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A Helminth Cathelicidin-like Protein Suppresses Antigen Processing and Presentation in Macrophages via Inhibition of Lysosomal vATPase

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Non-standard abbreviations:

vATPase:	vacuolar ATPase
HDM:	helminth defence molecule
HDP:	host defence peptide
AMP:	antimicrobial peptide
CT-B:	cholera toxin subunit B
Clq:	chloroquine
LUVs:	Large uni-lamellar liposome vesicles
POPC:	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
POPS:	1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylserine
POPE:	1-palmitoyl-2-oleoyl- sn-glycero-3-phosphatidylethanolamine
DOPC:	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
BMDMs:	Bone marrow derived macrophages
hCatL:	Human Cathepsin L

ABSTRACT

We previously reported the identification of a novel family of immunomodulatory proteins (termed helminth defence molecules; HDMs) that are secreted by medically-important trematode parasites. Since HDMs share biochemical, structural and functional characteristics with mammalian cathelicidin-like host defence peptides (HDPs), we proposed that HDMs modulate the immune response via molecular mimicry of host molecules. In the present study, we report the mechanism by which HDMs influence the function of macrophages. We show that the HDM secreted by *Fasciola hepatica* (FhHDM-1) binds to macrophage plasma membrane lipid rafts via selective interaction with phospholipids and/or cholesterol, before being internalised by endocytosis. Following internalisation, FhHDM-1 is rapidly processed by lysosomal cathepsin L to release a short C-terminal peptide (containing a conserved amphipathic helix that is key to HDM function) which then prevents the acidification of the endolysosomal compartments by inhibiting vacuolar (v) ATPase activity. The resulting endolysosomal alkalisation impedes macrophage antigen processing and prevents the transport of peptides to the cell surface in conjunction with MHC class II for presentation to CD4+ T cells. Thus, we have elucidated a novel mechanism by which helminth pathogens alter innate immune cell function to assist their survival in the host.

Key Words: Trematode, *Fasciola hepatica*, macrophage, lysosomal acidification, host defence peptides

INTRODUCTION

The innate immune response represents the first line of defence against invading pathogens. In addition to roles in direct recognition and engulfment of pathogens, macrophages promote antigen-specific adaptive immune responses. Pathogen antigens phagocytosed by macrophages undergo partial proteolysis by lysosomal peptidases before being loaded onto major histocompatibility complex (MHC) class II molecules for presentation to $CD4^+$ T cells (1), thereby generating both effector and memory immune responses. Thus, macrophages are critical in establishing communication between the innate and adaptive immune systems, and, in doing so largely determine the nature of the adaptive immune responses mounted which ensures that pathogens are either eliminated or their pathological effects are minimised.

Host defence peptides (HDPs; also referred to as antimicrobial peptides (AMPs) when they possess direct antimicrobial activity) represent an evolutionarily conserved component of innate immunity (2). To date, over 1000 naturally-occurring HDPs/AMPs have been identified displaying great diversity in sequence lengths, structures and activities (3). Since HDPs/AMPs share only limited sequence identity, they are broadly classified on the basis of homologous secondary structure as cathelicidins (linear α-helical peptides), defensins (β-strand peptides connected by disulfide bonds), and bactenecins (loop peptides) (4). Despite such variation, HDPs/AMPs are generally cationic with hydrophobic faces (5,6), allowing many of them to interact with, and disrupt, negatively charged microbial cell membranes (7).

Helminth parasites have devised many mechanisms for manipulating the responses of their hosts to allow their survival for long periods, and these often involve the secretion of specific molecules (8). We recently reported the identification of a novel immunomodulatory molecule secreted by the animal and human pathogen *Fasciola hepatica* (termed helminth defence molecule; FhHDM-1) that exhibits structural, biochemical and functional characteristics similar to mammalian cathelicidin-like HDPs (9). Phylogenetic analysis revealed that FhHDM-1 represented an archetypal member of a family of HDMs conserved throughout the Trematoda which includes major parasitic helminths of humans such *Schistosoma mansoni*, *S. japonicum*, *Opisthorchis viverrini*, *Clonorchis sinensis* and *Paragonimus westermani*. We isolated native and recombinant FhHDM-1 and showed that, like the mammalian cathelicidins, the precursor parent molecule is proteolytically processed (in this case by the parasite endopeptidase, cathepsin L1), to release a 34-residue C-terminal peptide (FhHDM p2) that forms an amphipathic helix. We proposed that the secretion of

HDMs represented an exquisite example of parasite immunomodulation mediated by the secretion of a host-like molecular mimic that influences host innate immune responses. Indeed, we demonstrated that both FhHDM-1, and FhHDM-1 p2 protected mice against LPS-induced inflammation by preventing the activation of macrophages (9).

In the present study, we investigated the mechanism by which HDMs influence the function of macrophages. Our studies show that FhHDM-1 binds to macrophage plasma membrane lipid rafts, via selective interaction with phospholipids and/or cholesterol before being internalised by endocytosis. Following internalisation, FhHDM-1 is rapidly processed by lysosomal cathepsin L to release a 27 amino acid C-terminal peptide (containing the conserved amphipathic helix), which then prevents the acidification of the endolysosomal compartments by inhibiting vacuolar (v) ATPase activity. Preventing endolysosomal acidification impedes macrophage antigen processing by proteases, such as cathepsin L, preventing the presentation of peptides at the cell surface in conjunction with MHC class II to CD4+ T cells. Thus, we have elucidated a novel mechanism by which helminth pathogens alter innate immune cell function to prevent the development of effective adaptive immune responses, thereby ensuring prolonged parasite survival in the host.

MATERIALS AND METHODS

Recombinant FhHDM-1 and synthetic peptide analogues

Recombinant FhHDM-1 was expressed in *E. coli* and purified as previously described (9). Residual bacterial endotoxin was removed from all samples using RP-HPLC. Final endotoxin levels were measured using the Chromo-LAL assay kit (Associates of Cape Cod, East Falmouth, MA, USA) and shown to be 0.0029 EU/µg.

A number of peptides were prepared based on the C-terminal alpha amphipathic helix of FhHDM-1 (Fig. 1). FhHDM-1 peptide 1 (FhHDM-1 p1) corresponds to residues 51-80, and represents a truncation of the C-terminal amphipathic helix. FhHDM-1 p2 corresponds to residues 56-89 and matches the C-terminal fragment of FhHDM-1 containing the complete amphipathic helix that is released following cleavage by FhCL1 (9). FhHDM-1 2Pro also corresponds to residues 56-89, but contains two proline substitutions (I69P and L80P) designed to disrupt the α-helix. Similarly, FhHDM-1 nonHP corresponds to residues 56-89 but contains six substitutions (I69R, I73Q, I75K, L76R, L84T and Y87K) designed to abolish the hydrophobic face of the amphipathic helix whilst preserving the α-helix. FhHDM-1 p3

matches the C-terminal fragment of FhHDM-1 containing the complete amphipathic helix that is released following cleavage by lysosomal cysteine peptidases as identified in the present study. The molar concentration of the HDM-derived peptides used is presented in Fig. S1.

HDM peptides were labelled with Cy5 (GE Healthcare, Pittsburgh, PA) or Alexa Fluor 488 (Life Technologies, Vic, Australia) according to the manufacturer`s instructions. All peptides were synthesised endotoxin-free by GL Biochem (Shanghai, China).

Primary human macrophages

Monocytes were isolated from buffy coats obtained from normal healthy adult donors and supplied by the Australian Red Cross Blood Service (Sydney, Australia). Mononuclear cells were purified from buffy coats by Ficoll-paque separation then CD14+ monocytes were enriched by positive selection (Miltenyi Biotec). Monocytes were differentiated into macrophages for up to 7 days in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 2 % v/v human serum and their phenotype confirmed (expression of CD206) using an LSR II flow cytometer (BD Bioscience).

Confocal laser scanning microscopy

For confocal laser scanning microscopy (CLSM) experiments, human primary macrophages ($1x10^6$) derived from blood monocytes (Australian Red Cross, Sydney, Australia) were treated with 10 μg/mL of recombinant FhHDM-1 at 37ºC then washed and fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X/PBS. After blocking, cells were incubated for 1 h at room temperature with primary antibody (1:2000) specific for His_6 tag (Life Technologies, Vic Australia). Cells were then washed and incubated with Alexa Fluor 488-conjugated (Life Technologies, Vic Australia) secondary antibody. To follow internalisation of FhHDM-1, macrophages ($7x10^5$) were simultaneously incubated with 10 μg/mL of Alexa Fluor 488-labelled FhHDM-1 and 60 nM LysoTracker (Life Technologies, Vic, Australia) for 45 min at 37ºC.

To examine the effect of FhHDM-1 on lysosome formation, macrophages ($1x10^6$) were incubated with either recombinant FhHDM-1 (10 μg/mL) or vehicle (PBS) for 20 h at 37ºC. After washing, cells were incubated with *E. coli* LPS (50 ng/mL; serotype 111:B4; Sigma, NSW Australia) for 2 h and stained with LysoTracker, according to the

manufacturer`s instructions. The cells were mounted in VectaShield® (Vector Laboratories, Burlingame, CA, USA) and examined with a Nikon A1 confocal scanning laser microscope. Images were rendered and analysed using NIS Elements software (Nikon, Melville, NY, USA).

3D-Structured Illumination Microscopy

Human primary macrophages ($1x10^6$) were treated with recombinant FhHDM-1 (10 μg/mL) for 2 h. Cells were incubated with 4 μg/mL of cholera toxin subunit B Alexa Fluor 594-conjugate (CT-B) for 20 min at 4°C, then fixed, and stained for FhHDM-1 using the His_6 antibody as described above. Imaging was performed using a DeltaVisionOMX 3D-Structured Illumination Microscope®, version 3 (OMX 3D-SIM, Applied Precision Inc, Issaquah, USA). Solid state lasers (405, 488, 593 nm) were captured simultaneously using 3 Photometrics Cascade (Photometrics, Tucson, USA) back- illuminated EMCCD cameras (>90% QE) with a 512 x 512 CCD, and on-chip charge multiplication. All data capture used an Olympus UPlanSApo 100x 1.4NA oil objective and standard excitation and emission filter sets (in nm, 405 EX / 419-465 EM, 488 EX / 500-550 EM and 592.5 EX / 608-648 EM). 3D-SIM images were sectioned using a 125 nm Z-step size. Raw 3-phase images were reconstructed as previously described (10,11) and the reconstructed images were rendered in 3D, with interpolation, using IMARIS v.7 software (Bitplane Scientific, Zurich, Switzerland).

Flow cytometry

RAW264.7 macrophages ($1x10^6$/well) were cultured in 24-well plates with or without 0.25 μg/mL trypsin (Life Technologies) for 1 h at 37°C. After washing, cells were incubated with Cy5-labelled recombinant FhHDM-1 (10 μg/mL) for 30 min at 4°C. Binding of Cy5-FhHDM-1 and was assessed by flow cytometry. Cells were also stained for the surface markers CD11b and F4/80 using directly conjugated FITC-monoclonal antibodies (BD Bioscience, San Jose, CA, USA).

Sodium carbonate membrane extraction

RAW264.7 macrophages ($2x10^7$) were incubated with 20μg/mL of recombinant FhHDM-1 at 37°C for 2 h followed by cross-linking with 1% paraformaldehyde for 30 minutes. After

sonication, membranes were prepared by centrifugation at 20,000g for 1 h. The membrane pellet was sequentially extracted with 0.1M Na_2CO_3 (pH 11) and 1 % Triton X100 (TX100) to produce peripheral and integral membrane protein fractions respectively (12). Samples were separated by non-reducing SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-FhHDM-1 (9), anti-actin, and anti-calnexin antibodies (Sigma, NSW Australia).

HDM-liposome interaction assay

Large uni-lamellar liposome vesicles (LUVs) composed of the phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylserine (POPS) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE) in a 3:1:1 molar ratio (Avanti Polar Lipids, Alabaster, AL) were made via the extrusion method (13) in 10% sucrose. HDMs (50 μM) were incubated with the LUVs for 2 h at 37°C before separation overnight on a discontinuous sucrose gradient (100,000g) at 4°C. Fractions were taken from the top (LUVs) and bottom of the tube and run in reducing 4-12% NuPage Bis-Tris gels (Life Technologies, Vic, Australia). Gels were silver-stained and imaged with a PharosFX laser imaging system (Bio-Rad, NSW Australia).

Phospholipid ELISA

PolySorp 96-well plates (Nunc, Roskilde, Denmark) were coated, in triplicate, with 200 μL of 100μg/mL lipids (sphingomyelin, POPC, POPS, POPE, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) as well as cholesterol) dissolved in ethanol and dried under nitrogen gas overnight (14). Plates were washed then incubated with serially diluted biotinylated FhHDM-1 p2 for 90 min at 37°C. Plates were washed and bound proteins were detected with ExtrAvidin-AP with pNPP substrate (Sigma, NSW, Australia).

Phagocytosis assays

Human primary macrophages or RAW264.7 cells were treated with HDMs (10 μg/ml) for 20 h or 1 h with folimycin (1 μM; Merck, NSW, Australia) or NH_4Cl (20 mM). In some experiments, cells were treated with E-64 (10 μM; Sigma, NSW, Australia) for 30 min prior to stimulation with recombinant FhHDM-1 for 3 h. Cells were then washed and incubated with 125μg/ml *E. coli* pHrodo Bioparticles (Life Technologies, Vic, Australia) for 2 h at

37°C and fluorescence from phagocytosed bioparticles was read using a Bio-Tek KC4 microfluorometer (532 nm excitation and 585 nm emission). Net phagocytosis was calculated relative to PBS-treated cells (equivalent to 100% phagocytosis). Phagocytosis of zymosan particles by RAW264.7 cells was determined using the CytoSelect 96-Well Phagocytosis Assay (Cell Biolabs Inc. San Diego, CA, Australia), according to the manufacturer`s instructions. To visualise phagocytosis, cells ($1x10^6$) were treated with or without Alexa Fluor 488-conjugated FhHDM-1 (50 μg/mL) for 2 h at 37ºC followed by Alexa Fluor 647-conjugated dextran (10 μg/mL; Life Technologies, Vic, Australia) for 2 h at 37ºC. Cells were washed and fixed before imaging by CLSM.

Enrichment of lysosomal membranes

The method of Cohen *et al*. (15) was used to prepare a fraction enriched 10x for lysosomes. Briefly, THP-1 or RAW264.7 macrophages ($4x10^7$) were sonicated on ice and a post-nuclear supernatant further centrifuged at 100,000g for 20 min to pellet lysosomes and endosomes. The lysosome pellet was extracted with water for 5 min then centrifuged at 100,000g for 20 min. The supernatant was collected as the lysosome-soluble fraction (Ly-S) and the pellet as lysosome-insoluble membrane fraction (Ly-M). The Ly-M pellet was resuspended in 10 mM Tris acetate (pH 7.0) overnight at 4°C. The Ly-S and Ly-M fractions were analysed by Western blot, for LAMP1 expression, using a specific antibody (Abcam, Cambridge, MA, USA).

Purification of phagosomes using latex beads

The method of Shui *et al*. (16) was used to isolate phagosomes. Briefly, RAW264.7 macrophages were incubated with 0.8 μM deep blue-dyed latex beads (Sigma, NSW, Australia) and allowed to internalize for 2 h at 37°C. The bead-containing phagosomes were purified using a sucrose gradient as described by Desjardins *et al*. (17). Soluble and membrane protein fractions were prepared from the washed phagosomes as described above.

ATPase assay

ATPase activity was detected in macrophage Ly-M fractions using the ATPase Assay kit (Innova Biosciences, Babraham, Cambridge, UK) according to the manufacturer`s instructions. For inhibition assays, serial dilutions of HDMs were pre-incubated with Ly-M in 0.1 M Tris-HCl (pH 7.5) for 1 h at 37°C prior to incubation with the ATP substrate.

Processing of FhHDM-1 by macrophage lysosomal proteases

Lysosomal cathepsin activity in macrophage extracts was confirmed by incubating the soluble extract fraction (Ly-S) with the fluorogenic substrate Z-Leu-Arg-NHMec (Bachem, Bubendorf, Switzerland). Assays were performed (± 10 μM E-64) at pH 5.5 as previously described (18).

Processing of FhHDM-1 was analysed by mixing 25 μg of the recombinant with RAW264.7 macrophage-derived Ly-S (1 μg protein) in 0.1 M sodium acetate (pH 5.5) containing 1 mM EDTA and 1 mM DTT for up to 5 h at 37 °C. Reactions were stopped by the addition of E-64 (10 μM). Digests were also performed using purified native human cathepsin L (Abcam, Cambridge, MA, USA). Samples were run on 4–12% Bis-Tris gels (Life Technologies, Vic Australia), transferred to nitrocellulose membranes and probed with an anti-FhHDM-1 antibody (9).

To identify peptides resulting from proteolytic processing of FhHDM-1, samples of the 0 h, 2 h and 5 h Ly-S digests were analysed by Matrix Assisted Laser Desorption Ionisation (MALDI) mass spectrometry at the Australian Proteome Analysis Facility as previously described (9). Spectra were acquired in positive mode in the mass range 2,000-20,000 Da with a mass accuracy of ± 50 Da.

Antigen processing assays

Bone marrow-derived macrophages (BMDMs) were generated from the femurs of BALB/c mice and confirmed to be $F4/80^{+}CD11b^{+}$ by flow cytometry. FITC-conjugated casein (50 μg/ml) was fed to RAW264.7 macrophages or BMDMs (3×10^{6}) with or without HDMs (5, 20 and 50 μg/ml) or chloroquine (20 μM). After 3 h incubation at 37°C, the cells were washed then lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche, NSW, Australia). Lysates were normalised for protein content and run on 4-12 % Bis-Tris gels (Life Technologies, Vic, Australia). Gels were scanned in the FITC channel using a PharosFX laser imaging system and normalized spot quantities were determined using PDQuest version 8.01 software (Bio-Rad, NSW, Australia).

Antigen presentation assays

BMDMs (1 x 10^5) were treated with HDMs (10-100 μg/ml) for 2 h then pulsed overnight with 10 mg/ml full-length ovalbumin (OVA; Sigma, NSW, Australia) or pre-processed $OVA^{323-339}$ peptide (Anaspec, Fremont, CA, USA). The HDM-treated OVA-pulsed BMDMs were then co-cultured with 1 x 10^5 DO-11-10 T cell hybridomas (ECACC) for 24 h and levels of IL-2 in the culture medium measured by ELISA (BD Pharmingen, NSW, Australia).

RESULTS

FhHDM-1 binds to the macrophage plasma membrane via interaction with lipid rafts

To investigate how the cathelicidin-like molecule secreted by *F. hepatica* interacts with macrophages, we first incubated primary human macrophages with recombinant FhHDM-1 and examined its cellular localisation pattern using an anti-His_6 tag antibody. No fluorescence was observed in PBS-treated cells stained with the anti-His_6 tag antibody (Fig. 2A), which confirms the specificity of the antibody for recombinant FhHDM-1. After 30 min incubation, fluorescence could be seen around the periphery of the cells, the intensity of which became stronger and distinctly punctate after 1 and 2 h (Fig. 2A). Localisation of the FhHDM-1 fluorescence at the macrophage plasma membrane was confirmed by counterstaining the cells with the lipophilic dye, DiI (Fig. 2A). When cells were incubated with an alternative His-tagged recombinant protein (FhCL1) (19), no surface labeling was observed, which demonstrates the specificity of the interaction of FhHDM-1 with the plasma membrane (Figs. S2A and S2B). Furthermore, OMX 3D-SIM microscopy revealed strong co-localisation of FhHDM-1 with the fluorescent lipid raft marker, CT-B (Fig. 2B; yellow fluorescence), indicating that FhHDM-1 interacts specifically with lipid rafts within the plasma membrane of primary human macrophages. FhHDM-1 also co-localised with CT-B on the surface of murine RAW264.7 macrophages (data not shown) demonstrating that its association with lipid rafts is not confined to human cells.

FhHDM-1 makes electrostatic interactions with the macrophage plasma membrane that are not associated with protein/receptor-binding

To investigate the biochemical nature of the FhHDM-lipid raft interaction, we performed an extraction of membranes, prepared from RAW264.7 macrophages treated with FhHDM-1. When membrane proteins are associated through electrostatic forces (typical of both receptor–ligand interactions and peripheral membrane proteins), these complexes are easily

disrupted and dissociated from the membrane. If, however, the protein is integral and associates with the membrane through hydrophobic interactions with the acyl core of the lipid bilayer, it will be highly resilient to dissociation. As expected, the peripheral membrane protein actin was only recovered in the Na_2CO_3 aqueous fraction of the RAW264.7 macrophages (Fig. 3A). By contrast, calnexin, which is an integral membrane protein, was found predominantly in the TX100 detergent fraction (Fig. 3A). FhHDM-1 was only recovered in the Na_2CO_3 aqueous fraction (Fig. 3A) suggesting that it interacts with plasma membrane/lipid raft components via electrostatic interactions. A similar result was obtained using membranes isolated from human THP-1 macrophages (Fig. S2C).

Having established that FhHDM-1 binds the macrophage plasma membrane via electrostatic interactions, we next investigated whether this was indicative of protein/receptor-binding. To investigate this, RAW264.7 macrophages were incubated ± trypsin for 1 h at 37°C and the surface binding of Cy5-FhHDM-1 determined by flow cytometry. Immunostaining for the macrophage surface markers, CD11b and F4/80, was used to demonstrate the success of trypsinisation. As expected, CD11b and F4/80 fluorescence was markedly decreased in trypsinised macrophages compared with undigested cells (Fig. 3B) showing that trypsin effectively degraded surface expressed membrane proteins. In contrast, the ability of FhHDM-1 to bind to the macrophage surface was unaffected following trypsinization as the level of Cy5-fluorescence was almost identical in digested and undigested cells (Fig. 3B), suggesting that FhHDM-1 does not associate with surface-expressed macrophage proteins (at least those susceptible to trypsinization).

Having established that FhHDM-1 did not likely interact with proteins expressed on the macrophage surface we examined its potential to bind directly to phospholipid or other non-protein components of the plasma membrane. To assess whether FhHDM-1 interacts with membranes, LUVs composed of common phospholipids, were used as model membranes. As shown by silver-stained SDS-PAGE, FhHDM-1 bound directly to the LUVs, whereas the control protein BSA was found at the bottom of the gradient (Fig. 3C).

A peptide corresponding to the conserved amphipathic helix of FhHDM-1 binds to liposomes

FhHDM-1 p2 is a 34 amino acid peptide containing the complete amphipathic helix of FhHDM-1 and is specifically released by proteolytic processing of the protein by the parasite protease FhCL1 (9). Like the parent molecule, this peptide bound to LUVs. In contrast, FhHDM-1 nonHP, which is similar to FhHDM-1 p2 but has six amino acid substitutions that

abolish the hydrophobic face of the amphipathic helix, did not associate with the LUV fraction but was instead detected in the bottom fraction of the sucrose gradient (Fig. 3C). This demonstrates the importance of the C-terminal amphipathic helix of FhHDM-1 for the association of this protein with cell-surface lipids.

We next investigated if the amphipathic helix preferentially bound particular phospholipid species. Monolayers of common phospholipids and cholesterol were prepared in ELISA plates and the binding of serially diluted biotinylated FhHDM-1 p2 was detected with a streptavidin-AP conjugate. The highest level of binding by FhHDM-1 p2 was to POPS, followed by DOPC and cholesterol (Fig. 3D). In contrast, FhHDM-1 p2 showed weaker binding to POPE and little or no reactivity with POPC and sphingomyelin (Fig. 3D). Sphingomyelin, POPC, and DOPC represent disaturated, monosaturated, and unsaturated phosphocholine lipids, respectively. FhHDM-1 p2 bound to DOPC (unsaturated) but also showed a high level of binding to POPS (monosaturated). This suggests that the selective phospholipid-binding properties of FhHDM-1 p2 are not simply due to lipid saturation status or to the presence of specific charged head groups in a particular lipid. Rather, binding ability appears to be related to the differential spacing of the head groups in the phospholipid bilayer, and also to the degree of oligomerisation of FhHDM-1 upon association with various phospholipid species (9,20). Whilst sphingomyelin and cholesterol are both key components of lipid rafts (21,22), FhHDM-1 p2 showed a much higher level of binding to cholesterol compared with sphingomyelin. Taken together, these data suggest that FhHDM-1 could bind to lipid rafts via interactions with cholesterol.

FhHDM-1 is internalised into endolysosomes and prevent their acidification via inhibition of vATPase activity

Using CSLM we found that Alexa Fluor 488-conjugated FhHDM-1 is internalised into the endolysosomal system of macrophages. FhHDM-1 co-localised with cytoplasmic vesicles that were visualised with LysoTracker (a lysosomotropic fluorescent dye that concentrates in acidic compartments) (see below and Fig. 7A). FhHDM-1 also co-localised with Alexa Fluor 647-conjugated dextran (when co-incubated with RAW264.7 cells), which further confirms that FhHDM-1 enters the endolysosomal pathway (Fig. 5A).

To investigate its effect on lysosome function, primary human macrophages were incubated with recombinant FhHDM-1 or vehicle (PBS) prior to treatment with bacterial LPS to induce lysosome formation. Following this, the cells were stained with LysoTracker. In 51

% of the PBS-treated macrophages, numerous acidic (LysoTracker-positive) vesicles were distributed throughout the cytoplasm following incubation with LPS (Fig. 4A, top panel). In contrast, only 25 % of the macrophages pre-treated with FhHDM-1 showed any LysoTracker-positive staining in response to LPS with very few acidic compartments visible within the cytoplasm (Fig. 4A, bottom panel). In addition, incubation of macrophages with FhHDM-1, prior to the addition of *E. coli* bioparticles conjugated to a pH-sensitive fluorogenic dye (that increases in fluorescence with decreasing pH), resulted in a significant reduction of fluorescence (36%; p=0.01) in comparison to vehicle (PBS)-treated control cells (Fig. 4A). FhHDM-1 p2 exhibited a significantly greater effect on the fluorescence levels (66%; p=0.006), while FhHDM-1 nonHP had no effect on fluorescence levels compared to control cells. Strikingly, the vATPase inhibitor, folimycin showed a similar level of inhibition (59%; p=0.007) to FhHDM-1 p2 (Fig. 4B). Treatment of the cells with NH_4Cl, which raises lysosomal pH (23), effectively abolished *E. coli* bioparticle fluorescence (p=0.004). Additional experiments using non-opsonised zymosan particles and Alexa Fluor 647-conjugated dextran showed that FhHDM-1 does not inhibit non-specific phagocytosis or endocytosis (Fig. 5B) confirming that the reduced *E. coli* bioparticle fluorescence resulted from FhHDM-induced endolysosomal alkalisation. When macrophages were pre-incubated with the cysteine peptidase inhibitor E-64, the inhibitory effect of FhHDM-1 on pH-dependent *E. coli* bioparticle fluorescence was abolished (Fig. 4C).

FhHDM-p2, but not the parent FhHDM-1molecule, inhibits vATPase activity.

The above data shows that FhHDM-1 and FhHDM-p2 prevented the acidification of vesicles within the macrophage. We next investigated whether this was due to the inhibition of vATPase, which acts as the major regulating factor for the acidification of the endolysosomal lumen (24). A lysosome-enriched fraction was prepared from THP-1 or RAW264.7 macrophages (both showed similar results as described below). The insoluble (membrane; Ly-M) component of this fraction was positive for the lysosomal marker, LAMP-1, by immunoblot (Fig. S3A). Furthermore, soluble extracts (Ly-S) from this fraction were strongly positive for lysosomal cathepsin L activity using the diagnostic fluorescent substrate Z-Leu-Arg-NHMec (Fig. S3B). The Ly-M fractions were treated with HDMs and assayed for ATPase activity. As shown in Figure 6, FhHDM-1 p2 inhibited ATPase activity associated with lysosomal membranes (equivalent to vATPase activity) in a concentration-dependent manner (IC50 = 0.9 μM). Strikingly, full-length recombinant FhHDM-1, as well as synthetic

peptide analogues with a truncated C-terminal amphipathic helix (FhHDM-1 p1), disrupted helical structure (FhHDM-1 2Pro) or where the hydrophobic face of the C-terminal amphipathic helix has been abolished (FhHDM-1 nonHP), did not inhibit ATPase activity even at the highest concentrations tested (Fig. 6). Similar results were obtained using membranes isolated from phagosomes purified using latex beads (data not shown).

FhHDM-1 is internalised by macrophages and processed by lysosomal cathepsin L to release vATPase inhibitory peptides

Observations that FhHDM-1-derived peptides inhibit vATPase, while the full-length parent molecule does not, may have been attributable to the processing of FhHDM-1 by endogenous proteases to release the C-terminal amphipathic helix following internalisation into the endolysosome. Our data showing that the cysteine peptidase inhibitor, E-64, could inhibit the effect of FhHDM-1 on endolysosome acidification indicated an involvement of this class of peptidase (see above). Therefore, full-length FhHDM-1 was incubated with the Ly-S fraction (i.e. native contents of macrophage lysosomes) at pH 5.5 and the reaction products were analysed by Western blot, using an anti-FhHDM-1 antibody. The blots showed that the 9.2 kDa band corresponding to full-length recombinant FhHDM-1 had slightly decreased in intensity concurrent with the appearance of a number of smaller protein fragments over time (Fig. 7B). These fragments were not detected when the reaction was performed in the presence of E-64 demonstrating that the full-length protein had been cleaved by a lysosomal cysteine peptidase. When the digests were performed using purified human cathepsin L (hCatL) endopeptidase (a key cysteine peptidase involved in antigen processing within the lysosome) a similar pattern of FhHDM-1 peptide fragments was observed (Fig. 7B).

The FhHDM-1 Ly-S digests (0, 2 and 5 h reactions) were analysed by MALDI TOF MS (Table 1; Fig. S3C). The only major mass detected in the 0 h reaction was matched to full-length recombinant FhHDM-1 (*m/z* 9232; S/N ratio 341). The intensity of this peak decreased considerably after 2 h (not detected) and 5 h (S/N ratio 13). The 5 h digest also contained a peak that matched to the recombinant FhHDM-1 minus the C-terminal His-tag (*m/z* 8399; S/N ratio 53), suggesting that processing of the recombinant molecule by mammalian peptidases involves initial removal of the His-tag as previously observed during processing of FhHDM-1 by FhCL1 (9). The dominant mass detected in the 5 h sample (*m/z* 3357; S/N ratio 361) matched to a 29 amino acid C-terminal peptide (minus the His-tag, but including the LE added by the expression vector) created by cleavage after Asp^{63} (native

peptide numbering). Interestingly, hCatL cleaved FhHDM-1 after Leu^{60} (Table 1) suggesting that the C-terminal peptide is processed further by lysosomal aminopeptidases or dipeptidyl/tripeptidyl aminopeptidase creating the final cleavage site at Asp^{63} observed following digestion with the native lysosomal extract (Ly-S).

Based on the putative hCatL cleavage site, a 27-residue synthetic peptide (FhHDM-1 p3) corresponding to the C-terminal region of the native molecule was designed for subsequent analysis. This peptide is eight residues shorter at the N-terminus and one residue longer at the C-terminal compared to FhHDM p2 (see Fig. 1). Strikingly, FhHDM-1 p3 inhibited vATPase activity in a concentration-dependent manner as effectively as FhHDM-1 p2 (Fig. 7C).

We also designed a number of synthetic peptides with various N- and C-terminal truncations of the amphipathic helix in order to determine the minimal sequence required for vATPase inhibition (Fig. 1). However, none of these truncated peptides inhibited vATPase activity (Fig. 7C). Of particular note is peptide FhHDM-1 48-70, which is shorter than FhHDM-1 p3 at the N-terminal end by only three amino acids (Asn^{64}-Leu^{65}-Gly^{66}) and indicates that this motif may be important for vATPase inhibition.

FhHDM-1 inhibits endolysosomal antigen processing and MHC class II antigen presentation

By preventing the acidification of endolysosomes, via inhibition of vATPase activity, we hypothesised that FhHDM-1 would inhibit antigen processing by macrophages as the activity of lysosomal proteases and hence the degradation of foreign proteins, is optimal at low pH (25). We therefore exposed murine macrophages to the MHC class II antigen casein (conjugated to FITC to enable fluorescent in-gel detection), and analysed its degradation by SDS-PAGE. Whilst FITC-casein was rapidly degraded in PBS-treated cells, antigen degradation was blocked when cells were co-incubated with chloroquine (Clq) a weak base known to elevate endolysosomal pH (23). Similarly, FhHDM-1 blocked processing of FITC-casein in a concentration-dependent manner (Fig. 8A-B). Whilst FhHDM-1 p3 had no measurable effect on FITC-casein degradation, FhHDM-1 p2 had a small inhibitory effect at higher concentrations that was not seen with peptide nonHP (Fig. 8A-B). It is interesting that FhHDM-1 showed a greater inhibitory effect on FITC-casein processing than FhHDM-1 p2 (Figure 8A) whilst FhHDM-1 p2 had a greater effect than FhHDM-1 on endolysosomal alkalization (Figure 4B). Whilst unexpected, these observations are reproducible and may be due to differences in the cell type used and assay sensitivity.

We next determined if FhHDM-1 could effect antigen presentation by macrophages. To do this we used OVA-specific T cell hybridoma DO-11-10 cells whose proliferative response to $OVA^{323–339}$ peptide, presented with MHC class II, can be quantified by measuring the levels of secreted interleukin-2 (IL-2) (26,27). DO-11-10 T cells activated *in vitro* by OVA-pulsed murine bone marrow derived macrophages, that were pre-treated with FhHDM-1, produced significantly less IL-2 ($p = 0.001$) than those exposed to PBS-treated macrophages (Fig. 8C). This effect was not observed when the pre-processed $OVA^{323–339}$ peptide was used as antigen or when OVA-pulsed macrophages were pre-treated with peptide nonHP (data not shown). Therefore, FhHDM-1 modulated the ability of macrophages to present antigen.

DISCUSSION

In response to stimulation by microbes and pro-inflammatory mediators, mammalian innate immune cells release an array of host defence peptides (HDPs) that work cooperatively to perform broad-spectrum antimicrobial activities (28-30). In addition to their direct antimicrobial activity, recent studies have shown that some HDPs (in particular the human cathelicidin-derived peptide LL-37) also regulate aspects of innate immunity that can protect against excessive inflammation stimulated by the LPS endotoxin (reviewed in (31)). They achieve this by interrupting key stages of LPS-mediated cell signalling, thereby preventing activation of macrophages and dendritic cells via toll-like receptors (TLR) (32-34). These mechanisms include direct binding and sequestration of LPS, preventing its interaction with TLRs and blocking the LPS-LPS-binding protein (LBP) interaction, both important early stage events in the recognition of microbial infections (35-37). We previously demonstrated that the helminth pathogen *F. hepatica* secreted a small molecule, FhHDM-1, which exerted similar effects on LPS signalling, including direct binding of LPS, thereby reducing its interaction with both LBP and to the macrophage surface (9). FhHDM-1 exhibited a striking ability to protect mice against LPS-induced inflammation by preventing the release of inflammatory mediators from macrophages (9). We proposed that HDMs are secreted by the helminth parasites to protect the host against excessive LPS-induced inflammation that would occur following disruption of the intestinal epithelial barrier (and concomitant systemic transfer of luminal bacteria), during parasite invasion and migration (9). Thus, by preventing the activation of innate immune responses normally induced by LPS, the helminth enhances the survival of the host and, accordingly, its own longevity. However, since we discovered

that HDMs are also rapidly internalised into macrophages we surmised that they have an additional, novel, mechanism of action that influences macrophage function.

Confocal microscopy revealed that the initial interaction of FhHDM-1 with macrophages occurs through association with lipid rafts on the cell surface. Despite extensive trypsinization of cell surface proteins, the interaction between FhHDM-1 and the macrophage was not affected, indicating that FhHDM-1 binds to non-proteinaceous lipid raft components. One putative binding partner for FhHDM-1 is cholesterol, a key sterol component of lipid rafts (22), since this molecule contains a cholesterol-binding motif, (L/V-$[X]_{1-5}$-Y-$[X]_{1-5}$-R/K, spanning residues V^{45}TKAYEKAR^{53}) that is highly conserved across all known cholesterol-binding proteins (38). However, FhHDM-1 p2, which lacks the cholesterol binding motif due to an N-terminal truncation, nevertheless binds cholesterol in a concentration-dependent manner. While the motif may enhance the specificity of the FhHDM-1-cholesterol interaction, or perhaps dictate the orientation of HDM-binding at the cell surface, it alone is clearly not essential for cholesterol-binding.

It is also probable that FhHDM-1 binds to lipid rafts via the HDM C-terminal amphipathic helix since a similar structural element of the tyrosine kinase interacting protein (Tip) of *Herpesvirus saimiri* has been shown to target Tip to lipid rafts and directs its lysosomal trafficking (39). The human cathelicidin-derived peptide, LL-37, also interacts with lipid rafts on the cell surface prior to endocytosis by CHO-K1 cells (40). However, LL-37 cannot bind to cholesterol (indeed its presence strongly reduces the ability of LL-37 to interact with phospholipid membranes; 41,42) and, therefore, more likely interacts with the $P2X_7$ receptor, which is found within lipid rafts (43). While LL-37 directly induces the release of cytokines from monocytes (notably IL-1β), via direct activation of $P2X_7$ receptors (44), we have found that FhHDM-1 p2 does not activate this receptor (data not shown). This highlights key functional differences between the two molecules and how they interact with macrophages.

Following initial binding to lipid rafts, FhHDM-1 was internalised into LysoTracker-positive vesicles and co-localised with fluorescently-labelled dextran, which is known to enter cells via the endolysososmal system (45). Pre-incubation of macrophages with FhHDM-1 led to a reduction of fluorescence associated with *E. coli* bioparticles that were conjugated to a pH-sensitive fluorogenic dye such that they only fluoresce upon delivery into acidified phagolysosomal vesicles. Experiments using non-opsonised zymosan particles and fluorescently-labelled dextran confirmed that the reduced *E. coli* bioparticle fluorescence was

not simply due to inhibition of non-specific uptake by FhHDM-1. The effect of FhHDM-1 on lysosomal pH was also demonstrated using the pH sensitive dye, LysoTracker as pre-incubation of macrophages with FhHDM-1 significantly reduced the numbers of LysoTracker-positive (acidic) vesicles throughout the cell in response to LPS stimulation. Collectively, these experiments show that FhHDM-1 causes the collapse of the endo/phagolysosomal pH gradient in macrophages. Proteomics analysis of FhHDM-1-treated primary human macrophages supports this finding, indicating that the parasite molecule has a suppressive effect on components of the lysosomal pathway (M.W. Robinson and R. Alvarado, unpublished data).

Acidification of endolysosomal compartments is primarily mediated by vATPase, a transmembrane complex comprised of cytosolic (V_1) and membrane bound (V_0) subunits (46). We found that FhHDM-1 p2 inhibited ATPase activity associated with macrophage endolysosomal membranes in a concentration-dependent manner. Since in assembled, functional vATPase complexes, ATP hydrolysis by the cytoplasmic V_1 domain drives proton transport through the membrane bound V_0 domain from the cytoplasm to the endolysosomal lumen (46) prevention of this process provides a novel, and direct, biochemical mechanism by which the secreted parasite molecule impairs acidification of endolysosomal vesicles. Consistent with the data derived from our *E. coli* bioparticle assays, vATPase inhibition required an intact HDM amphipathic helix (peptide nonHP was non-inhibitory), which further highlights the importance of the C-terminal tail of FhHDM-1. It is interesting to note that the human salivary HDP histatin-5, which possesses anti-*Leishmania* activity also targets a proton pump, the mitochondrial F_1F_0-ATPase (ATP synthase), of the parasite (47). However, this peptide does not adopt an amphipathic structure (48) and does not display primary sequence identity with FhHDM-1 p2, although both peptides possess short α-helices.

While the full-length parent FhHDM-1 molecule was capable of uncoupling endolysosomal acidification in macrophages, it was unable to directly inhibit vATPase activity. For this, FhHDM-1 needs to be proteolytically processed within the macrophage. MALDI-TOF MS analysis revealed that processing of FhHDM-1 by native lysosomal peptidases released a 29-residue C-terminal fragment (*m*/*z* 3357) created by cleavage after Asp^{63}. Purified native human cathepsin L released an almost identical 30-residue C-terminal fragment (*m*/*z* 3400) from FhHDM-1 created by cleavage after Leu^{60}, suggesting that this enzyme is responsible for FhHDM-1 cleavage *in vivo*. A 27-residue synthetic peptide analogue (FhHDM-1 p3) based on the sequence of the released peptides inhibited

macrophage vATPase activity, in a concentration-dependent manner, as effectively as the 34-residue peptide FhHDM-1 p2. Experiments using various N- and C-terminal truncations of FhHDM-1 p3, showed that FhHDM-1 p3 itself was the shortest peptide tested that retained vATPase inhibitory activity and highlighted an Asn^{64}-Leu^{65}-Gly^{66} motif located just upstream of the amphipathic helix, as important for inhibition. It appears that following raft-dependent endocytosis, FhHDM-1 is processed by macrophage lysosomal cathepsin L to release a ~27-residue peptide from its C-terminus that directly inhibits vATPase leading to impaired acidification of endolysosomal compartments. Our observation that pre-incubation of macrophages with the cysteine peptidase inhibitor, E-64, abolished FhHDM-1-induced endolysosomal alkalisation supports this hypothesis.

One of the major roles of macrophages is to present peptides derived from exogenous antigens in the context of MHC class II to $CD4^+$ T cells (1). Efficient degradation of antigenic peptides by peptidases within endolysosomal compartments can only occur at low pH (pH 5.0-5.5) (25) and agents such as chloroquine, that elevate the pH of the endolysosomal lumen, have a significant suppressive effect on antigen processing and subsequent presentation (49). Indeed, we found that FhHDM-1-induced endolysosomal alkalisation led to the inhibition of MHC class II antigen (FITC-casein) processing by macrophages and significantly reduced presentation of OVA to $CD4^+$ T cells. Type 2 cystatins secreted by various parasitic nematodes have been shown to impair MHC class II-restricted antigen processing, via inhibition of mammalian lysosomal cysteine peptidases (cathepsins B, H, L, S and asparaginyl endopeptidase) in antigen presenting cells (50,51). However, given that the low pH microenvironment within the endolysosomal lumen ensures the activity of around 40 types of acid hydrolases, including peptidases, lipases, phosphatases, nucleases and glycosidases (46), and that pH regulates the release of internalised ligands from their endosomal receptors (52), is it probable that FhHDM-1-induced uncoupling of endolysosomsal acidification will have a broad span of effects on macrophage function.

The targeting of vATPase by HDMs represents a novel mechanism of immunomodulation by a secreted parasite molecule. The inhibition of antigen processing and presentation by macrophages by uncoupling endolysosomal acidification has not previously been described as a mechanism by which a helminth parasite evades the protective responses of the host (see Fig. 9 for an overview of this process). In the context of parasite infection, our data suggest that the secretion of FhHDM-1 by juvenile worms would suppress the

development of parasite-specific immune responses generated in response to subsequent developmental stages. The broader, detrimental, consequences of this would be to prevent the development of antigen-specific responses to unrelated third-party antigens, such as a co-infecting microbial pathogen or delivery of a vaccine, as is often the case for human populations in areas of endemic helminth infection. On the other hand, a deeper understanding of the mechanism of action of HDMs could be of great benefit, potentially providing a therapeutic where the inhibition of vATPase or lysosomal acidification halts the progress of pathology, such as in cancer or osteoarthritis (53,54).

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FIGURE LEGENDS

Figure 1. Primary sequence alignment of the HDMs used in the present study. The region forming the 21-residue amphipathic helix of FhHDM-1 and the mutated residues in peptides FhHDM-1 nonHP and FhHDM-1 2Pro are boxed in grey. The putative cholesterol-binding motif is underlined.

Figure 2. FhHDM-1 binds to the macrophage plasma membrane via interaction with lipid rafts. (A) FhHDM-1-treated (10 μg/ml) or PBS-treated (control) primary human macrophages were immunostained for the presence of FhHDM-1 (green). Cells were also stained with DAPI (blue) to detect nuclei or with the lipophilic dye DiI (red) to visualise the plasma membrane and examined by CLSM. FhHDM-1 fluorescence was observed around the periphery of the cells in association with the plasma membrane. Scale bars = 3 μm. (B) Primary human macrophages were treated with FhHDM-1 (10 μg/ml) for 2 h then immunostained for the presence of FhHDM-1 (green) and stained with Alexa Fluor 594-CT-B conjugate (lipid raft marker; red) and with DAPI (blue) to detect nuclei. Cells were examined with a DeltaVisionOMX 3D-Structured Illumination Microscope®. Strong co-localisation of FhHDM-1 with CT-B (yellow) suggests that FhHDM-1 interacts specifically with lipid rafts within the plasma membrane of primary human macrophages. Scale bar = 3 μm.

Figure 3. FhHDM-1 binds membranes via selective interaction with phospholipids and cholesterol. (A) RAW264.7 macrophages were treated with FhHDM-1 (20 μg/ml) for 2 h. Cell membranes were then prepared and either washed with Na_2CO_3 or solubilised with TX100 and the extracts subjected to SDS-PAGE. Western blots were probed with antibodies specific for FhHDM-1, calnexin or actin. (B) RAW264.7 macrophages were incubated with (dashed line) or without (solid line) trypsin and the binding of Cy5-labelled FhHDM-1 (10 μg/ml) to the cell surface determined by FACS following 30 min incubation at 4°C. The macrophage markers CD11b and F4/80 were used as positive controls for trypsinization. (C) HDMs (50 μM) were incubated with LUVs (as model phospholipid membranes) for 2 h and separated by centrifugation on sucrose gradients. LUVs and bottom fractions were analysed by SDS-PAGE and visualized by silver staining. LL-37 and BSA were used as positive and negative controls respectively. (D) Phospholipid-coated microtiter wells were incubated with serial dilutions of biotinylated FhHDM-1 p2 for 90 min and assessed for binding by detection

with streptavidin-AP. Data are the mean absorbance values ± SD of three independent experiments.

Figure 4. FhHDM-1 prevents the acidification of endolysosomes. (A) Primary human macrophages were pre-incubated with FhHDM-1 (10 μg/mL) or PBS for 20 h and then treated with bacterial LPS (50 ng/mL) for 2 h to induce lysosome formation. Cells were then stained for lysosomes (red). Scale bar = 10 μm. (B) Primary human macrophages were incubated with HDMs (10 μg/mL) for 20 h prior to the addition of *E. coli* fluorescent bioparticles. FhHDM-1 and FhHDM-1 p2 significantly inhibited the pH-dependent fluorescence of the bioparticles whilst peptide FhHDM-1 nonHP did not. The vATPase inhibitor folimycin (1 μM) showed a similar level of inhibition to FhHDM-1 p2. NH_4Cl (20 mM), which raises lysosomal pH, effectively abolished *E. coli* bioparticle fluorescence. Data are the mean ± SD of three separate experiments. (C) RAW264.7 macrophages were incubated with or without the cysteine peptidase inhibitor E-64 (10 μM) for 30 min prior to treatment with PBS or FhHDM-1 (10 μg/ml) for 3 h. Fluorescent *E. coli* bioparticles were then added to the cells for 2 h at 37°C. The inhibition of the pH-dependent bioparticle fluorescence shown by FhHDM-1 was abolished when the cells were pre-treated with E-64. Data are the mean ± SD of two separate experiments.

Figure 5. FhHDM-1 does not inhibit non-specific phagocytosis by macrophages. (A) RAW264.7 macrophages were incubated ± Alexa Fluor-488-conjugated FhHDM-1(50 μg/mL) for 2 h prior to the addition of Alexa Fluor 647-conjugated dextran (10 μg/mL for 2 h). The cells were then analysed by confocal microscopy. Treatment with FhHDM-1 (green fluorescence) had no effect on phagocytosis of the dextran (red fluorescence). Co-localisation of dextran and FhHDM-1 can be seen as yellow fluorescence. Scale bar = 5 μm. (B) RAW264.7 macrophages were treated with recombinant 10 μg/mL FhHDM-1 for 2 h or 20 h prior to the addition of non-opsonised zymosan particles. Whilst cytochalasin D (Cy-D) strongly inhibited zymosan uptake, FhHDM-1 had no significant effect on phagocytosis of the zymosan particles. Data are the mean ± SD of three separate experiments.

Figure 6. FhHDM-p2, but not the parent FhHDM-1 molecule, inhibits vATPase activity. The effect of HDMs on ATPase activity associated with lysosomal membranes prepared from

RAW264.7 macrophages was assessed using the ATPase Assay kit (Innova Biosciences). Data are the mean values ± SD of three independent experiments.

Figure 7. FhHDM-1 is internalised by macrophages and processed by lysosomal cathepsin L. (A) RAW264.7 macrophages were incubated with 10 μg/mL Alexa Fluor 488-conjugated FhHDM-1 (HDM-488; green fluorescence), stained with LysoTracker (red fluorescence) and examined by CLSM. Co-localisation of FhHDM-1 with LysoTracker (yellow fluorescence) shows that FhHDM-1 enters the endolysosmal pathway in macrophages. Scale bar = 5 μm. (B) To investigate whether lysosomal peptidases can process FhHDM-1, 25 μg recombinant FhHDM-1 was incubated with 1 μg native lysosomal contents (Ly-S) or purified human cathepsin L (hCatL) at pH 5.5. Reactions were performed for up to 5 h at 37 °C and stopped by the addition of E-64 (10 μM). Samples were analysed on 4–12 % Bis-Tris gels and blots were probed with an anti-FhHDM-1 antibody. 5+E64; 5 h digest performed in the presence of 10 μM E-64. (C) The 5 h Ly-S digest shown in (B) was analysed by MALDI-TOF MS (Figure S3C; Table 1). The dominant mass detected (*m/z* 3357) matched to a 29-residue C-terminal fragment of FhHDM-1 created by cleavage after Asp^{63}. A synthetic peptide FhHDM-1 p3, based on this sequence, inhibited macrophage vATPase activity in a concentration-dependent manner, similar to FhHDM-1 p2. Truncated peptides based on FhHDM-1 p3 (see Figure 1) did not inhibit vATPase. Data are the mean ± SD of three separate experiments.

Figure 8. FhHDM-1 inhibits endolysosomal antigen processing and MHC II antigen presentation. (A) SDS-PAGE of RAW264.7 macrophages that phagocytosed FITC-casein for 3 h in the presence or absence of HDMs (5, 20 and 50 μg/ml) and 20 μM chloroquine (Clq). (B) Fluorescent imaging of the gels and densitometry shows the amount of unprocessed intracellular FITC-casein as a percentage of the PBS-treated control cells. Data are representative of three independent experiments. (C) IL-2 production by DO-11-10 T cells in response to activation by OVA-pulsed BMDMs pre-treated for 2 h with FhHDM-1 (10, 50 and 100 μg/ml).

Figure 9. Overview of the proposed mechanism of action of FhHDM-1. Exogenous antigen is taken up by macrophages and routed to the endocytic pathway where it is degraded

by lysosomal proteases and loaded to MHC class II for presentation to CD4+ T cells at the cell surface. (1) Endolysosomal acidification is mediated by vATPase which creates optimal conditions for lysosomal protease activity. (2) Following entry into the endolysosomal compartments, FhHDM-1 is cleaved by lysosomal proteases to release the C-terminal fragment (containing an amphipathic helix, shown in red), which then inhibits vATPase activity. The resulting endolysosomal alkalisation leads to an inhibition of antigen processing and MHC class II presentation.

Supplemental Data

Fig. S1. Peptide molar concentrations. The molar concentrations for HDM peptides (derived from the µg/ml amounts used in this study) are shown.

Fig. S2. Binding of FhHDM-1 to the macrophage surface. (A) RAW264.7 macrophages were incubated with 20 ug/ml (solid line) or 50 ug/ml (dashed line) of recombinant FhHDM-1 or FhCL1 for 1 h at 4°C. The filled grey histogram shows untreated cells. Bound recombinants were detected with a mouse anti-His antibody and goat anti-mouse Alexa Fluor 488. (B) RAW264.7 macrophages were treated with 20 µg/ml FhHDM-1 or FhCL1 for 1 h then stained using a mouse anti-His antibody. Cells were also stained with DAPI (blue) to detect nuclei or with the phalloidin (red) to visualise the actin cytoskeleton and examined by CLSM. FhHDM-1 staining (green fluorescence) can be seen along the plasma membrane. Scale bars = 5 µm. (C) THP-1 macrophages were treated with FhHDM-1 (20 µg/ml) for 2 h. Cell membranes were then prepared and either washed with Na_2CO_3 or solubilised with TX100 and the extracts subjected to SDS-PAGE. Western blots were probed with antibodies specific for FhHDM-1, calnexin or actin.

Fig. S3. Biochemical characterisation of macrophage lysosomal fractions and processing of FhHDM-1 by the Ly-S fraction. (A) Western blot of RAW264.7 macrophage lysosomal membranes (Ly-M), soluble lysosomal contents (Ly-S) and a cell cytoplasm fraction (Cyt) probed with an anti-LAMP-1 antibody. (B) Lysosomal cathepsin activity within the soluble Ly-S fraction was confirmed using the fluorogenic cathepsin substrate Z-Leu-Arg-NHMec. The assay was performed at pH 5.5 and peptidolytic activity was measured by monitoring the release of the fluorogenic leaving group (-NHMec) over 30 min at 37°C ± the cysteine

peptidase inhibitor E-64 (10 μM). (C) MALDI TOF MS analysis of the FhHDM-1 Ly-S digest. The 5 h Ly-S digest of FhHDM-1 (Figure 7B) was analysed by MALDI-TOF MS. The dominant mass detected (*m/z* 3357) was matched to a 29-residue C-terminal fragment of FhHDM-1 created by cleavage after Asp^{63}.

Table 1. MALDI TOF MS analysis of FhHDM-1 Ly-S and hCatL digests.

Digests of FhHDM-1 with Ly-S and hCatL (0 h, 2 h and 5 h) were analysed by MALDI TOF MS. The major masses detected correspond to full-length recombinant FhHDM-1 (*m*/*z* 9272) and C-terminal peptide fragments released following cleavage by Ly-S (*m*/*z* 3329) and hCatL (*m*/*z* 3400). [1]recombinant FhHDM-1 numbering.

					Digest time point					
					0 h		2 h		5 h	
Sample	***m*/*z* submitted**	***m*/*z* matched**	**Delta (Da)**	**HDM peptide[1]**	**Area**	**S/N**	**Area**	**S/N**	**Area**	**S/N**
Ly-S digest	9232 3357	9272 3329	+40 -28	1-79 44-73	736,009 8,752	341 55	ND 288	ND 17	21,109 26,911	13 361
hCatL digest	9229 3400	9272 3400	+43 0	1-79 42-71	67,977 ND	94 ND	7,732 37,293	15 70	ND 6,422	ND 7