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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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ABSTRACT We previously reported the identification of a novel family of immunomodulatory proteins, termed helminth defense molecules (HDMs), that are secreted by medically important trematode parasites. Since HDMs share biochemical, structural, and functional characteristics with mammalian cathelicidin-like host defense 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 internalized by endocytosis. Following internalization, FhHDM-1 is rapidly processed by lysosomal cathepsin L to release a short C-terminal peptide (containing a conserved amphipathic helix that is a key to HDM function), which then prevents the acidification of the endolysosomal compartments by inhibiting vacuolar ATPase activity. The resulting endolysosomal alkalization 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.—Robinson, M. W., Alvarado, R., To, J., Hutchinson, A. T., Dowdell, S. N., Lund, M., Turnbull, L., Whitchurch, C. B., O’Brien, B. A., Dalton, J. P., Donnelly, S. A helminth cathelicidin-like protein suppresses antigen processing and presentation in macrophages via inhibition of lysosomal vATPase. FASEB J. 26, 4614–4627 (2012). www.fasebj.org

Key Words: trematode · Fasciola hepatica · lysosomal acidification · host defense peptides

The innate immune response represents the first line of defense 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), which thereby generates 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 minimized.

Host defense 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, >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 based on 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), which allows 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 F. hepatica helminth defense molecule 1 (FhHDM-1), which 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 as Schistosoma mansoni, Schistosoma 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-1 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 internalized by endocytosis. Following internalization, FhHDM-1 is processed rapidly by lysosomal cathepsin L to release a 27-aa C-terminal peptide (containing the conserved amphipathic helix), which then prevents the acidification of the endolysosomal compartments by inhibiting vacuolar ATPase (vATPase) activity. Preventing endolysosomal acidification impedes macrophage antigen processing by proteases, such as cathepsin L, which prevents 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 Escherichia coli and purified as described previously (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 α-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 2 proline substitutions (I69P and L80P) designed to disrupt the α helix. Similarly, FhHDM-1 nonHP corresponds to residues 56–89 but contains 6 substitutions (I69R, L76R, L84T, and Y87K) designed to abolish the hydrophobic face of the amphipathic helix while 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 Supplemental Fig. S1. HDM peptides were labeled with Cy5 (GE Healthcare, Pittsburgh, PA, USA) or Alexa Fluor 488 (Life Technologies, Mulgrave, VIC, Australia) according to the manufacturer’s instructions. All peptides were synthesized endotoxin-free by GL Biochem (Shanghai, China).

FhHDM-1, FhHDM-1 p1, FhHDM-1 p2, FhHDM-1 nonHP, FhHDM-1 2Pro, FhHDM-1 p3, FhHDM-1 37–49, FhHDM-1 48–70, FhHDM-1 48–68, FhHDM-1 50–70, FhHDM-1 50–68

**Figure 1.** Primary sequence alignment of the HDMs used in the present study. Region forming the 21-residue amphipathic helix of FhHDM-1 and the mutated residues in peptides FhHDM-1 nonHP and FhHDM-1 2Pro are shaded. Putative cholesterol-binding motif is underscored.
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, NSW, Australia). Mononuclear cells were purified from buffy coats by Ficoll-paque separation, and CD14+ monocytes were enriched by positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were differentiated into macrophages for up to 7 d in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 2% v/v human serum and their phenotype confirmed (expression of CD206) using an LSR II flow cytometer (BD Bioscience, San Jose, CA, USA).

Confocal laser scanning microscopy

For confocal laser scanning microscopy (CLSM) experiments, human primary macrophages (1×10⁶) derived from blood monocytes (Australian Red Cross, Sydney, NSW, Australia) were treated with 10 μg/ml of recombinant FhHDM-1 at 37°C, 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 His6 tag (Life Technologies). Cells were then washed and incubated with Alexa Fluor 488-conjugated (Life Technologies) secondary antibody. To follow internalization of FhHDM-1, macrophages (7×10⁴) were simultaneously incubated with 10 μg/ml of Alexa Fluor 488-labeled FhHDM-1 and 60 nM LysoTracker (Life Technologies) for 45 min at 37°C.

To examine the effect of FhHDM-1 on lysosome formation, macrophages (1×10⁶) were incubated with either recombinant FhHDM-1 (10 μg/ml) or vehicle (PBS) for 20 h at 37°C. After washing, cells were incubated with E. coli LPS (50 ng/ml; serotype 111:B4; Sigma, Sydney, 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 (Nikon, Melville, NY, USA). Images were rendered and analyzed using NIS Elements software (Nikon).

3D-structured illumination microscopy

Human primary macrophages (1×10⁶) were treated with recombinant FhHDM-1 (10 μg/ml) for 2 h. Cells were then washed with 4 μg/ml of cholera toxin subunit B (CT-B) Alexa Fluor 594-conjugate (Life Technologies) for 20 min at 4°C, then fixed and stained with His6 antibody as described above. Imaging was performed using a DeltaVision OMX 3D-structured illumination microscope, version 3 (OMX 3D-SIM; Applied Precision Inc., Issaquah, WA, USA). Solid-state lasers (405, 488, and 593 nm) were captured simultaneously using 3 Photometrics Cascade (Photometrics, Tucson, AZ, USA) back-illuminated EMCCD cameras (>90% QE) with a 512 × 512 CCD and on-chip charge multiplication. All data capture used an Olympus UPPlanSapo ×100 1.4NA oil objective and standard excitation and emission filter sets (λem/λex: 405/419–465, 488/500–550, and 592/608–648 nm; Olympus, Tokyo, Japan). 3D-SIM images were sectioned using a 125-nm Z-step size. Raw 3-phase images were reconstructed as described previously (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 (1×10⁶/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-labeled 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).

Sodium carbonate membrane extraction

RAW264.7 macrophages (2×10⁶) were incubated with 20 μg/ml of recombinant FhHDM-1 at 37°C for 2 h, followed by cross-linking with 1% paraformaldehyde for 30 min. After sonication, membranes were prepared by centrifugation at 20,000 g for 1 h. The membrane pellet was sequentially extracted with 0.1 M Na₂CO₃ (pH 11) and 1% Triton X-100 (TX100) to produce peripheral and integral membrane protein fractions, respectively (12). Samples were separated by nonreducing SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-FhHDM-1 (9), anti-actin, and anti-calnexin antibodies (Sigma).

HDMLiposome interaction assay

Large unilamellar liposome vesicles (LUVs) composed of the phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidycholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-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, USA) 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 (100,000 ρg) 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). Gels were silver stained and imaged with a PharsysFX laser imaging system (Bio-Rad, Sydney, NSW Australia).

Phospholipid ELISA

PolySorp 96-well plates (Nunc, Roskilde, Denmark) were coated, in triplicate, with 200 μl of 100 μg/ml lipids [spin-gomyelin, POPC, POPS, POPE, and 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 biotinylated FhHDM-1 for 90 min at 37°C. Plates were washed, and bound proteins were detected with ExtrAvidin-AP with pNPP substrate (Sigma).

Phagocytosis assays

Human primary macrophages or RAW264.7 cells were treated with HDMs (10 μg/ml) for 20 h or 1 h with folicin (1 μM; Merck, Frenchs Forest, NSW, Australia) or NH₄Cl (20 mM). In some experiments, cells were treated with E-64 (10 μM; Sigma) for 30 min prior to stimulation with recombinant FhHDM-1 for 3 h. Cells were then washed and incubated with 125 μg/ml E. coli pHrodo bioparticles (Life Technologies) for 2 h at 37°C, and fluorescence from phagocytosed bioparticles was read using a Bio-Tek KC4 microfluorometer (λex/λem: 532/585 nm). 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, USA), according to the manufacturer’s instructions. To visualize phagocytosis, cells (1×10⁶)
were treated with or without Alexa Fluor 488-conjugated FhHDM-1 (50 μg/ml) for 2 h at 37°C, followed by Alexa Fluor 647-conjugated dextran (10 μg/ml; Life Technologies) for 2 h at 37°C. 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 10× for lysosomes. Briefly, THP-1 or RAW264.7 macrophages (4×10^7) were sonicated on ice, and a postnuclear supernatant was further centrifuged at 100,000 g for 20 min to pellet lysosomes and endosomes. The lysosome pellet was extracted with water for 5 min, then centrifuged at 100,000 g for 20 min. The supernatant was collected as water-soluble (Ly-S) fraction, and the pellet as lysosome-insoluble membrane (Ly-M) fraction. 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 analyzed 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) 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, UK), according to the manufacturer’s instructions. For inhibition assays, serial dilutions of HDMs were preincubated 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 (with or without 10 μM E-64) at pH 5.5 as described previously (18).

Processing of FhHDM-1 was analyzed 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 (hCatL; Abcam). Samples were run on 4–12% Bis-Tris gels (Life Technologies), 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-, 2-, and 5-h Ly-S digests were analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) at the Australian Proteome Analysis Facility (Macquarie Park, NSW, Australia), as described previously (9). Spectra were acquired in positive mode in the mass range 2000–20,000 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 (CQ; 20 μM). After 3 h incubation at 37°C, the cells were washed, then lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche, Castle Hill, NSW, Australia). Lysates were normalized for protein content and run on 4–12% Bis-Tris gels (Life Technologies). Gels were scanned in the FITC channel using a PhorosFX laser imaging system, and normalized spot quantities were determined using PDQuest 8.01 software (Bio-Rad).

**Antigen presentation assays**

BMDMs (1×10^6) were treated with HDMs (10–100 μg/ml) for 2 h, then pulsed overnight with 10 mg/ml full-length ovalbumin (OVA; Sigma) or preprocessed OVA (N235–330) peptide (Anspec, Fremont, CA, USA). The HDM-treated OVA-pulsed BMDMs were then cocultured with 1×10^5 DO-11-10 T-cell hybridomas [European Collection of Cell Cultures (ECACC), Salisbury, UK] for 24 h, and levels of interleukin-2 (IL-2) in the culture medium were measured by ELISA (BD Pharmingen, North Ryde, 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 localization pattern using an anti-His<sub>6</sub> tag antibody. No fluorescence was observed in PBS-treated cells stained with the anti-His<sub>6</sub> 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). Localization 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; ref. 19), no surface labeling was observed, which demonstrates the specificity of the interaction of FhHDM-1 with the lipid raft marker, CT-B (Fig. 2A; yellow fluorescence), indicating that FhHDM-1 interacts specifically with lipid rafts within the plasma membrane of primary human macrophages. FhHDM-1 also colocalized 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.
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 visualize the plasma membrane and examined by CLSM. FhHDM-1 fluorescence was observed around the periphery of the cells in association with the plasma membrane. 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 DeltaVision OMX 3D-SIM. Strong colocalization 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 bars = 3 µm.

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 Na2CO3 aqueous fraction. As expected, the peripheral membrane protein is integral and associates with the membrane and dissociated from the membrane. If, however, the 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 Na2CO3 aqueous fraction (Fig. 3A). By contrast, calnexin, which is an integral membrane protein, was found predominantly in the TX100 detergent fraction (Fig. 3A). FhHDM-1 was recovered only in the Na2CO3 aqueous fraction (Fig. 3A), which suggests 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 (Supplemental Fig. S2G).

Having established that FhHDM-1 binds the macrophage plasma membrane via electrostatic interactions, we next investigated whether this condition was indicative of protein/receptor-binding. To investigate this, RAW264.7 macrophages were incubated with or without trypsin for 1 h at 37°C, and the surface binding of Cy5-FhHDM-1 was determined by flow cytometry. Immunostaining for the macrophage surface markers CD11b and F4/80 was used to demonstrate the success of trypsinization. As expected, CD11b and F4/80 fluorescence was decreased markedly in trypsinized macrophages compared with undigested cells (Fig. 3B), which suggests 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), which suggests 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 nonprotein 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-aa 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 6 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 whether 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 finding 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 oligomerization of FhHDM-1 on association with various phospholipid species (9, 20). While 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 internalized into endolysosomes and prevents acidification via inhibition of vATPase activity

Using CSLM, we found that Alexa Fluor 488-conjugated FhHDM-1 is internalized into the endolysosomal system of macrophages. FhHDM-1 colocalized with cytoplas-
mic vesicles that were visualized with LysoTracker (a lysosomotropic fluorescent dye that concentrates in acidic compartments; see below and Fig. 7A). FhHDM-1 also colocalized with Alexa Fluor 647-conjugated dextran (when coincubated with RAW264.7 cells), which further confirms that FhHDM-1 enters the endolysosomal pathway (see Fig. 4A).

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. 5A, top panel). In contrast, only 25% of the macrophages pretreated with FhHDM-1 showed any LysoTracker-positive staining in response to LPS, with very few acidic compartments visible within the cytoplasm (Fig. 5A, 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-treated control cells (Fig. 5A). 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. 5B). Treatment of the cells with NH₄Cl, which raises lysosomal pH (23), effectively abolished E. coli bioparticle fluorescence (P=0.004). Additional experiments using nonopsonised zymosan particles and Alexa Fluor 647-conjugated dextran showed that FhHDM-1 does not inhibit nonspecific phagocytosis or endocytosis (Fig. 4B), confirming that the reduced E. coli bioparticle fluorescence resulted from FhHDM-induced endolysosomal alkalization. When macrophages were preincubated with the cysteine peptidase inhibitor E-64, the inhibitory effect of FhHDM-1 on pH-dependent E. coli bioparticle fluorescence was abolished (Fig. 5C).

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

The above data show that FhHDM-1 and FhHDM-1 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 (which 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 (Supplemental 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 (Supplemental Fig. S3B). The Ly-M fractions were treated with HDMs and assayed for ATPase activity. As shown in Fig. 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),

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**Figure 4. FhHDM-1 does not inhibit nonspecific phagocytosis by macrophages.** A) RAW264.7 macrophages were incubated with or without 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). Cells were then analyzed by confocal microscopy. Treatment with FhHDM-1 (green fluorescence) had no effect on phagocytosis of the dextran (red fluorescence). Colocalization of dextran and FhHDM-1 showed yellow fluorescence. Scale bars = 5 μm. B) RAW264.7 macrophages were treated with recombinant 10 μg/ml FhHDM-1 for 2 or 20 h prior to the addition of nonopsonised zymosan particles. While cytochalasin D (Cy-D) strongly inhibited zymosan uptake, FhHDM-1 had no significant effect on phagocytosis of the zymosan particles. Data are means ± sd of 3 separate experiments.
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 internalized 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 internalization 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 analyzed 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, which raises lysosomal pH, effectively abolishing E. coli bioparticle fluorescence shown by FhHDM-1 was abolished when the cells were pretreated with E-64. Data are means ± sd of 2 separate experiments.

![Figure 6.](image)

**Figure 6.** FhHDM-1 p2, but not the parent FhHDM-1 molecule, inhibits vATPase activity. 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 means ± sd of 3 independent experiments.

The FhHDM-1 Ly-S digests (0-, 2-, and 5-h reactions) were analyzed by MALDI–time-of-flight (TOF) MS (Table 1 and Supplemental Fig. S3C). The only major mass detected in the 0-h reaction was matched to full-length recombinant FhHDM-1 [m/z 9232; signal/noise (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), which suggests 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-aa C-terminal peptide (minus the His tag, but including the LE added by the expression vector) created by cleavage after Asp63 (native peptide numbering). Interestingly, hCatL cleaved FhHDM-1 after Leu60 (Table 1), which suggests that the C-terminal peptide is processed further by lysosomal aminopeptidases or dipeptidyl/tripeptidyl aminopeptidase, creating the final cleavage site at Asp63 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 8 residues shorter at the N terminus and one residue longer at the C terminus compared to FhHDM p2 (see Fig. 1). Strikingly, FhHDM-1 p3 inhibited vATPase activity in a concentration-dependent manner, similar to FhHDM-1 p2. Truncated peptides based on FhHDM-1 p3 (see Fig. 1) did not inhibit vATPase. Data are the means ± sd of 3 separate experiments.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>m/z submitted</th>
<th>m/z matched</th>
<th>Δ (Da)</th>
<th>HDM peptide</th>
<th>Area (Da)</th>
<th>S/N</th>
<th>Area (Da)</th>
<th>S/N</th>
<th>Area (Da)</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly-S digest</td>
<td>9232</td>
<td>9272</td>
<td>+40</td>
<td>1–79</td>
<td>736,009</td>
<td>341</td>
<td>ND</td>
<td>ND</td>
<td>21,109</td>
<td>13</td>
</tr>
<tr>
<td>hCatL digest</td>
<td>3357</td>
<td>3357</td>
<td>−28</td>
<td>44–73</td>
<td>8,752</td>
<td>55</td>
<td>288</td>
<td>17</td>
<td>26,911</td>
<td>361</td>
</tr>
<tr>
<td>hCatL digest</td>
<td>9229</td>
<td>9272</td>
<td>+43</td>
<td>1–79</td>
<td>67,977</td>
<td>94</td>
<td>7,732</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>hCatL digest</td>
<td>3400</td>
<td>3400</td>
<td>0</td>
<td>42–71</td>
<td>37,293</td>
<td>70</td>
<td>6,422</td>
<td>7</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Digests of FhHDM-1 with Ly-S and hCatL (0, 2, and 5 h) were analyzed by MALDI-TOF MS. 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 3357) and hCatL (m/z 3400). HDM peptides are indicated by recombinant FhHDM-1 numbering. S/N, signal/noise; ND, not detected.
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 analyzed its degradation by SDS-PAGE. While FITC-casein was rapidly degraded in PBS-treated cells, antigen degradation was blocked when cells were coincubated with 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). While 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 (Fig. 8A), while FhHDM-1 p2 had a greater effect than FhHDM-1 on endolysosomal alkalization (Fig. 5B). While unexpected, these observations are reproducible and may be due to differences in the cell type used and assay sensitivity.

We next determined whether FhHDM-1 could affect antigen presentation by macrophages. To do this, we used OVA-specific T cell hybridoma DO-11-10 cells, whose proliferative response to OVA\textsuperscript{323–339} peptide, presented with MHC class II, can be quantified by measuring the levels of secreted IL-2 (26, 27). DO-11-10 T cells activated \textit{in vitro} by OVA-pulsed murine bone marrow-derived macrophages, which were pretreated 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 preprocessed OVA\textsuperscript{323–339} peptide was used as antigen or when OVA-pulsed macrophages were pretreated 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 proinflammatory mediators, mammalian innate immune cells release an array of 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 ref. 31). They achieve this by interrupting key stages of LPS-mediated cell signaling, thereby preventing activation of macrophages and dendritic cells via toll-like receptors (TLRs; refs. 32–34). These mechanisms include direct binding and sequestration of LPS, which prevents 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 \textit{F. hepatica} secreted a small molecule, FhHDM-1, which exerted similar effects on LPS signaling, including direct binding of LPS, and 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 internalized 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 nonproteinaceous 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]nY-[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; refs. 41, 42) and, therefore, more likely interacts with the P2X7 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 P2X7 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 internalized into LysoTracker-positive vesicles and co-localized with fluorescently labeled dextran, which is known to enter cells via the endolysosomal system (45). Preincubation 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 on delivery into acidified phagolysosomal vesicles. Experiments using nonopsinized zymosan particles and fluorescently labeled dextran confirmed that the reduced *E. coli* bioparticle fluorescence was not simply due to inhibition of nonspecific uptake by FhHDM-1. The effect of FhHDM-1 on lysosomal pH was also demonstrated using the pH-sensitive dye LysoTracker, as preincubation 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 (unpublished results).

Acidification of endolysosomal compartments is primarily mediated by vATPase, a transmembrane complex consisting of cytosolic (V1) and membrane bound (V0) subunits (46). We found that FhHDM-1 p2 inhibited ATPase activity associated with macrophage endolysosomal membranes in a concentration-dependent manner. In assembled functional vATPase complexes, ATP hydrolysis by the cytoplasmic V1 domain drives proton transport through the membrane-bound V0 domain from the cytoplasm to the endolysosomal lumen (46). Hence, 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 noninhibitory), 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 F1F0-ATPase (ATP synthase) of the parasite (47). However, this peptide does not adopt an amphipathic structure (48), nor does it display primary sequence identity with FhHDM-1 p2, although both peptides possess short α-helices.

While the full-length parent FhHDM-1 molecule could uncouple endolysosomal acidification in macrophages, it could not 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 Asp63. Purified native hCatL released an almost identical 30-residue C-terminal fragment (m/z 3400) from FhHDM-1 created by cleavage after Leu60, which suggests that this enzyme is responsible for FhHDM-1 cleavage in vivo. A 27-residue synthetic peptide analog (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 Asn64-Leu65-Gly66 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 preincubation of macrophages with the cysteine peptidase inhibitor E-64 abolished FhHDM-1-induced endolysosomal alkalization 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; ref. 25); agents such as Clq, which 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 alkalization 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 ~40 types of acid hydrolases, including peptidases, lipases, phosphatases, nucleases, and glycosidases (46), and that pH regulates the release of internalized ligands from their endosomal receptors (52), it is probable that FhHDM-1-induced uncoupling of endolysosomal 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. On one hand, the broader, detrimental consequences of this would be to prevent the development of antigen-specific responses to unrelated third-party antigens, such as a coinfecting 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).

Figure 9. Overview of the proposed mechanism of action of FhHDM-1. 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. 2) Endolysosomal acidification is mediated by protease activity. 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 alkalization leads to an inhibition of antigen processing and MHC class II presentation.
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of TNF-α by blocking the binding of LPS to CD14+ cells. *J. Immunol.* **167**, 3329–3338

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