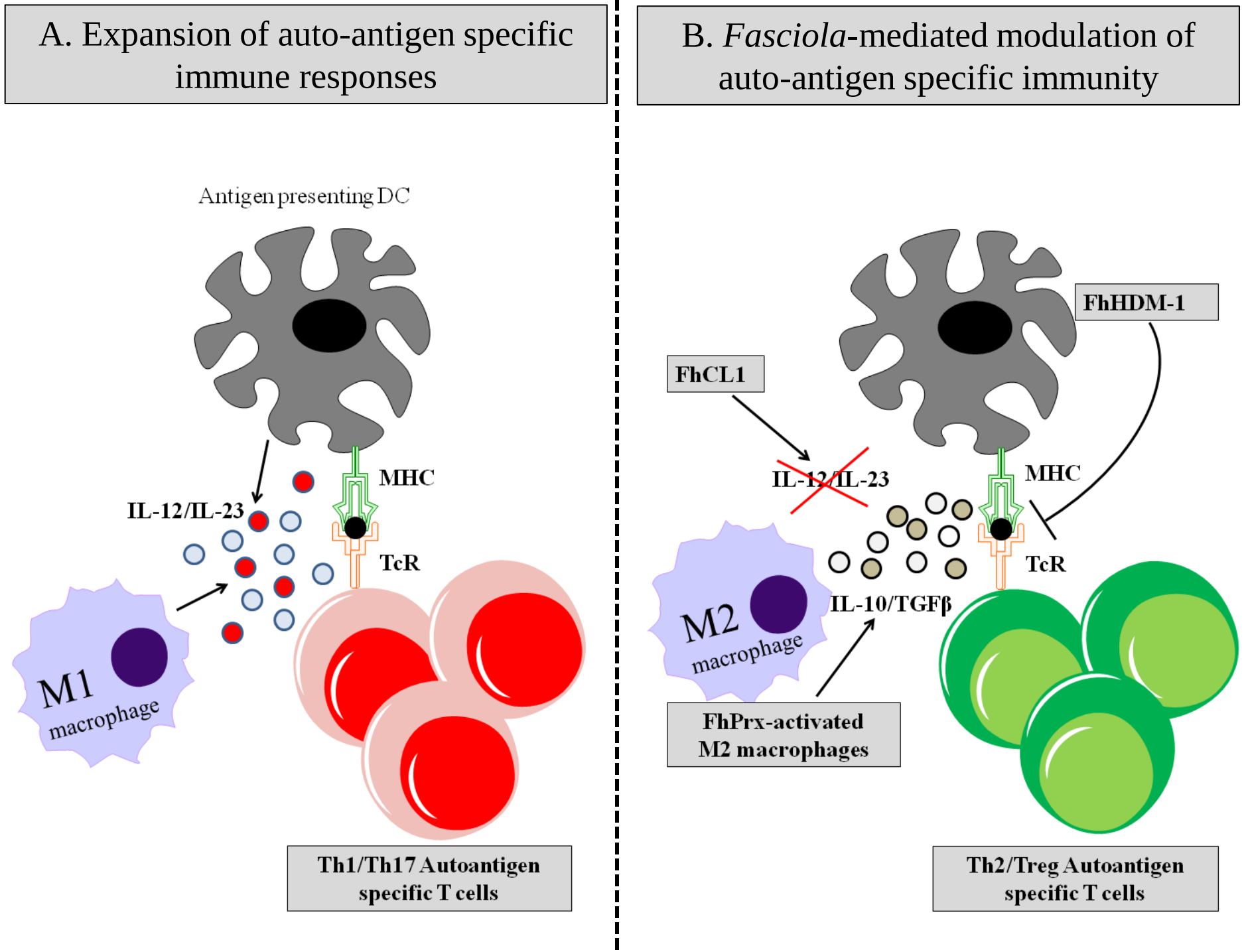


A



B

	Protein	Accession No.	Matched peptides	% cover	Peaks Score %
Band 1	Peroxiredoxin	O76944	5	17	98
	Prolylcarboxypeptidase[a]	Fhep43g08.q1k	5	19	99
	Sphingomyelin phosphodiesterase[a]	Fhep53a06.q1k	6	15	92
Band 2	Peroxiredoxin	O76944	5	12	99
Band 3	Peroxiredoxin	O76944	13	15	99
	GST sigma-class	DQ974116	6	20	97
Band 4	Helminth Defence Molecule (HDM)[a,b]	Fhep21e05.q1k	3	10	68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B. *Fasciola*-mediated modulation of auto-antigen specific immunity
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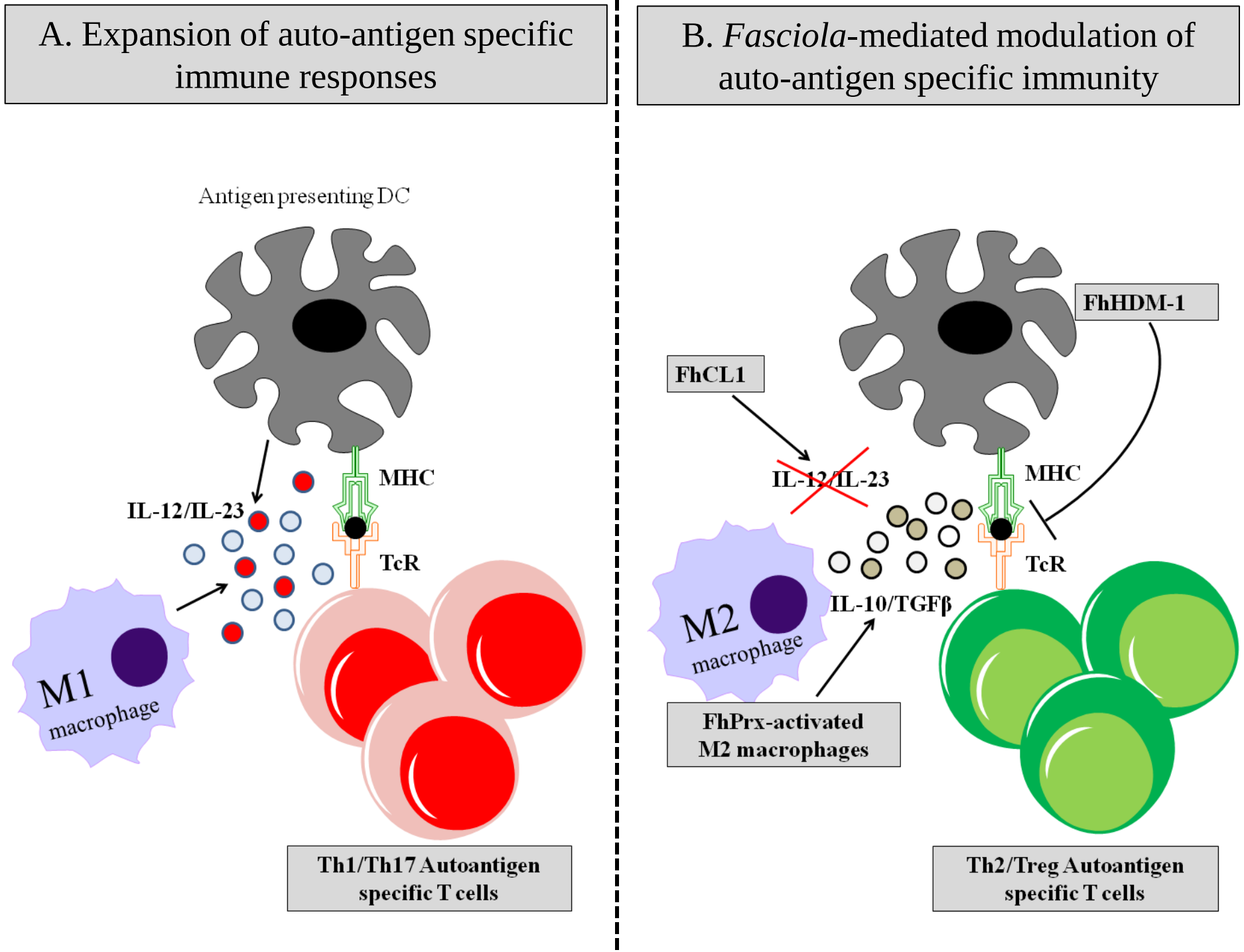
IL-12/IL-23
TcR
M1 macrophage
Th1/Th17 Autoantigen specific T cells
FhHDM-1
FhCL1
IL-12/IL-23
MHC
TcR
IL-10/TGFβ
M2 macrophage
FhPrx-activated M2 macrophages
Th2/Treg Autoantigen specific T cells

Highlights

- Within hours of infection, *Fasciola hepatica* suppresses host protective immune responses.
- Administration of *Fasciola* excretory/secretory products mimics the immune modulatory properties of live infection.
- *Fasciola* excretory/secretory products prevent the development of type 1 diabetes in mice.
- Cathepsin L1 secreted by *F. hepatica* prevents the activation of pro-inflammatory macrophages.
- *Fasciola hepatica* peroxiredoxin converts macrophages to an M2 phenotype.
- *Fasciola* helminth defence molecule-1 inhibits the processing and presentation of antigen by macrophages.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