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REGULAR ARTICLE

Homolog of allograft inflammatory factor-1 induces macrophage migration during innate immune response in leech

Tilo Schorn · Francesco Drago · Gianluca Tettamanti · Roberto Valvassori · Magda de Eguileor · Jacopo Vizioli · Annalisa Grimaldi

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Abstract Allograft inflammatory factor-1 (AIF-1) is a 17-kDa cytokine-inducible calcium-binding protein that, in vertebrates, plays an important role in the allograft immune response. Its expression is mostly limited to the monocyte/macrophage lineage. Until recently, AIF-1 was assumed to be a novel molecule involved in inflammatory responses. To clarify this aspect, we have investigated the expression of AIF-1 after bacterial challenge and its potential role in regulating the innate immune response in an invertebrate model, the medicinal leech (*Hirudo medicinalis*). Analysis of an expressed sequence tag library from the central nervous system of *Hirudo* revealed the presence of the gene *Hmaif-1*/alias *Hmiba1*, showing high homology with vertebrate *aif-1*. Immunohistochemistry with an anti-*Hm*AIF-1 polyclonal antibody revealed the constitutive presence of this protein in spread $CD68^{+}$ macrophage-like cells. A few hours after pathogen (bacterial) injection into the body wall, the amount of these immunopositive cells co-expressing *Hm*AIF-1 and the common leucocyte marker CD45 increased at the injected site. Moreover, the recombinant protein *Hm*AIF-1 induced massive angiogenesis and was a potent chemoattractant for macrophages. Following r*Hm*AIF-1 stimulation, macrophage-like cells co-expressed the macrophage marker CD68 and the surface glycoprotein CD45, which, in vertebrates, seems to have a role in the integrin-mediated adhesion of macrophages and in the regulation of the functional responsiveness of cells to chemoattractants. CD45 is therefore probably involved in leech macrophage-like cell activation and migration towards an inflammation site. We have also examined its potential effect on *Hm*AIF-1-induced signalling.

Keywords Macrophage-like cells · *Hm*AIF-1 · Innate immune response · *Hirudo medicinalis*

Introduction

The allograft inflammatory factor-1 (AIF-1) is a "calcium-binding protein" of 17 kDa. Its expression is limited to the monocyte/macrophage line and it is strongly induced by cytokines such as interferon-γ (Alkassab et al. 2007). AIF-1 was identified and cloned for the first time in rat cardiac transplant subject to chronic rejection (Utans et al. 1995). Subsequently, AIF-1-like factors sharing a well-preserved amino-acid sequence and functional role have been described in other groups of metazoans. Data in the literature have indeed shown that AIF-1 expression increases significantly after transplantation, wound healing or bacterial infections, both in vertebrate and in invertebrate species (Utans et al. 1995; Kruse et al. 1999; Watano et al. 2001; de Zoysa et al. 2010; Zhang et al. 2011) suggesting that this factor is involved in the inflammatory response and in immune system regulation. Despite extensive investigation focused on both the molecular characteristics and the expression level of AIF-1 during the inflammatory response or wound healing, the physiological role of this protein in the regulation of the immune system remains unclear. In particular, AIF-1 has previously been reported to be a modulator of the innate immune response during macrophage activation (Utans et al. 1995; Alkassab

Tilo Schorn and Francesco Drago contributed equally to this work.

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T. Schorn · G. Tettamanti · R. Valvassori · M. de Eguileor · A. Grimaldi (✉)
Department of Biotechnology and Life Sciences, University of Insubria, Via J.H. Dunant 3, 21100 Varese, Italy
e-mail: annalisa.grimaldi@uninsubria.it

F. Drago · J. Vizioli
Laboratoire PRISM (Protéomique, Réponse Inflammatoire, Spectrométrie de Masse), U 1192 INSERM Université Lille 1, Bât. SN3, Cité Scientifique, 59650 Villeneuve d'Ascq, France

et al. 2007). However, the direct relationship between AIF-1 expression and macrophage activation/migration during the inflammation phase after injury or bacterial infection is unknown.

In order to improve our understanding of these points, we have focused our research on the possible role of AIF-1 during the immune response in an invertebrate model, the leech *Hirudo medicinalis* (Annelida, Hirudinea), after challenge by bacterial pathogen. The interest in using this experimental model is linked to its anatomical and physiological features that allow the observation and study of the events linked to wound healing and tissue repair (de Eguileor et al. 2003, 2004; Tettamanti et al. 2004). The cellular immune response, in particular, can be easily and unambiguously evaluated in the leech body wall, which is a predominantly avascular muscular region containing a few immunocompetent cells of myeloid origin, i.e. macrophages, granulocytes and natural killer cells (de Eguileor et al. 1999). The effects of lesion or bacterial challenge in this area are rapidly induced (24 h) and can be studied by morphological and histochemical analyses (de Eguileor et al. 1999). In addition, our previous work (de Eguileor et al. 2000a, 2000b; Grimaldi et al. 2004, 2006) and that of others (Macagno et al. 2010) has indicated the existence, in leech, of several CDs (cluster of differentiation) proteins, commonly used as immune cell markers, similar to mammalian CDs.

All these features allow the easy detection and monitoring of immunocompetent cell migration and of the angiogenic process. In *H. medicinalis*, new vessel formation is a sequential process involving an initial vasculogenic step, followed by angiogenesis. The new vessels act as a "piping" system to transfer precursors of immunocompetent cells into lesioned or bacterially challenged areas. After transendothelial migration, these cells leave the circulating fluid, disperse in the surrounding connective tissue and differentiate into mature leucocytes that mediate the inflammatory response (Grimaldi et al. 2006). Thus, the basic steps of the immune responses can be easily analysed in leeches, which lack the complex feed-back control systems typical of Vertebrates.

Recently, a gene showing high similarity with vertebrate *aif-1*, named *Hmiba1/alias Hmaif-1* (GenBank accession number KF437461), has been identified (Drago et al. 2014) and characterized in the central nervous system (CNS) of the leech. Here, we have observed that *Hm*AIF-1 is constitutively expressed in the leech body wall and that the production of the protein is not induced by the injection of phosphate-buffered saline (PBS) or the Gram-negative bacterium *Aeromonas hydrophila*. Interestingly, in leeches challenged with lipopolysaccharides (LPS) of Gram-negative bacteria or with the environmental bacteria pathogen *Micrococcus nishinomiyaensis*, we have observed important recruitment of $CD68^+$ macrophage-like cells into the infection area. Moreover, we have demonstrated that the recombinant *Hm*AIF-1 induces massive angiogenesis and acts also as a potent chemoattractant for these macrophage-like cells. These recruited $CD68^+$ cells co-express *Hm*AIF-1 and the common leucocyte marker CD45. CD45 is cell surface glycoprotein that, in vertebrates, has been implicated in the integrin-mediated adhesion of macrophages (Roach et al. 1997; Zhu et al. 2011; St-Pierre and Ostergaard 2013) and is thought to play a role in regulating the functional responsiveness of cells to chemoattractants (Roach et al. 1997; Mitchell et al. 1999). We have therefore hypothesized that, in the leech, CD45 plays a key role in macrophage activation, maturation and migration towards the inflammation site. We have also examined the potential effect on *Hm*AIF-1-induced signalling.

Materials and methods

Recombinant *Hm*AIF-1 production

The cDNA encoding the *Hm*AIF-1 (GenBank accession no. KF437461) was amplified by the polymerase chain reaction (PCR) from total leech CNS cDNA, as previously described (Drago et al. 2014). Amplification was performed by using specific forward (5′-CCCCGGATCCATGAGTTTGG ACCTCAAAGAC-3′) and reverse (5‘-CCCCCTCG AGTCACTTCGTCCCACTGTTG-3′) primers containing *Bam*HI and *Nde*I restriction sites, respectively, for cloning into the expression vector. A single PCR product was obtained and ligated into the *Bam*HI/*Nde*I-digested pET16b vector (Novagen, Madison, Wis., USA) carrying an N-terminal His-tag sequence. The construct was transformed into an *Escherichia coli* JM109 (Promega, Madison, Wis., USA) strain for plasmid amplification and cloning steps were verified by DNA sequencing of both strands. The plasmid was then transformed into BL21 (DE3)pLysS *E. coli* competent cells (Promega). Recombinant protein expression was induced in log-phase cultured bacteria (2 h at 37 °C, OD_{600}=0.6) by adding 1 mM isopropyl thiogalactoside (Promega) and leaving the culture to grow for a further 3 h at 37 °C. Samples removed from uninduced cultures were used for controls. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed on culture medium and cell pellet lysates to establish the cellular localization of the recombinant protein and to estimate its relative amount in these two fractions. The bulk of the product was observed in cell-free culture medium that was collected and used for the following immunoaffinity purification steps. The recombinant His-tagged *Hm*AIF-1 (r*Hm*AIF-1) was purified from the transformant by using the Dynabeads His-Tag Isolation & Pulldown system (Life Technologies, Carlsbad, USA) in accordance with manufacturer's protocol. The presence of r*Hm*AIF-1 in the eluted fraction was confirmed by SDS-PAGE analyses and MALDI MS/MS mass spectrometry (orbitrap; data not shown).

Animals and treatments

Leeches (*H. medicinalis*, Annelida, Hirudinea, from Ricarimpex, Eysines, France) measuring 10 cm were kept in water at 20 °C in aerated tanks and fed weekly with calf blood. Each treatment (PBS, bacterial or recombinant protein injection) was performed at the level of the 20th metamere on leeches anaesthetized with a 10 % ethanol solution. Treated and untreated (control) animals were anesthetized and then dissected to remove body wall tissues at specific time points. Animals were randomly divided into separate experimental groups (five individuals for each time point) and submitted to various protocols and treatments.

Group 1 T6 h, T24 h, T48 h, T72 h, T7 days: control samples injected with 100 μl sterilized PBS consisting of 138 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, (pH 7.4).

Group 2 T24 h, T48 h, T72 h, T7 days: samples injected with 100 μl sterilized PBS containing 20 ng r*Hm*AIF-1 functionally to characterize cells migrating under the influence of this factor. The best concentration of r*Hm*AIF-1 required to induce significant cell migration was determined based on our previous work on leeches (Tettamanti et al. 2003; Grimaldi et al. 2011). We tested three different concentrations (5 ng, 10 ng and 20 ng) and obtained the best response with the highest amount.

Group 3 T4 h, T48 h, T72 h, T7 days: samples injected with 100 μl sterilized PBS containing 20 ng r*Hm*AIF-1 pre-incubated for 30 min at room temperature with 1 μg specific polyclonal anti-*Hm*AIF-1 raised in rabbit (New Zealand White, SPF) against a synthetic peptide corresponding to the predicted N-terminal region of the *Hm*Iba1 protein (Proteogenix, France; Drago et al. 2014) to perform antibody-mediated neutralization experiments.

Group 4 T24 h: samples injected with 100 μl PBS containing 20 ng r*Hm*AIF-1 pre-incubated for 30 min at room temperature with 1 μg polyclonal rabbit anti-CD45 antibody (Twin Helix, Milano, Italy) to inhibit macrophage-like cell responsiveness to r*Hm*AIF-1.

Group 5 Samples injected with 100 μl sterilized PBS containing, respectively, 100 ng/ml LPS from *E. coli* (Serotype 0111:B4, Sigma, St. Louis, Mo., USA) or heat-killed Gram-positive (*M. nishinomiyaensis*) and Gram-negative (*A. hydrophila*) bacteria (3×10^7 colony-forming units/ml) for immune stimulation assays. Bacteria were isolated from the natural environment of *H. medicinalis*. Challenged animals were sacrified 6 h after injection and treated as indicated above.

Optical and electron microscopy

Leech tissues, dissected from the area of the injection, were fixed for 2 h in 0.1 M cacodylate buffer at pH 7.4, containing 2 % glutaraldehyde. Specimens were then washed in the same buffer and postfixed for 1 h with 1 % osmium tetroxide in cacodylate buffer, pH 7.4. After standard serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica, Wien, Austria). Semi-thin sections (0.75 μm in thickness) were stained by conventional methods (crystal violet and basic fuchsin, according to Moore et al. 1960) and subsequently observed under a light microscope (Nikon Eclipse Ni, Nikon, Tokyo, Japan). Data were recorded with a DS-5 M-L1 digital camera system (Nikon). Ultrathin sections (80 nm in thickness) were placed on copper grids, stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol, Tokyo, Japan). Data were recorded with a MORADA digital camera system (Olympus, Tokyo, Japan).

Immunohistochemistry

Serial cryosections (7 μm in thickness) were stained by crystal violet and basic fuchsin for a morphological view or used for immunofluorescence staining. Sections, rehydrated with PBS for 5 min, were pre-incubated for 30 min with PBS containing 2 % bovine serum albumin (BSA) before incubation in primary polyclonal antibodies (1 h at 37 °C). The primary antibodies used were: rabbit anti-*Hm*AIF-1 (Drago et al. 2014), diluted 1:1000; rabbit anti-human CD68 (Santa Cruz Biotechnology, Calif., USA), which reacts, as previously demonstrated, with leech macrophage-like cells (de Eguileor et al. 2003), diluted 1:100; rabbit anti-human CD45 (Twin Helix), which reacts, as previously demonstrated, with leech hematopoietic precursor cells and myeloid leucocyte cells (Grimaldi et al. 2006), diluted 1:100. The washed specimens were incubated for 1 h at room temperature with the appropriate secondary antibodies diluted 1:200 (Abcam, Cambridge, UK): goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (excitation 493 nm, emission 518 nm), goat anti-rabbit Cy3-conjugated antibody (excitation 562 nm, emission 576 nm), goat-anti rabbit Cy5-conjugated antibody (excitation 650 nm, emission 672 nm). Double-labelling experiments were performed as described: (1) to detect *Hm*AIF-1, *Hm*AIF-1/CD45 or *Hm*AIF-1/CD68, the anti-*Hm*AIF-1 was applied first and then sections were incubated with the secondary antibody (goat anti-rabbit FITC-conjugated antibody). After being washed, the cells were incubated with the anti-CD45 or anti-CD68 antibody and, subsequently, with the secondary Cy5-conjugated goat anti-rabbit antibody; (2) to detect CD45/CD68, the anti-CD45 was applied first and then sections were incubated with the

secondary Cy5-conjugated goat anti-rabbit antibody. After being washed, the cells were incubated with the anti-CD68 antibody and subsequently with the secondary antibody (goat anti-rabbit FITC-conjugated antibody). Nuclei were counterstained by incubating the sections for 15 min with 4,6-diamidino-2-phenylindole (DAPI, 0.1 mg/ml in PBS, excitation 340 nm, emission 488 nm). The slides were mounted in Citifluor (Citifluor, London, UK) with coverslips and examined with a Nikon fluorescence microscope or with a confocal laser microscope (Leica TCS SP5). Images were combined with Adobe Photoshop (Adobe Systems). In all controls, primary polyclonal anti-*Hm*AIF-1 antibody was substituted with rabbit pre-immune serum (1:20,000) or primary antibodies were omitted and sections were treated with BSA-containing PBS and incubated only with the secondary antibodies.

Biochemical procedures

Protein extract preparation

H. medicinalis tissues from the unstimulated body wall or from injected areas were frozen in liquid nitrogen and then homogenized with a mortar. For SDS-PAGE, leech homogenates were suspended in extraction buffer (2× Laemmli's buffer in the presence of a protease inhibitor cocktail; Sigma, Milan, Italy). The particulate material was removed by centrifugation at 13,000 rpm for 10 min at 4 °C in a refrigerated Eppendorf Minispin microcentrifuge. Supernatants were denatured at 100 °C for 10 min.

SDS-PAGE procedure

Equal amounts of protein extracts were separated in 10 % acrylamide minigels by analytical SDS-PAGE. Molecular weights were determined by concurrently running broad-range standards from Bio-Rad (Richmond, Mass., USA).

Western blot

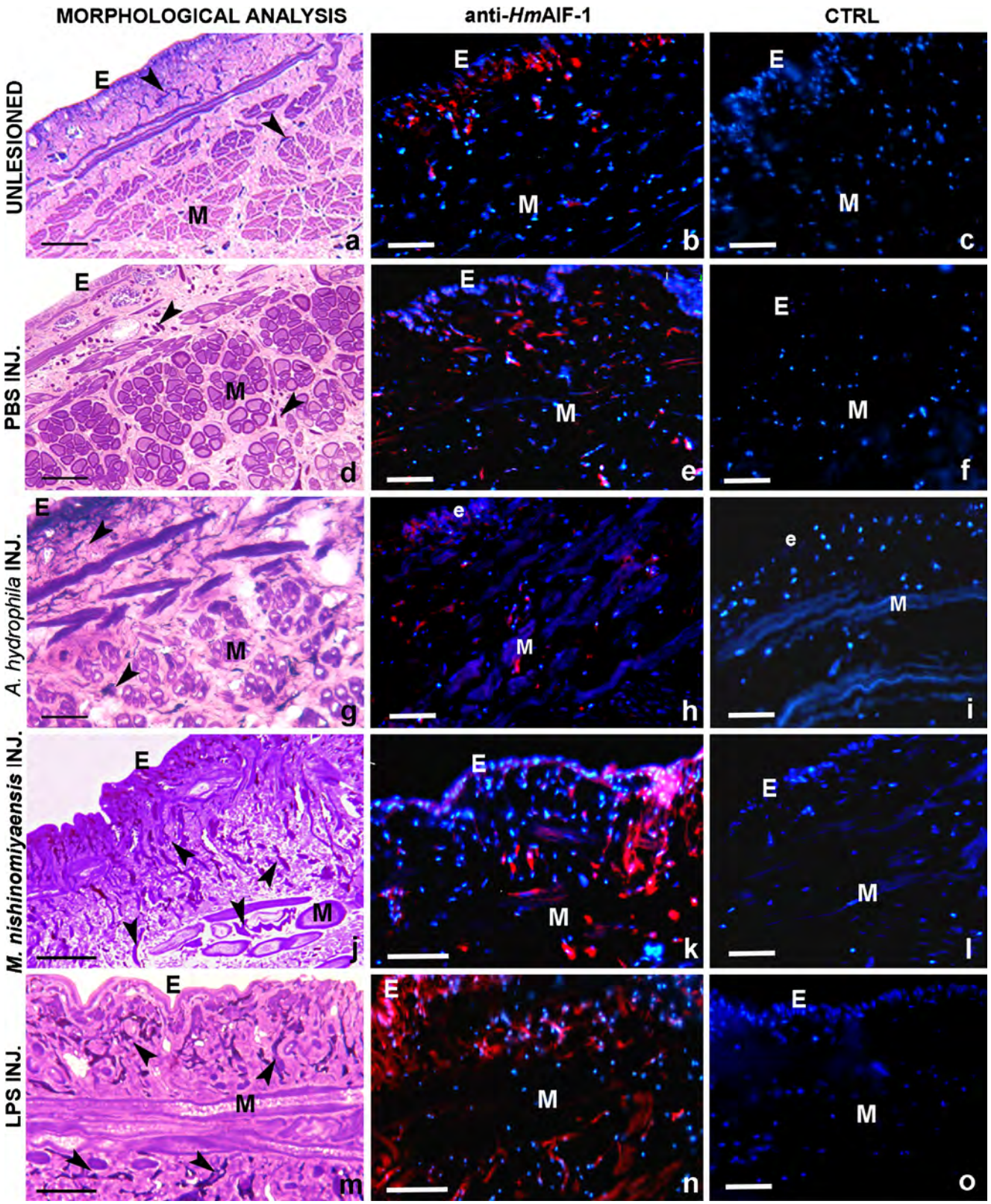
Proteins separated by SDS-PAGE were transferred onto Bio-Rad nitrocellulose filters. Membranes were then saturated with 5 % non-fat dried milk in TRIS-buffered saline (TBS, 20 mM TRIS–HCl buffer, 500 mM NaCl, pH 7.5) at room temperature for 2 h and incubated for 90 min with rabbit polyclonal anti-*Hm*AIF-1 antibody (1:5000 dilution in 5 % TBS-milk). After the membrane was washed three times with TBS-Tween 0.1 %, the antigens were revealed with a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, USA), diluted 1:5000. After a washing step, immunocomplexes were revealed with luminol LiteAblot PLUS Enhanced Chemiluminescent Substrate (EuroClone, Pero, Italy). In control experiments, anti-*Hm*AIF-1 antibody was substituted with rabbit pre-immune serum (1:20,000; not shown). Bands were normalized by using the ImageJ software package (http://rsbweb.nih.gov/ij/download.html), with the housekeeping protein D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was detected with a rabbit polyclonal anti-human GAPDH IgG (Proteintech, Chicago, USA), diluted 1:2000. The expression level of *Hm*AIF-1 in treated leeches was reported relative to control unlesioned animals. Experiments were performed in triplicate and data represent mean values±SEM. Statistical significance was assessed by an unpaired Student's *t*-test.

Results

*Hm*AIF-1 expression in unlesioned and microbially challenged leeches

AIF-1 has previously been reported to be significantly up-regulated in response to microbe infection both in vertebrates and in invertebrates. To determine whether the same effect occurred in the leech, we performed immunocytochemical analysis with a specific *Hm*AIF-1 antibody on cryosections from unlesioned, PBS-injected or immune-challenged animals injected with the symbiont Gram-negative bacterium *A. hydrophila*, with the pathogen Gram-positive bacterium *M. nishinomiyaensis* or with LPS. Our data showed that *Hm*AIF-1 was constitutively expressed in unlesioned animals (Fig. 1a-c). This factor was mainly expressed in cells located in the connective tissue underlying the body wall epithelium and surrounding the fields of muscle fibres. A similar pattern was observed in samples analysed 6 h after PBS treatement, indicating that the mechanical stress induced by the injection or the vehicule solution alone did not exert a significant effect on *Hm*AIF-1 expression in the body wall of challenged animals (Fig. 1d-f). Similarly, the injection of heat-killed *A. hydrophila* appeared ineffective with respect to the presence of *Hm*AIF-1$^+$

Fig. 1 Morphological (optical microscopy) and immunohistochemical (fluorescence microscopy) analysis of cryosections from *Hirudo medicinalis* body wall: unlesioned (**a–c**), PBS-injected (*PBS INJ.*; **d-f**) or immune-challenged by injection of *A. hydrophila* (**g–i**), *M. nishinomiyaensis* (**j–l**) or lipopolysaccharides (*LPS INJ.*; **m–o**). Localization of allograft inflammatory factor-1 (AIF-1). Note the population of resident and migrating immune-responsive cells (*arrowheads*) located under the epithelium (*E*) and among the muscle fibres (*M*). Immunohistochemistry was performed with a rabbit polyclonal anti-*Hm*AIF-1 antibody (*red*); nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; *blue*). Negative control experiments were performed with primary anti-*Hm*AIF-1 pre-immune serum (**c**) or secondary antibody alone (**f**, **i**, **l**, **o**). *Bars* 100 μm (**a-i**), 50 μm (**j-o**)



cells in the challenged area (Fig. 1g-i). Interestingly, 6 h after *M. nishinomiyaensis* (Fig. 1j-l) or LPS (Fig. 1m-o) injection, numerous cells were clearly recognizable in the challenged area. These cells were *Hm*AIF-1^{+} and mainly localized under the epithelium and among the muscle fibre fields. No signal was detected in negative control experiments in which the

primary antibody was substituted with rabbit pre-immune serum (Fig. 1c) or in which the primary polyclonal anti-*Hm*AIF-1 antibody was omitted and sections were incubated only with the secondary antibody (Fig. 1f, i, l, o).

The expression profile of *Hm*AIF-1 in unlesioned, PBS and bacterially challenged animals was confimed by Western blot assay (Fig. 2). According to the results obtained from leech CNS, immunoblot analysis on body wall protein extracts confirmed the presence of an immunoreactive product at about 18 kDa. As observed by immunohistochemistry analysis (Fig. 1), the amount of *Hm*AIF-1 protein did not significantly change 6 h after PBS or *A. hydrophila* injection with respect to the basal expression level, but highly increased upon *M. nishinomiyaensis* or LPS challenge (Fig. 2a, b). GAPDH was used as an internal reference and band intensity appeared to be homogeneously distributed in the loaded samples. No specific signals were observed in the negative control experiments performed by using primary rabbit pre-immune serum (data not shown).

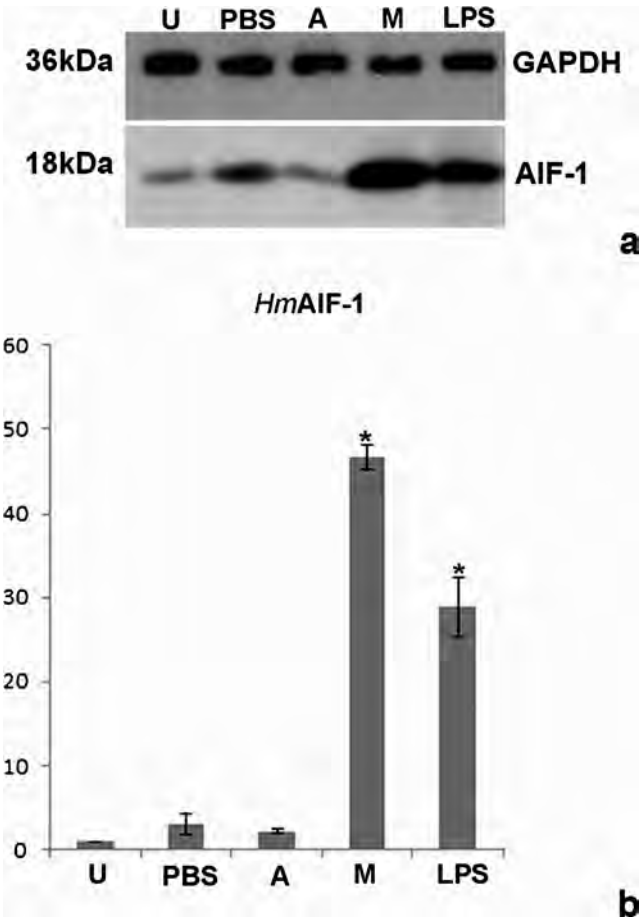


Fig. 2 Western blot analysis. **a** Protein extracts of unlesioned leech body walls (*U*) and body walls injected with PBS, *A. hydrophila* (*A*), *M. nishinomiyaensis* (*M*) or LPS were probed with the anti-*Hm*AIF-1 antibody. The housekeeping protein D-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. In all samples, the anti-*Hm*AIF-1 detected specific immunoreactive bands of about 18 kDa, according to the molecular weight ladder (kDa). **b** *Hm*AIF-1 protein was quantified by densitometry from three experiments. *$P<0.05$ compared with unlesioned leeches

Double-immunolocalization of *Hm*AIF-1 and of leucocyte and macrophage markers in bacterially challenged leeches

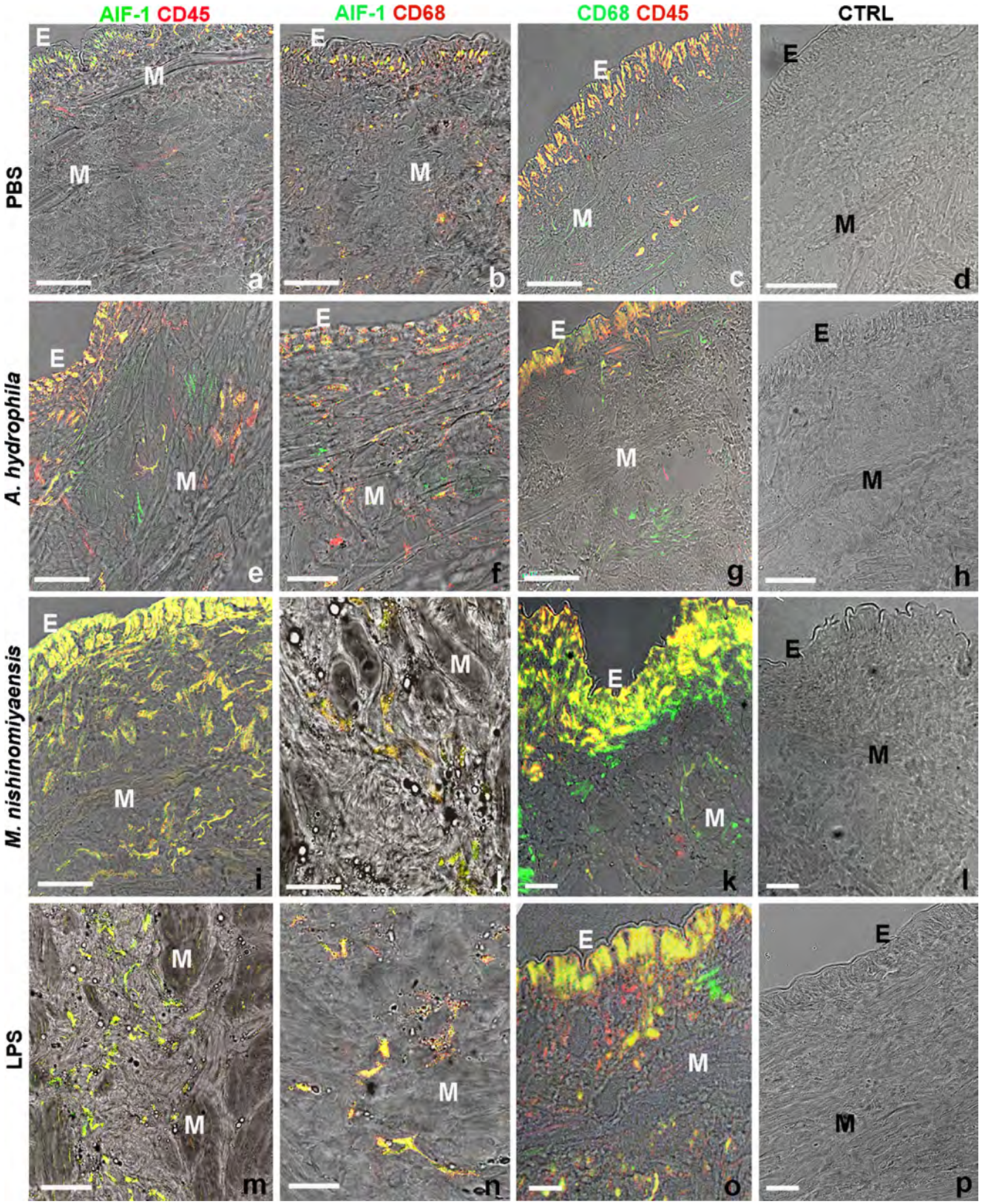
To characterize the resident and migrating cell expressing *Hm*AIF-1 described above, we performed double-staining experiments coupling anti-*Hm*AIF-1 to antibodies directed against the common human leucocyte marker CD45 and the specific human macrophage marker CD68 (Fig. 3). In leeches injected with PBS or *A. hydrophila*, cells that were *Hm*AIF-1$^+$/CD45$^+$ (Fig. 3a, e) or *Hm*AIF-1$^+$/CD68$^+$ (Fig. 3b, f) and CD45$^+$/CD68$^+$ (Fig. 3c, g) were more concentrated in the sub-epithelial area of the body wall, whereas among the muscle fibres, a few resident immunocompetent cells that were AIF-1$^+$/CD45$^+$ (Fig. 3a, e) or AIF-1$^+$/CD68$^+$ (Fig. 3b, f) and CD45$^+$/CD68$^+$ (Fig. 3c, g) were visible. A larger amount of cells gathered at the challenged area was observed 6 h after the treatment with *M. nishinomiyaensis* (Fig. 3i-k) or LPS (Fig. 3m-o). Numerous CD68$^+$/CD45$^+$ cells were visible lying underneath the epithelium and migrating among the muscle fibres (Fig. 3k, o). These cells also expressed *Hm*AIF-1 (Fig. 3i, j, m, n). This is the first report of a macrophage-specific marker in leech. Moreover, our data were in accordance with previous reports on vertebrates, indicating that CD45 is involved in macrophage-like cell differentiation, activation and migration.

Control experiments performed in the absence of the anti-*Hm*AIF-1 primary antibody (Fig. 3d, h) or with the pre-immune serum (Fig. 3l, p), were negative in all the samples.

Analysis of leech tissues injected with *Hm*AIF-1 recombinant protein (r*Hm*AIF-1)

In order to assess the possible involvement of *Hm*AIF-1 in the regulation of the innate immune response of *H. medicinalis* and, in particular, in macrophage activation and migration, we evaluated the effects of the injection of the recombinant

Fig. 3 Double-immunolocalization of *Hm*AIF-1 and macrophage markers in cryosections of control PBS-injected (**a–c**) and bacterially challenged (**e–o**) leech body wall. At 6 h following *M. nishinomiyaensis* or LPS injection, numerous *Hm*AIF-1$^+$/CD45$^+$, *Hm*AIF-1$^+$/CD68$^+$, CD68$^+$/CD45$^+$ macrophages (*yellow*) migrating towards the injected area were detectable under the epithelium (*E*) and among the muscle fibres (*M*). Double-immunostaining was performed with anti-*Hm*AIF-1 (*green*) and anti-CD45 (*red*) or anti-CD68 (*red* in **b**, **f**, **j**, **n**; *green* in **c**, **g**, **k**, **o**) antibodies. **d**, **h**, **l**, **p** No signal was detected in negative control experiments in which primary antibodies were omitted (**d**, **h**) or performed with primary anti-*Hm*AIF-1 pre-immune serum (**l**, **p**). *Bars* 100 μm (**a-d**), 50 μm (**e-h**, **i**, **k**, **l**, **m**, **o**, **p**), 25 μm (**j**, **n**)



protein r*Hm*AIF-1 in the leech body wall at various time points following treatment.

At T24 h (Fig. 4a), T48 h (Fig. 4b) and T72 h (data not shown) after injection of 20 ng r*Hm*AIF-1, a large number of

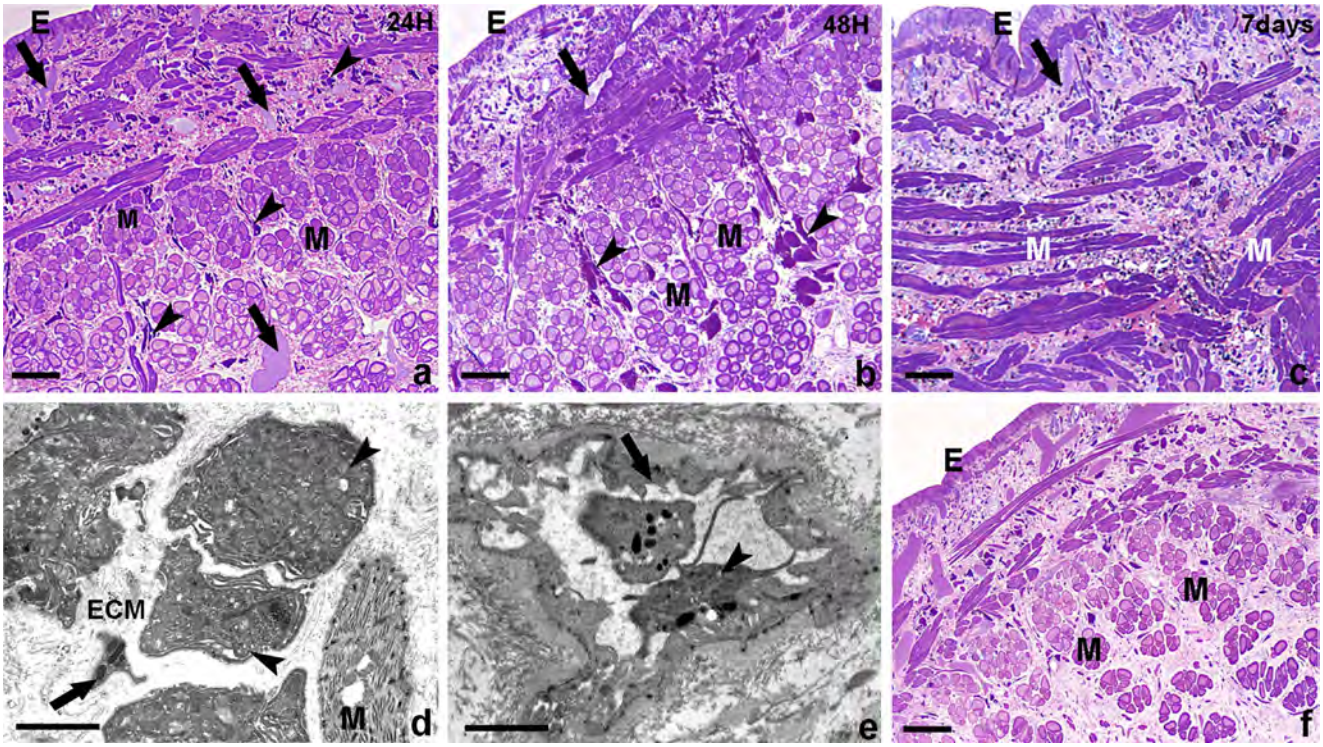


Fig. 4 Morphological analysis of leech body wall after injection of r*Hm*AIF-1 (**a–e**) or of both r*Hm*AIF-1 and function-blocking anti-*Hm*AIF-1 antibody (**f**). **a**, **b** After 24 h (**a**) and 48 h (**b**), numerous neo-vessels (*arrows*) and migrating cell (*arrowheads*) are found among muscles (*M*) and under the epithelium (*E*). **c** At 7 days after r*Hm*AIF-I injection, vessels (*arrows*) are still visible in the leech body wall. **d**, **e** Images obtained by transmission electron microscopy. **d** Detail of macrophages (*arrowheads*) and extravasated haematopoietic precursor cells (*arrow*) localized in the extracellular matrix (*ECM*) surrounding the muscle fibres (*M*). **e** Haematopoietic precursor cells (*arrowhead*) in the lumen of a neo-vessel (*arrow*). **f** At 24 h following r*Hm*AIF-1+anti-*Hm*AIF-1 antibody injection, reduced infiltration of cells and vessels among the groups of muscle fibres (*M*) and under the epithelium (*E*) is evident. *Bars* 100 μm (**a-c**, **f**), 2 μm (**d**), 400 nm (**e**)

migrating cells were readily recognizable among the muscle fibres. Interestingly, the migration of these cells was associated with an angiogenic process and with the appearance of a network of novel blood vessels in the space among the fields of muscle (Fig. 4a-c). At 7 days after injection (Fig. 4c), the number of migrating cells was highly reduced, and only some vessels were still detectable under the epithelium. As observed by transmission electron microscopy (TEM), at 48 hours after r*Hm*AIF-1 injection (Fig. 4d), the entire body wall was heavily infiltrated by cells showing the typical features of leech macrophage-like cells as described in our previous work. Neo-vessels, in the lumen of which circulating cells were clearly recognizable, were also present (Fig. 4e). These cells extravasate in the connective tissue and differentiate into mature leucocytes involved in the innate immune response of leech. Extravasated haematopoietic precursor cells were indeed visible in the extracellular matrix surrounding the macrophages and muscle fibres (Fig. 4d).

Function-blocking experiments were performed by injecting r*Hm*AIF-1 pre-incubated with the specific anti-*Hm*AIF-1 polyclonal antibody (Fig. 4f). The results showed a reduced infiltration of cells and vessels among the groups of muscle fibres and under the epithelium confirming the responsiveness of leech leucocytes and endothelial cells to *Hm*AIF-1.

Double immunolocalization of *Hm*AIF-1 and leucocyte and macrophage markers in rHmAIF-1-injected leeches

In order to characterize the cell types recruited to the site of r*Hm*AIF-1 injection and to confirm our hypothesis that *Hm*AIF-1 influences the mobilization of leech macrophage-like cells, double-staining experiments were performed on cryosections of tissues collected 24 h after injection (Fig. 5) of anti-*Hm*AIF-1, anti-CD68 and anti-CD45 antibodies. Double-immunofluorescence experiments confirmed the accumulation of macrophage-like cells, positive for both CD45 and CD68, at the injection site of the recombinant protein (Fig. 5a). A large number of *Hm*AIF-1/CD45-positive cells, mainly distributed in the sub-epithelial region, were also visible in the injected area (Fig. 5b). These data indicated that r*Hm*AIF-1 not only promoted the migration of $CD45^{+}/CD68^{+}$ macrophages, but also exerted a positive feed-back effect on recruited cells inducing the expression of the factor.

Function-blocking experiments showed a reduced number of macrophage-like cells co-expressing the two markers CD45

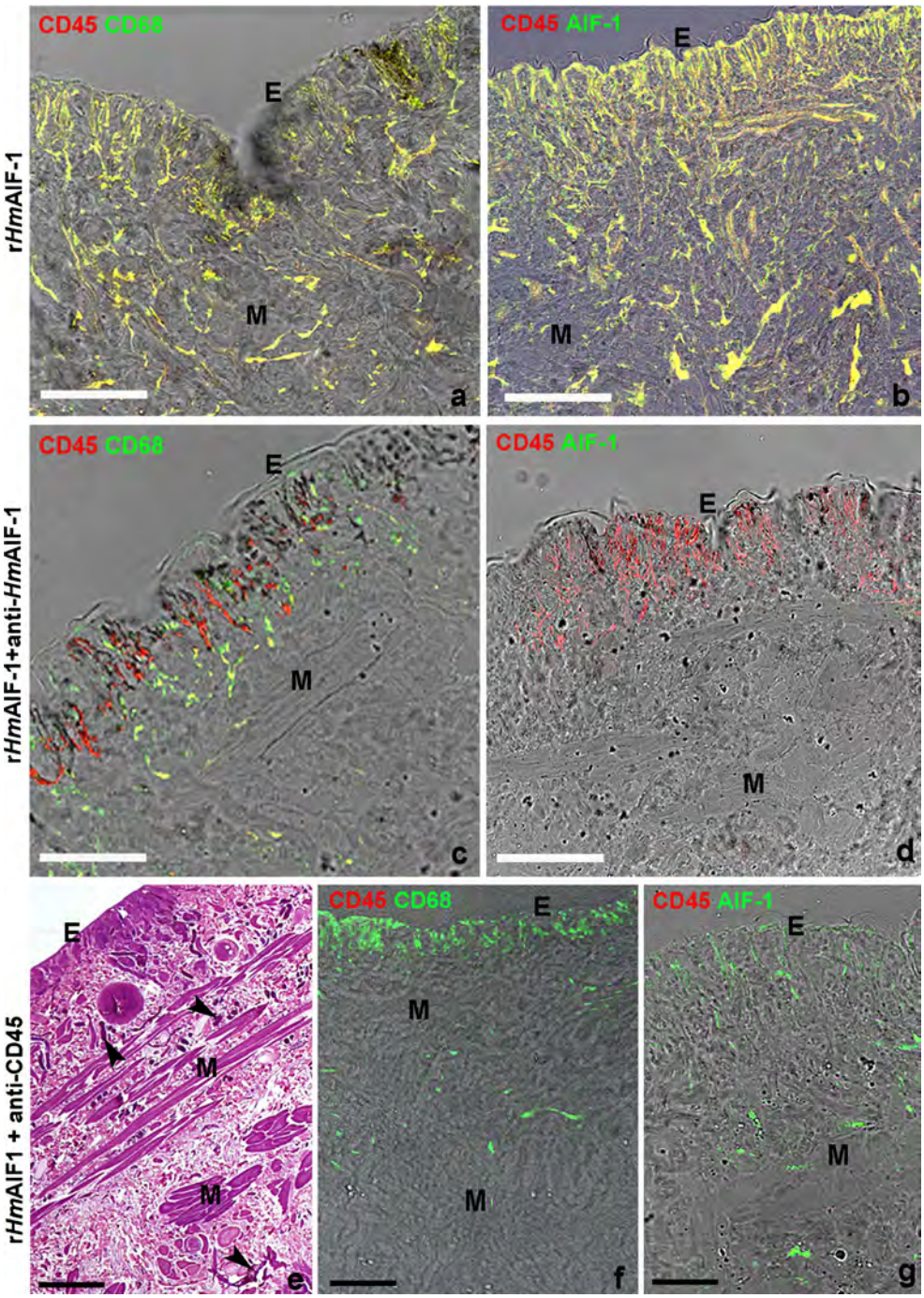


Fig. 5 **a**, **b** At 24 h after r*Hm*AIF-1 injection, numerous migrating macrophages (*yellow*) are double-stained by the antibodies for CD45 and CD68 and are $CD45^{+}/AIF\text{-}1^{+}$. **c**, **d** At 24 h after injection of an r*Hm*AIF-1+anti-*Hm*AIF-1 mixture, a few activated macrophages co-expressing CD45 (*red* in **c**) and CD68 (*green* in **c**) or CD45 (*red* in **d**) and *Hm*AIF-1 (*green* in **d**) are present. **e**–**g** After injection of the antibody against CD45 together with the r*Hm*AIF-1, only a few macrophages (*arrowheads* in **e**) that are $CD45^{+}/CD68^{+}$ (**f**) are found underneath the epithelium (*E*) and among muscles (*M*). **g** Injection of antibody against CD45 inhibits CD45 expression and a few resident macrophages are $CD45^{-}/AIF\text{-}1^{+}$. *Bars* 50 μm

and CD68 in the body wall of leeches injected with both the r*Hm*AIF-1 and the anti-*Hm*AIF-1 (Fig. 5c). Furthermore, the number of cells expressing *Hm*AIF-1 under the same experimental conditions was highly reduced and the $CD45^{+}$ cells were *Hm*AIF-1-negative (Fig. 5d), confirming a direct link between the injection of the recombinant protein and the induction of the factor in activated cells. Control experiments performed in the absence of primary antibodies were negative for all samples (data not shown).

Anti-CD45 polyclonal antibody treatment

To determine a possible role of CD45 on macrophage responsiveness to *Hm*AIF-1, we injected the r*Hm*AIF-1 together with the anti-CD45 antibody. Morphological (Fig. 5e) and immunocytochemical (Fig. 5f, g) analysis showed that the anti-CD45 treatment inhibited the *Hm*AIF-1-induced responses described above, reducing the number of $CD45^{+}/CD68^{+}$ macrophage-like cells gathered at the injection site. In addition, double-immunocytochemical experiments showed that

CD45 expression was inhibited in the body wall of r*Hm*AIF-1/anti-CD45-injected leeches (Fig. 5g) and only a few resident macrophage-like cells that were $CD68^+$ (Fig. 5f) and *Hm*AIF-1^+ (Fig. 5g) were still detectable, suggesting a direct role of CD45 in the migration capability of the *Hm*AIF-1-activated macrophages.

Discussion

In the present study, we demonstrate that, in the leech *H. medicinalis*, the inflammatory factor *Hm*AIF-1 is involved in the regulation of the innate immune response by inducing angiogenesis and macrophage-like cell migration. Our results show that, in leeches, recombinant *Hm*AIF-1 has strong chemotactic activity and, in particular, it promotes macrophage-like cell and vessel migration towards the stimulated area, exhibiting a similar function to AIF-1 of vertebrates. Moreover, we demonstrate that, in leeches, as in vertebrates, this molecule is constutively expressed in untreated animals but is dramatically enhanced after microbial infection.

*Hm*AIF-1 is expressed by leech macrophage-like cells

Even though AIF-1 was originally cloned from active macrophages in human and rat atherosclerotic allogenic heart grafts undergoing chronic transplant rejection (Utans et al. 1995), its transcripts have subsequently been demonstrated to be up-regulated in leucocytes upon LPS stimulation both in vertebrates (Miyata et al. 2001; Wang and Wu 2007) and in invertebrates (Zhang et al. 2011; Wang et al. 2013). These results clearly suggest that AIF-1 plays a key role not only in various host responses to infammatory stimuli, but in the whole-host immune defence reaction. We have thus hypothesized that *Hm*AIF-1 could also be involved in innate immune response in leeches and that its expression would be enhanced in a context of bacterial infection. For this reason, we have investigated the tissue-specific and temporal expression profile of *Hm*AIF-1 factor after challenging leeches with the environmental bacteria *A. hydrophila* and *M. nishinomiyaensis*. The first is a Gram-negative bacterium symbiont of *H. medicinalis*, present in the bacterial flora of leech gut where it plays an essential role in the digestion of blood (Graf et al. 2006; Patel et al. 2013). The second is a Gram-positive bacterial pathogen in leeches (Schikorski et al. 2008). Finally, we also injected a lipopolysaccharide (LPS) solution, in order to test the effect on *Hm*AIF-1 expression induced by a pathogenic Gram-negative bacterial infection. The action of LPS on leech tissues is well known and has previously been described. Leeches pricked and injected with LPS show a large number of migrating $CD68^+$ macrophage-like cells characterized by pseudopodia, phagolysosomes in their cytoplasm and strong lysosomal activity (de Eguileor et al. 2000a, 2000b).

Leeches challenged with *M. nishinomiyaensis* or stimulated with LPS exhibited a larger number of migrating *Hm*AIF-1^+ cells than undamaged, PBS- or *A. hydrophila*-injected leeches. These assays, confirmed by Western blot quantitative analysis, demonstrated that the increase in *Hm*AIF-1^+ cells was caused by the immune activation of LPS or Gram-positive bacterial challenge and not by the mechanical stress linked to the injection or to the mere presence of symbiont bacteria. Characterization of the migrating cells was achieved by both ultrastructural analysis and immunohistochemistry by using polyclonal antibodies directed against human macrophage and leucocyte markers CD68 and CD45. The use of antibodies generated against mammalian CD antigens to detect leucocytes and macrophage-like cells in the leech is supported by data from the literature concerning leeches (de Eguileor et al. 2000a; Grimaldi et al. 2004, 2006) and animals phylogenetically related to Annelids such as Molluscs and Sipunculids (Cossarizza et al. 1996; Blanco et al. 1997). Double-staining experiments highlighted that *Hm*AIF-1^+ cells co-expressed both CD45 and CD68. *Hm*AIF-1 positivity indicated that this factor, as described for mammals, was expressed in activated macrophages. This is the first report of a macrophage-specific marker in leech. Numerous $CD68^+$ and $CD45^+$ macrophage-like cells were mainly detected in animals injected with LPS or with the pathogen *M. nishinomiyaensis* suggesting that, as in mammals, the immune challenge in leech body wall induced the migration and accumulation of macrophage-like cells at the injury site. Moreover, *M. nishinomiyaensis* and LPS caused remarkable inflammatory effects, inducing massive angiogenesis and *Hm*AIF-1^+/$CD45^+$/$CD68^+$ macrophage migration. The immune challenge of leeches thus gives rise to an occurrence consistent with that obtained after wounding (de Eguileor et al. 2003; Tettamanti et al. 2003; Grimaldi et al. 2004, 2006), in agreement with the finding that the wound healing process, in *H. medicinalis*, is enhanced by a controlled bacterial infection. This suggests that, in leech, the immune response is based on the same molecules involved in wound healing and regenerative process (Schikorski et al. 2008). On the other hand, the effects of *A. hydrophila* are similar to those of PBS injection. These bacteria, being symbionts of the leech digestive tract, probably do not induce a strong immune response, even if injected in a different area of the leech body, namely the body wall. The observed differences in reactivity to bacteria and to LPS injections might be related to the different macrophage responses induced by Gram-negative and Gram-positive bacterial infection. Indeed, leech macrophage-like cells are known to produce antimicrobial proteins and peptides, such as *Hm*-theromacin and destabilase, that cleave the cell wall components of Gram-positive bacteria (Schikorski et al. 2008; Hildebrandt and Lemke 2011), thus

resulting in a more effective defence against Gram-positive than Gram-negative bacteria.

*Hm*AIF-1 is involved in recruitment of leech macrophages

The relationship between the functional responsiveness of macrophage-like cells to *Hm*AIF-1 was assessed by injecting the recombinant factor in the body wall of leeches. We observed, at 6 h after r*Hm*AIF-1 administration, the formation of an extensive vessel network spanning throughout the avascular body wall of the animal and the accumulation of numerous migrating cells localized among the fields of muscle fibres. Indeed, the injection of the recombinant protein together with a function-blocking anti-*Hm*AIF-1 antibody highly reduced angiogenesis and cell migration. Moreover, morphological analysis showed that macrophage recruitment induced by r*Hm*AIF-1 was higher in the first 48 h after injection and decreased starting from 72 h to 7 days after treatment. These data are in accordance with the reports from vertebrates in which AIF-1 is involved in macrophage activation at early stages, rather than at late stages of the inflammatory process (Autieri et al. 2000; Deininger et al. 2000, 2002; Zhang et al. 2011). In particular, at 24 h after r*Hm*AIF-1 injection, a high immunoreactive signal was detected in the cytoplasm of migrating cells, most of which were $CD68^+$. In addition, the r*Hm*AIF-1 injection not only promoted the migration of $CD45^+/CD68^+$ macrophage-like cells, but also induced an enhancement of *Hm*AIF-1 expression in the recruited cells. This suggests that the migrated cells, once chemoattracted to the stimulated area, express this factor to substain the recruitment of further macrophage-like cells or to maintain their accumulation at the injured site. Despite this positive feedback in *Hm*AIF-1 expression, its mechanisms of action on its target cells remains largely unexplored.

The $CD68^+$ cells also co-express the leucocyte common antigen CD45, which shares 55 % identity with human CD45 (Macagno et al. 2010). Notably, this transmembrane glycoprotein in vertebrates regulates integrin-mediated adhesion and is required to maintain macrophage adhesion. Once macrophages adhere to the extracellular matrix, they mature and respond to environmental stimuli (Trowbridge and Thomas 1994; Roach et al. 1997). Our data show that the injection of r*Hm*AIF-1 pre-incubated with specific anti-*Hm*AIF-1 antibody reduces the number of migrating cells, most of which no longer co-express the CD45 and CD68 markers. Since CD45 has been reported to regulate chemokine receptor expression in myeloid leucocytes and since it can modulate leucocyte traffic by regulating responses to chemokines (Mitchell et al. 1999), we hypothesize that CD45 expression in leech is involved in the maturation and functional responsiveness of macrophage-like cells to *Hm*AIF-1. Indeed, the injection of r*Hm*AIF-1 together with the anti-CD45 polyclonal antibody reduces the migration of macrophage-like cells. Although further experiments will be necessary, this result suggests that the recruitment of these migrating cells is induced by a close interaction between *Hm*AIF-1 and the CD45 protein expressed at the surface of responsive cells.

Concluding remarks

The results presented here reveal that the expression of *Hm*AIF-1 significantly increases during the early phases of the inflammatory response and that this is mainly exerted by activated macrophage-like cells. We demonstrate, for the first time, that *Hm*AIF-1 is not only a potent angiogenic factor, but also a potent chemoattractant for macrophage-like cells. During bacterial infections, *Hm*AIF-1 might be involved in the activation of these migrating cells whose role is to rid the area of bacteria. This process is probably linked to the interaction between *Hm*AIF-1 and CD45 to promote the integrin-mediated adhesion of macrophage-like cells to the extracellular matrix.

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