Ph.D. Course in Cellular and Molecular Biology
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Identification and modulation of the Allograft Inflammatory Factor-1 (AIF-1) homologous in the medicinal leech *Hirudo medicinalis*

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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 allografts immune response and its expression is mostly limited to the monocyte/macrophage lineage. Recently it was assumed that AIF-1 was a novel molecule involved in inflammatory responses. To better clarify this aspect in the present study we investigated the expression of AIF-1 after bacterial challenge, wounds and tissue transplants and its potential role in regulating the innate immune response in an invertebrate model, the medicinal leech (*Hirudo medicinalis*). The analysis of an EST library from *H. medicinalis* CNS, revealed the presence of a gene, named *Hmaif-1*/alias *Hmiba1*, showing a high homology with vertebrate *aif-1*. Immunohistochemistry using an anti-*Hm*AIF-1 polyclonal antibody showed that this protein is constitutively present in spread, $CD68^+$ macrophage-like cells. A few hours after pathogen bacterial injection in the body wall, the amount of these immunopositive cells increases at the injected site, co-expressing *Hm*AIF-1 and the common leukocyte marker CD45. A similar overview was observed in the early stages of wound healing and in transplants, with many immunopositive cells around the lesion site or around the graft. Moreover, here we

demonstrated that the recombinant protein *Hm*AIF-1 induced a massive angiogenesis and it was a potent chemoattractant for macrophages. After r*Hm*AIF-1 stimulation, macrophage-like cells co-expressed the macrophage marker CD68 and the surface glycoprotein CD45, which in Vertebrates is involved in the integrin-mediated adhesion of macrophages and plays a key role in regulating the functional responsiveness of cells to chemoattractants. We therefore hypothesized that CD45 could play a role for leech macrophage-like cells activation and migration towards the inflammation site and we examined its potential effect on *Hm*AIF-1-induced signaling.

INTRODUCTION

Allograft Inflammatory Factor-1

The Allograft Inflammatory Factor-1 (AIF-1), also called MRF-1, Iba1, daintain and IL-19, is a "calcium-binding protein" cytoplasmic cytokine of 17 kDa and 143 aa, IFN-γ-inducible, with a Ca^{2+}-binding EF-hand domain of 12 aa, and has been identified first in chronic rejection of rat cardiac allografts. In humans, AIF-1 is encoded within the HLA class III genomic region, located on chromosome 6p21, moreover AIF-1-like factors, with aminoacid structure and with very well preserved functional role, have been described in other groups of metazoans. Data in literature have indeed shown that the expression of AIF-1 increases significantly after transplantation, wounds or bacterial infections both in vertebrates (Utans et al., 1995; Deininger et al., 2000; Watano et al., 2001; Deininger et al., 2002; Autieri & Chen, 2005; Alkassab et al., 2007) and in invertebrates, such as Sponges (Kruse et al., 1999), Mollusks (de Zoysa et al., 2010; Zhang et al., 2011; Li et al., 2012b; Zhang et al., 2013) and Echinoderms (Ovando et al., 2012), suggesting that it could play a key role in the inflammatory response and in the immune system regulation. Its expression is in fact mainly associated with infiltrating macrophages in the allografts, inflammatory

lesions of central nervous system, tumors, bacterial infections and mechanical or biochemical vascular lesions (Deininger et al., 2000; Autieri & Carbone, 2001; Deininger et al., 2002; Autieri & Chen, 2005; Zhang et al., 2011; Li et al., 2012a; Ovando et al., 2012; Zhang et al., 2013; Drago et al., 2014). Furthermore, it appears to be closely related to vasculogenesis and angiogenesis and increases in a dose dependent manner the proliferation and migration of macrophages and endothelial cells.

In particular AIF-1 expression was detected in cells expressing major histocompatibility complex class II molecules and in a subset of activated macrophages and microglial cells expressing MRP-8^{+} (macrophage-related protein-8), a Ca^{2+}-sensing proteins whose translocation from the cytoplasm to the cellular membranes constitutes an indicator of activation and phosphorylation of macrophages (Beschorner et al., 2000).

Since the release of AIF-1 is a Ca^{2+}-dependent mechanism, it seems that AIF-1 may play a role in cell-cell interactions under inflammatory conditions (Tanaka & Koike, 2002). In particular, the ability to bind calcium allows to develop distinct pathways of signal transduction, protein expression and cell cycle regulation during the activation of macrophages and microglial cells. Therefore AIF-1 results to be a modulator of the immune response

during macrophage activation and tissue regeneration (Alkassab et al., 2007; Pawlik et al., 2008).

Interesting to note is that AIF-1 shows the same functions and colocalizes with a leukocyte-specific member of the transmembrane PTPase family namely CD45, ubiquitously expressed on the surface of all nucleated cells of hematopoietic origin (Alkassab et al., 2007; Sommerville et al., 2012; Jeong et al., 2013; Li et al., 2013). CD45 is a cell surface glycoprotein that, in vertebrates, is implicated in integrin-mediated adhesion of macrophages (Roach et al., 1997; Zhu et al., 2011; St-Pierre & Ostergaard, 2013) and play a role in regulating the functional responsiveness of cells to chemoattractants (Roach et al., 1997; Mitchell et al., 1999), affecting the normal feedback mechanisms that are required to maintain adhesion and phagocytic activity. Indeed it has been reported that monocytes highly express AIF-1 and CD45, whereas resident microglia express AIF-1 but weakly and barely express CD45, confirming that both CD45 and AIF-1 might be involved in macrophage migration.

Despite extensive investigation focused on both molecular characteristics and 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 is still not clear. In particular, it has previously reported that AIF-1 is a modulator of the innate immune response during macrophage activation (Utans et al., 1995; Alkassab et al., 2007), but the direct relationship between AIF-1 expression and macrophage activation/migration during the inflammation phase after injury or bacterial infection remains unclear, probably because the study of the immune response in Vertebrates appears to be a difficult challenge, primarily due to the complexity of these organisms.

Choice of the experimental model and goal of the research

We focused our research on the possible role of AIF-1 during the immune response in an invertebrate model, the leech *H. medicinalis* (Annelida, Hirudinea), after challenge by bacterial pathogen, injury and grafts. Invertebrates, offering simpler systems, represent a great alternative. Indeed, basic steps of immune responses can be easily analyzed in leeches that might lack complex feed-back control systems typical of vertebrates (de Eguileor et al., 2000b; de Eguileor et al., 2001; de Eguileor et al., 2003; de Eguileor et al., 2004; Grimaldi et al., 2006; Grimaldi et al., 2009; Schikorski et al., 2009; Grimaldi et al., 2011).

Experimental model

The interest in using the leech as experimental model is linked to its anatomical and physiological features that allow to observe and study events linked to bacterial infection, wound healing and tissue repair (de Eguileor et al., 2003; de Eguileor et al., 2004; Tettamanti et al., 2004; Grimaldi et al., 2011). The cellular immune response, in particular, can be easily and unambiguously evaluated in leech's body wall, which is a predominantly avascular muscular district containing a few immunocompetent cells of myeloid origin, i.e. macrophages, granulocytes and NK (de Eguileor et al., 1999). *H. medicinalis* is in fact characterized by the absence of a true vascular system within the muscular body wall and by the presence of two specific tissues: the botryoidal tissue, located close to the digestive system, involved in myeloerythroid and hematopoietic cells production and in the formation of new vessels, and the vasofibrous tissue, located more externally and involved in tissue regeneration after injury (Huguet & Molinas, 1994; Huguet & Molinas, 1996; Grimaldi et al., 2011) (Fig. 1).

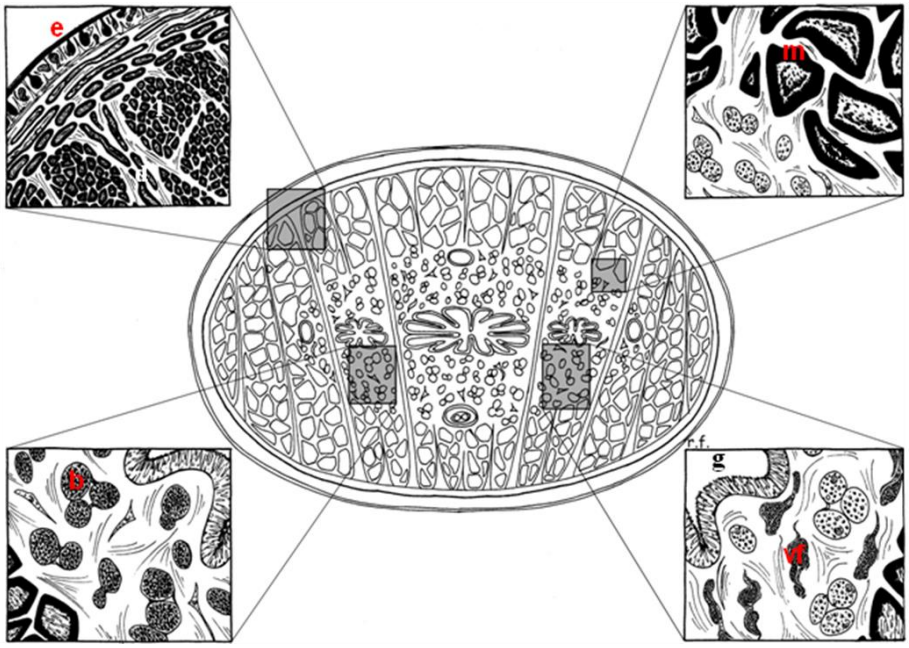

Fig. 1: Transverse section of *Hirudo*. **e:** epithelium; **m:** longitudinal muscles; **b:** botryoidal tissue; **vf:** vasofibrous tissue

The effects of bacterial challenge, lesion or grafts in the leech body wall are rapidly induced (24 hours) and can be studied by morphological and histochemical analyses. The inflammatory phase is characterized by an influx of macrophages that are responsible for phagocytosis and immune cytotoxicity and clean the stimulated area, and release various growth factors (de Eguileor et al., 1999; de Eguileor et al., 2000a, b; Grimaldi et al., 2006; Tettamanti et al., 2006). A second, proliferative phase consists of a process characterized by fibroplasia, with fibroblast migration into the wound and deposition of new collagen,

fibronectin and other matrix components (Tettamanti et al., 2004; Tettamanti et al., 2005). This phase is also characterized by angiogenesis, with an active endothelial cell multiplication and sprouting of new vascular capillaries from parent vessels (de Eguileor et al., 2003; Tettamanti et al., 2003b; de Eguileor et al., 2004; Tettamanti et al., 2006). In *H. medicinalis,* new vessels 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 injured or bacterial challenged areas. After a transendothelial migration, these cells leave the circulating fluid, disperse in the surrounding connective tissue and differentiate in mature leukocytes that mediate inflammatory response (Grimaldi et al., 2006). In addition our previous papers (de Eguileor et al., 2000a, b; Grimaldi et al., 2004; Grimaldi et al., 2006) and a recent report (Macagno et al., 2010) 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 easy detection and monitoring of immunocompetent cell migration and of the angiogenic process and the basic steps of immune responses can be easily analysed in

leeches that lack complex feedback control systems typical of Vertebrates.

Moreover a gene showing high similarity with Vertebrate AIF-1, named *Hm*Iba1/alias *Hm*AIF-1 (GenBank accession number KF437461), has been recently identified (Drago et al., 2014) and characterized in leech Central Nervous System (CNS). *Hm*AIF-1 genomic sequence consists of 7 exons and 6 introns, and the protein has a molecular weight of about 18 kDa. The aminoacid sequence presents an identity of 52% with the human AIF-1, and it shows a closer relationship with molluscan than with other metazoan AIF-1 molecules, as is expected on the basis of evolutionary proximity between Annelids and Mollusks (both Lophotrochozoan) (Drago et al., 2014). In addition a polyclonal antibody against *Hm*AIF-1 and the recombinant protein has been developed by the Jacopo Vizioli's group (University of Lille) with which we collaborate.

Goal of the research

In order to better understand the role of *Hm*AIF-1 during the innate immune response of leech after bacterial challenging, wound healing and graft stimulations, we investigated the effects of the AIF-1 overexpression by recombinant *Hm*AIF-1 protein injection and the tissue-specific and temporal expression of *Hm*AIF-1 after

injections of both symbiotic and pathogen bacteria, injuries and grafts.

Immunohistochemistry and western blot studies will be performed to determine the localization and the modulation of this gene in leech. The presence of *Hm*AIF-1 in uninjured, bacteria challenged, experimentally injured and grafted tissues will be established using the specific rabbit anti-*Hm*AIF-1 polyclonal antibody. Ultrastructural analysis at electron microscope, the acid phosphatase enzymatic histochemical reaction and immunohistochemical analysis using the polyclonal antibody anti-CD68 and anti-CD45 macrophage cell markers will be performed to characterize the cells involved in the immune response and expressing *Hm*AIF-1.

MATERIALS AND METHODS

Animals and Treatments

Leeches (*Hirudo medicinalis*, Annelida, Hirudinea, from Ricarimpex, Eysines, France) measuring 10 cm were kept in water at 20° C in aerated tanks. Animals were fed weekly with calf blood. Animals were randomly divided into separate experimental groups according to different protocols and treatments. Each treatment (bacterial or recombinant protein injections, wounds or tissue taking to be grafted) was performed at the level of the 20° segment. Before each experiment, leeches were anaesthetized with a 10% ethanol solution. Anesthetized leeches were then dissected and the body tissues were removed at specific time points after treatments.

Group 1: uninjured control leeches to provide information on normal body organization.

Group 2: (T6 h, T24 h, T48 h, T72 h, T7 days) control samples injected with 100 µl of sterilized phosphate buffer saline (PBS; 138 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

Group 3: (T24 h, T48 h, T72 h, T7 days) samples injected with 100 µl of sterilized PBS containing 20 ng of recombinant *Hm*AIF-1 (r*Hm*AIF-1) to functionally characterize cells migrating under the influence of this

factor. The best concentration of r*Hm*AIF-1 required to induce significant cells migration was determined basing on our previous works on leeches (Tettamanti et al., 2003b; Grimaldi et al., 2011). We tested three different concentrations (5 ng, 10 ng and 20 ng) and we obtained the best response with the higher amount.

Group 4: (T24 h, T48 h, T72 h, T7 days) samples injected with 100 µl of sterilized PBS containing 20 ng of r*Hm*AIF-1 pre-incubated 30 min at room temperature with 1 µg of a 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 5: (T24 h) samples injected with 100 µl of PBS containing 20 ng of r*Hm*AIF-1 pre-incubated 30 min at room temperature with 1 µg of pre-immune serum (1:20000) of the anti-*Hm*AIF-1 antibody.

Group 6: (T24 h) samples injected with 100 µl of PBS containing 20 ng of r*Hm*AIF-1 pre-incubated 30 min at room temperature with 1 µg of polyclonal rabbit anti-CD45 antibody (Twin Helix, Milano, Italy), to inhibit macrophage-like cells responsiveness to r*Hm*AIF-1.

Group 7: samples injected with 100 µl of sterilized PBS containing respectively 100 ng/ml of LPS from *E. coli*

(Serotype 0111:B4, Sigma, St. Louis, MO, USA) or heat-killed Gram-positive (*Micrococcus nishinomiyaensis*) and Gram-negative (*Aeromonas hydrophila*) bacteria ($3X10^7$ CFU/ml) for immune stimulation assays. Bacteria were isolated from the natural environment of *H. medicinalis.* Challenged animals were sacrificed 6 h after injection and treated as indicated above.

Group 8: (T24 h, T48 h, T72 h, T7 days) samples injured at about the 20° segment with a razor blade, in order to assess the modulation of *Hm*AIF-1 during the wound healing.

Group 9: (T24 h, T72 h, T7 days) samples used as hosts and donors for autografts and allografts. Surgical grafting was performed at the distal dorsal portion of leeches, about 2/3 from the oral extremity (at about the 20° segment): grafts were sutured with Dafilon® surgical synthetic monofilament (B. Braun) to avoid transplant loss due to contraction of the muscular body wall. Grafted leeches were kept in moist chambers for a post-surgical recovery period of 24 h, and subsequently placed in water tanks. The rate of successful transplantation experiments for all graft types was 90%. All leeches survived surgery and were able to move and feed following recovery from anesthesia. Autograft-bearing leeches: at about the 20° segment from the oral sucker, a block of 2mm×2mm×2mm

was excised and afterwards replaced in the same hollow; allograft-bearing leeches: *H. medicinalis* host received a block of 2mm×2mm×2mm body wall excised from the 20° segment of a co-specific leech. Grafts were sutured with Dafilon® surgical synthetic monofilament (B. Braun) in order to avoid the transplant loss due to contraction of the body wall.

Optical and Electron Microscopy

Leech tissues, dissected from the area of the injection, the wound or the graft, 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 post-fixed 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 fuchsine, according to Moore et al., 1960) and subsequently observed under the light microscope Nikon Eclipse Ni (Nikon, Tokyo, Japan). Data were recorded with a DS-5M-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). For immunogold cytochemistry, samples were fixed for 2 h with 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffered saline (PBS), and then washed in the same buffer. After a standard step of serial ethanol dehydration, they were embedded in an Epon-Araldite 812 mixture (Sigma, St. Louis, MO) and sectioned with a Reichert Ultracut S ultratome (Leica, Wien, Austria). Ultrathin sections (80 nm in thickness), after etching with NaOH 3% in absolute ethanol (Causton, 1984), were incubated for 30 min with PBS containing 2% bovine serum albumin (BSA) and then for 1 h with the primary rabbit anti-leech AIF-1 antibody (working dilution 1:50). Primary antibodies were visualized by immunochemical staining with secondary goat anti-rabbit IgG (H+L)-gold conjugate antibodies (GE Healthcare Amersham, Buckingamshire, UK) (particle size, 10 nm) diluted 1:40 (incubation 30 min at room temperature). Control sections were incubated in saturation buffer and then with secondary antibodies alone. Samples were counterstained with uranyl acetate in water and observed with a Jeol 1010 EX electron microscope (Jeol, Tokyo, Japan). Data were

recorded with a MORADA digital camera system (Olympus, Tokyo, Japan).

Acid Phosphatase Reaction (ACP)

Leech tissues, dissected from the area of the injection or wound, were embedded in Polyfreeze tissue freezing medium (OCT) (Polysciences, Eppelheim, Germany) and immediately frozen in liquid nitrogen. Cryosections (7 µm in thickness), obtained with a Leica CM 1850 cryotome, were rehydrated with PBS for 5 minutes, incubated with sodium acetate-acetic acid 0.1 M buffer for 5 minutes and then in the reaction mixture (sodium acetate-acetic acid 0.1 M buffer, 0.01% naphtol AS-BI phosphate, 2% NN-dimethylformamide, 0.06% Fast Red Violet LB and MnCl2 0.5nM) for 90 minutes at 37°C. After washings in PBS, the slides were mounted with PBS/glycerol 2:1 and observed with the light microscope Nikon Eclipse Ni (Nikon, Tokyo, Japan). Images were taken with the digital camera Nikon Digital Sight DS-SM (Nikon, Tokyo, Japan).

Indirect Immunofluorescence Staining

Serial cryosections (7 µm in thickness) were stained by crystal violet and basic fuchsine for a morphological view or used for immunofluorescence staining. Sections, rehydrated with PBS for 5 minutes, were pre-incubated for

30 min with PBS containing 2% bovine serum albumin (BSA) before the primary antibody incubation (1 h at 37°C). The primary antibodies used were: rabbit polyclonal anti-human CD45 (Twin Helix, Milano, Italy) which react with leech hematopoietic cells (de Eguileor et al., 2003) diluted 1:100, rabbit polyclonal anti-human CD68 (Santa Cruz Biotechnology) which react with leech macrophages (Grimaldi et al., 2006) diluted 1:100 and rabbit anti-*Hm*AIF-1 (Drago et al., 2014) diluted 1:1000. The use of antibodies generated against mammalian CD antigens to detect macrophages in leech is supported by several data from the literature on leeches (de Eguileor et al., 2000a, b; Grimaldi et al., 2004; Grimaldi et al., 2006) and on animals phylogenetically related to Annelids (Molluscs, Sipunculids) (Cossarizza et al., 1996; Blanco et al., 1997).

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 FITC-conjugated (excitation 493 nm, emission 518 nm), goat anti-rabbit Cy3-conjugated (excitation 562 nm, emission 576 nm), goat-anti rabbit Cy5-conjugated (excitation 650 nm, emission 672 nm). Double labelling experiments were performed as already described (Grimaldi et al., 2009): a) to detect AIF-1, AIF-1/CD45 or AIF-1/CD68 the anti AIF-1 was applied first, then sections

were incubated with the secondary antibody goat anti-rabbit FITC conjugated. After washing the cells were incubated with the antibody anti CD45 or anti CD68 and subsequently, with the secondary Cy5 conjugated goat anti-rabbit antibody; b) to detect CD45/CD68, the anti CD45 was applied first, then sections were incubated with the secondary Cy5 conjugated goat anti-rabbit antibody. After washing, the cells were incubated with the antibody anti CD68 and subsequently with the secondary antibody goat anti-rabbit FITC conjugated. Nuclei were stained by incubating for 15 min with 49,6-Diamidino-2-Phenylindole (DAPI, 0.1 mg/ml in PBS, excitation 340 nm, emission 488 nm). The slides were mounted in Citifluor (Citifluor Ltd, 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, Inc.).

In control samples, primary antibodies were omitted and sections were treated with BSA-containing PBS and incubated only with the secondary antibodies.

Biochemical procedures

H. medicinalis tissues from the unstimulated body wall or from injected or wounded areas were frozen in liquid nitrogen and then homogenized with a mortar. For SDS-

polyacrylamide gel electrophoresis (SDS-PAGE), leech homogenates were suspended in extraction buffer 2X Laemmli's Buffer in the presence of a protease inhibitor cocktail (Sigma, Milan, Italy); the particulate material was removed by centrifugation at 13000 rpm for 10 min at 4°C in a refrigerated Eppendorf Minispin microcentrifuge. Supernatants were denatured at 100°C for 10 min.

SDS-PAGE

Proteins were separated in analytical SDS-PAGE using 10% acrylamide minigels. Molecular weights were determined by concurrently running broad range standards from Bio-Rad (Bio-Rad, Richmond, MA, 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, 20mM Tris-HCl buffer, 500mM NaCl, pH 7.5) at room temperature for 2 hr, and incubated for 90 minutes with a rabbit anti-leech AIF-1 antibody (1:5000 dilution in 5% TBS-milk) or rabbit polyclonal anti-human CD45 IgG (Twin Helix) diluted 1:1000. After washing the membrane three times with TBS-Tween 0,1%, the antigens were revealed with the secondary anti-rabbit IgG antibody HRP-conjugated (Jackson ImmunoResearch Laboratories, Inc., West Grove, USA), diluted 1:5000. After washing, the

immunocomplexes were revealed with luminol LiteAblot® PLUS Enhanced Chemiluminescent Substrate (EuroClone S.p.A., Pero, Italy).

Bands were normalized, using the ImageJ software package (http://rsbweb.nih.gov/ij/download.html), with the housekeeping protein GAPDH, which were 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 relatively to control uninjured animals. Experiments were performed in triplicate and data represent the mean values ± SEM. Statistical significance was assessed by an unpaired Student's t test.

RESULTS

The role of *Hm*AIF-1 in immune response has been evaluated in leeches differently stimulated: 1) bacterial injection, 2) injury, 3) grafts, 4) injected with the recombinant protein r*Hm*AIF-1.

1) BACTERIAL INJECTIONS

Ultrastructural and enzyme histochemical analyses in bacterial challenged leeches

The uninjured leech body wall was essentially made up of an epithelium wrapping grouped muscle fibers embedded in a loose connective tissue. In normal uninjured body wall, few cells were observable immerse in the connective tissue surrounding the muscle fibers and underneath the epithelium (Fig. 1).

The enzymatic histochemical assay highlighted, in *M. nishinomiyaensis* or LPS challenged leech body wall cryosections, numerous cells under the epithelium and among muscle fibers characterized by a strong positivity for the acid phosphatase (ACP) reaction (Fig. 2a, b), which level increased during phagocytosis process (Räisänen et al., 2001). By contrast, the ACP signal was reduced in PBS or *A. hydrophila* injected leeches (Fig. 2c).

Ultrastructural examination at TEM of immune-challenged animals showed the presence of migrating macrophage-like cells among muscles fibers. A greater number cells was visible in tissues stimulated with the pathogen Gram-positive *M. nishinomiyaensis* or with LPS (Fig. 2d, e, g, h), compared to those stimulated with the symbiont Gram-negative bacterium *A. hydrophila* (Fig. 2f) or PBS-injected (Fig. 2i). The last tests were performed to unambiguously link the large increase in migrating cells with the immune activation of LPS and not with the injection of a solution.

*Hm*AIF-1 expression in uninjured and bacterial challenged leeches

It has been previously reported that AIF-1 is significantly up-regulated in response to microbes infection both in vertebrates (Miyata et al., 2001; Wang & Wu, 2007) and in invertebrates (Zhang et al. 2011; Wang et al., 2013). To determine whether the same effect was visible in the leech, we performed immunohistochemical analysis using a specific *Hm*AIF-1 antibody on cryosections from uninjured, 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 uninjured animals (Fig. 3a-c). This factor was mainly expressed in cells located in the connective tissue underlying the body wall epithelium and surrounding the fields of muscle fibers. A similar pattern was observed in samples analyzed 6 hours after PBS treatment, indicating that the mechanical stress induced by the injection or the vehicle solution alone did not exert a significant effect on *Hm*AIF-1 expression in the body wall of challenged animals (Fig. 3d-f). Similarly, the injection of heat killed *A. hydrophila* appeared ineffective respect to the presence of *Hm*AIF-1^{+} cells in the challenged area (Fig 3g-i). Interestingly, 6 hours after *M. nishinomiyaensis* (Fig. 3j-l) or LPS injection (Fig. 3m-o), 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 fibers fields. No signal was detected in negative controls experiments where the primary antibody was substituted with the rabbit pre-immune serum (Fig. 3c) or where the primary polyclonal anti-*Hm*AIF-1 antibody was omitted and sections were incubated only with the secondary antibody (Fig. 3f, i, l, o).

The expression profile of *Hm*AIF-1 in uninjured, PBS and bacterial challenged animals was confirmed by Western blot assay (Fig. 4). According to the results obtained on

leech CNS (Drago et al., 2014), immunoblotting analysis on body wall protein extracts confirmed the presence of an immune-reactive product at about 18 kDa (Fig. 4a). As observed in immunohistochemistry analysis, the amount of *Hm*AIF-1 protein did not significantly change 6 hours after PBS or *A. hydrophila* injection respect to the basal expression level, but highly increased upon *M. nishinomiyaensis* or LPS challenge (Fig 4b). GAPDH was used as internal reference and bands intensity appeared homogeneously distributed in the loaded samples (Fig. 4a). As previously reported (Drago et al., 2014), no specific signals were observed on the negative control experiments performed using primary rabbit pre-immune serum (data not shown).

Double immunolocalization of *Hm*AIF-1, leukocyte and macrophage markers in bacterial challenged leeches

To better characterize the resident and migrating cells acid phosphatase positive and expressing *Hm*AIF-1 described above, we performed double-staining experiments coupling anti-*Hm*AIF-1 to antibodies directed against the common human leukocytes marker CD45 and the specific human macrophage marker CD68 (Fig. 5). In PBS or *A*. *hydrophila* injected leeches, several *Hm*AIF-1^+/$CD45^+$ (Fig. 5a, e) or *Hm*AIF-1^+/$CD68^+$ (Fig. 5b, f) and

$CD45^+/CD68^+$ (Fig. 5c, g) were more concentrated in the sub-epithelial area of the body wall, while among the muscle fibers a few resident immunocompetent cells *Hm*AIF-$1^+/CD45^+$ (Fig. 5a, e) or *Hm*AIF-$1^+/CD68^+$ (Fig. 5b, f) and $CD45^+/CD68^+$ (Fig. 5c, g) were visible. A higher amount of cells gathered at the challenged area was observed 6h after the treatment with *M. nishinomiyaensis* (Fig. 5i-k) or LPS (Fig. 5m-o). Numerous $CD68^+/CD45^+$ were visible underneath the epithelium and migrating among the muscle fibers (Fig. 5k, o). These cells also expressed *Hm*AIF-1 (Fig. 5i, j, m, n) and it constitutes the first report of a macrophage-specific marker in leech. Moreover our data were in accordance with previous reports on Vertebrates (Trowbridge & Thomas, 1994; Roach et al., 1997), indicating that CD45 is implicated in macrophages differentiation, activation and migration.

Control experiments performed in the absence of the anti-*Hm*AIF-1 primary antibody (Fig. 5d, h) or with the pre-immune serum (Fig. 5l, p), were negative for all the samples.

The expression profile of CD45 in uninjured, PBS and bacterial challenged animals was also confirmed by Western blot assay (Fig. 6). Immunoblotting analysis on body wall protein extracts confirmed the presence of an immune-reactive product of 145 kDa (Fig. 6a). This

molecular weight corresponds to that of the murine counterparts. As observed in immunohistochemistry analysis, the amount of CD45 protein highly increased especially upon LPS challenge in respect to the basal expression level or 6 hours after PBS or *A. hydrophila* injection (Fig. 6b). As already described above, GAPDH was used as internal reference (Fig. 6a).

2) WOUND HEALING

Ultrastructural and enzyme histochemical analyses in wounded leeches

As already described in previous works (Grimaldi et al., 2004; Tettamanti et al., 2004; Grimaldi et al., 2006), following tissue damage, wound healing initiates with an inflammatory phase, characterized by a massive migration of immune cells, fibroblasts and myofibroblast-like cells towards the injured area. Wound repair and retraction was then obtained by the formation of a pseudoblastema region formed by the myofibroblast-like cells (Huguet & Molinas, 1994; Huguet & Molinas, 1996; Grimaldi et al., 2009; Grimaldi et al., 2011). Analysis of injured tissues at TEM highlighted the presence of numerous macrophages like cells in the connective tissue close to the pseudoblastema region. These cells were highly positive

for ACP reaction, confirming their macrophage activity (Fig. 7a-h).

*Hm*AIF-1 expression in wounded leeches

Sections of both uninjured, (Fig. 3a-c) and injured leeches analyzed after 24 h, 48 h, 72 h and 7 days from lesion (Fig. 8a-l), were immunostained with antibodies against *Hm*AIF-1. Cells expressing *Hm*AIF-1 were found dispersed in the extracellular matrix (ECM) surrounding the groups of muscle cells in the control uninjured animals (Fig. 3a-c) and close to the wound healing region of injured leeches (Fig. 8a-l). Our data showed that *Hm*AIF-1 expression dramatically increased in injured leeches, when a massive migration of cells towards the injured area was detectable (Fig. 8b, e, h, k). No signal was visible in negative controls experiments, in which primary antibody was omitted (Fig. 8c, f, i, l).

Western blot assay was performed to assess the expression profile of *Hm*AIF-1 in wounded leeches (Fig. 9a, b). Compared to the basal expression level detected in uninjured leeches, the amount of *Hm*AIF-1 protein significantly change in extracts of injured leech body wall, showing a peak of expression at 48 h from injury (Fig. 9b). GAPDH was used as internal reference (Fig. 9a).

Double immunolocalization of *Hm*AIF-1, leukocyte and macrophage markers in wounded leeches

Double immunofluorescent staining were then performed on cryosections of 24 h, 48 h, 72 h and 7 days injured leeches body wall using the following primary antibodies combination: *Hm*AIF-1/CD45, *Hm*AIF-1/CD68, CD45/CD68. Our data showed, in all sections, that the same cells expressing *Hm*AIF-1 factor and dispersed in the ECM surrounding the groups of muscle fibers (Fig. 10a, b) and close to the wound healing region (Fig. 10d, e) expressed also the common leukocyte marker CD45 and the macrophage cell markers CD68. Immunogold experiments confirmed the expression of *Hm*AIF-1 in $CD45^{+}/CD68^{+}$ macrophage-like cells (Fig. 10g-i).

Control experiments performed in the absence of the primary antibodies were negative for all the samples (Fig. 10c, f).

The expression profile of CD45 was analyzed by Western blot assay (Fig. 11a, b). Compared to the basal expression level detected in uninjured leeches, the amount of CD45 protein significantly change in body wall extracts of 48 and 72 h lesioned leech (Fig. 11b). GAPDH was used as internal reference and bands intensity appeared homogeneously distributed in the loaded samples (Fig. 11a).

3) GRAFTS

*Hm*AIF-1 expression in grafted leeches

Our previous data demonstrated that self-transplantation caused no rejection but only an inflammatory response, whereas host Hirudo leeches rejected both allo- and xenografts (Tettamanti et al., 2003a). In this work, we focused on a possible role of *Hm*AIF-1 in the rejection processes. Since *H. medicinalis* respond to allo- and xenografts in identical way, in terms of tissue reaction and cell populations involved, the results here presented were relative only to autografts and allografts experiments.

Leech responses to autograft: the grafted area of leeches was characterized by an acute inflammatory reaction involving cell migration among fields of muscle fibers (Fig 12a). These migrating cells, morphologically and functionally already described as macrophage-like cells (Tettamanti et al., 2003b) positively stained for ACP reaction (Fig. 12b) and showed a low level of *Hm*AIF-1/CD45 expression (Fig. 12c, d).

Leech responses to allograft: starting from 24 h after allograft, an acute inflammatory phase started with migrating immunocompetent cells. These cells, which migrated through the ECM, were involved in clot formation and in graft isolation from neighbouring tissues. In the

timespan of 7 days, non-self grafted tissue was completely surrounded and coated by host cells. Most of these cells were macrophages, which played a pivotal role with their phagocytotic activity directed to remove cell and matrix debris, positively stained for ACP reaction and highly co-expressed CD45 and *Hm*AIF-1 (Fig. 12e-g, i-k, m-o). No signal was detected in control negative experiments of immunolocalization, where primary antibody were omitted.

4) r*Hm*AIF-1 INJECTED LEECHES

Morphological and enzymatic histochemical analyses of leech tissues injected with r*Hm*AIF-1

In order to assess the possible involvement of *Hm*AIF-1 in the regulation of *H. medicinalis* innate immune response and in particular in macrophage activation and migration, we evaluated the effects of the injection of the recombinant protein r*Hm*AIF-1 in the leech body wall at different time points from treatment. At T24 h (Fig. 13d), T48 h (Fig. 13e) and T72 h (Fig. 13f) after r*Hm*AIF-1 injection, a large number of migrating cells, were readily recognizable among the muscle fibers. Interestingly, the migration of these cells was associated to an angiogenic process and to the appearance of a network of novel blood vessels in the space among the fields of muscle (Fig. 13d-f). After 7 days from injection (Fig. 13g), 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) analysis, 48 hours after r*Hm*AIF-1 injection (Fig. 13h), the entire body wall was heavily infiltrated by cells showing the typical features of leeches macrophages previously described (de Eguileor et al., 1999; de Eguileor et al., 2000a). Neovessels, in which lumen-circulating cells were clearly recognizable, were also visible (Fig. 13i). As previously described (Grimaldi et al., 2006), these cells extravasated in the connective tissue and differentiate in mature leukocytes involved in innate immune response of leech. Extravasated hematopoietic precursors cells were in fact visible in the extracellular matrix surrounding the macrophages and muscle fibers (Fig. 13h).

Function blocking experiments were performed by injecting the r*Hm*AIF-1 pre-incubated with the specific anti-*Hm*AIF-1 polyclonal antibody (Fig. 13c). Results showed a reduced infiltration of cells and vessels among the groups of muscle fibers and under the epithelium, as well as for the PBS inoculation and for uninjured leeches (Fig. 13a, b). This confirmed the responsiveness of leech leukocytes and endothelial cells to *Hm*AIF-1.

The enzymatic ACP reaction confirmed the presence of a larger number of migrating cells with macrophage activity

in leeches injected with r*Hm*AIF-1 (Fig. 14d, e) or both r*Hm*AIF and pre-immune serum (Fig. 14f, g), in respect with control uninjured (Fig. 14a), PBS injected (Fig. 14b) and both r*Hm*AIF and anti-*Hm*AIF-1 injected leeches (Fig. 14c).

Double immunolocalization of *Hm*AIF-1, leukocyte and macrophage markers in r*Hm*AIF-1 injected leeches

In order to characterize the cell types recruited at the site of r*Hm*AIF-1 injection and to confirm our hypothesis that *Hm*AIF-1 influences the mobilization of leech macrophages, double staining experiments were performed on cryosections of tissues collected 24 hours after injection using anti-*Hm*AIF-1, anti-CD68 and anti-CD45 antibodies. Both the experiments confirmed the accumulation of macrophage-like cells, positive for both CD45 and CD68, at the injection site of the recombinant protein (Fig. 15a). 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. 15b). This data indicated that r*Hm*AIF-1 not only promoted the migration of $CD45^{+}/CD68^{+}$ macrophages, but also exerted a positive feedback effect on recruited cells inducing the expression of the factor.

Function blocking experiments showed a reduced number of macrophages co-expressing both the two markers CD45 and CD68 in the body wall of leeches injected with both the r*Hm*AIF-1 and the anti-*Hm*AIF-1 (Fig. 15c). Furthermore, the number of cells expressing *Hm*AIF-1 upon the same experimental conditions was highly reduced and the $CD45^+$ cells were *Hm*AIF-1 negative (Fig. 15d), 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 the samples (data not shown).

Anti-CD45 polyclonal antibody treatment

To determine a possible role of CD45 on macrophages responsiveness to *Hm*AIF-1, we injected the r*Hm*AIF-1 together with the anti-CD45 antibody. Morphological (Fig. 15e) and immunohistochemical analysis (Fig. 15f, g) showed that the anti-CD45 treatment inhibited the *Hm*AIF-1-induced responses described above, reducing the number of macrophages $CD45^+/CD68^+$ gathered at the injection site. In addition, double immunohistochemical experiments showed that CD45 expression was inhibited in the body wall of r*Hm*AIF-1/anti-CD45 injected leeches

(Fig. 15f, g) and only a few resident macrophage $CD68^+$ (Fig. 15f) and *Hm*AIF-1^+ (Fig. 15g) were still detectable, suggesting a direct role of CD45 in migration capability of the *Hm*AIF-1-activated macrophages.

DISCUSSION

In both invertebrates and vertebrates, inflammation is an acute reaction triggered by different types of lesions and aimed to fulfill two functions: a cytotoxic function to kill infected microbes and a repair function to regenerate damaged tissues. It is mediated by specific cells such as macrophages and neutrophils that infiltrate the damaged tissue, removing tissue debris and controlling invading microorganisms. These cells synthesize different molecules such as growth factors and cytokines, inducing mesenchymal cell recruitment in the injured or infected area (Jeong et al., 2013).

In leeches as well proliferation and migration of immune cells are associated to important effects as angiogenesis and fibroplasia (Tettamanti et al., 2004; Grimaldi et al., 2006) and in the present study we demonstrate that in the leech *H. medicinalis* the inflammatory factor *Hm*AIF-1 is involved in the regulation of innate immune response by inducing angiogenesis and macrophages migration. Our results showed that, in leeches, the recombinant *Hm*AIF-1 has a strong chemotactic activity and in particular, it promotes macrophages and vessels migration towards the stimulated area, showing a similar function to AIF-1 of Vertebrates (Yamamoto et al., 2011). Moreover, here we show that in leeches, like in Vertebrates, this molecule is

constitutively expressed in untreated animals, but it is dramatically enhanced after microbial infection, mechanical injury or grafts.

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

Even if AIF-1 was originally cloned from active macrophages in human and rat atherosclerotic allogeneic heart grafts undergoing chronic transplant rejection (Utans et al., 1995), it has been later demonstrated that its transcripts were up-regulated in Vertebrates during macrophage and fibroblast migration (Yamamoto et al., 2011), in wound healing and in angiogenesis (Autieri et al., 2000; Autieri & Carbone 2001; Tian et al., 2009), both in Vertebrates and invertebrates in leukocytes upon LPS stimulation (Miyata et al., 2001; Zhang et al., 2011), and also in allograft rejection in sponges (Kruse et al., 1999). These results clearly suggest that AIF-1 could play a key role not only in different host responses to inflammatory stimuli, but also in the whole host immune defense reaction. We then hypothesized that *Hm*AIF-1 could be involved in innate immune response in leeches as well and that its expression would be enhanced in a context of bacterial infections, wound healing and grafts. For this

reason, we performed LPS, *A*. *hydrophila* and *M. nishinomiyaensis* injections, surgical wounds and grafts.

First of all we investigated the tissue-specific and temporal expression profile of *Hm*AIF-1 factor after challenging by the environmental bacteria *A*. *hydrophila,* a Gram-negative bacterium present in bacterial flora where plays an essential role in the digestion of blood (Graf et al., 2006; Patel et al., 2013), and *M. nishinomiyaensis*, a Gram-positive bacterium pathogen for leeches (Schikorski et al., 2008). Finally, we also injected a lipopolysaccharides (LPS) solution, in order to test the effect on *Hm*AIF-1 expression induced by a pathogenic Gram-negative bacterium infection. The action of LPS on leech tissues is well known and already described in previous works. Leeches pricked and injected with LPS showed a large amount of migrating $CD68^{+}$ macrophages characterized by pseudopodia, phagolysosomes in their cytoplasm and a strong lysosomial activity (de Eguileor et al., 2000a, b).

Leeches challenged with the *M. nishinomiyaensis* or stimulated with LPS showed a larger amount 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^{+} and in $CD45^{+}$ cells was caused by the immune

activation of LPS or Gram-positive bacterium challenge and not by the mechanical stress linked to the injection or to the presence of symbiont bacteria. The reason for the lower expression of CD45 than *Hm*AIF-1 in *M. nishinomiyaensis* injected leeches could be explained by the greater efficacy against Gram-positive bacteria of the antimicrobial proteins in *H. medicinalis* saliva and tissues.

Characterization of migrating cells was achieved by ultrastructural analysis, acid phosphatase reaction and immunohistochemistry using polyclonal antibodies directed against human macrophage and leukocytes markers CD68 and CD45. The use of antibodies generated against mammalian CD antigens to detect leukocytes and macrophages in leech is supported by several data from the literature on leeches (de Eguileor et al., 2000a; Grimaldi et al., 2004; Grimaldi et al., 2006) and on 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, is expressed in activated macrophages. It constitutes the first report of a macrophage-specific marker in leech. Numerous CD68^{+} and CD45^{+} macrophages were mainly detected in animals injected

with LPS or with the pathogen *M. nishinomiyaensis*. The ultrastructural morphology and acid phosphatase reaction positivity of migrating cells give further confirmation of the macrophage identity of migrating cells. It confirms that, like in Mammals, the immune challenge induces in leech body wall the migration and the accumulation of macrophage-like cells at the injected site. Moreover, *M. nishinomiyaensis* and LPS caused remarkable inflammatory effects, inducing a massive angiogenesis and macrophages *Hm*AIF-1^{+}/CD45^{+}/CD68^{+} migration. On the other hand, the effects of *A. hydrophila* were similar to those of PBS injection. These bacteria, being symbionts of leech digestive tract, probably did not induce a strong immune response even if injected in a different area of leeches body. The observed differences in reactivity to bacteria and LPS injections could be related to a different macrophage response induced by Gram-negative and Gram-positive bacterial infection. It is in fact known that leeches macrophages produce antimicrobial proteins and peptide, such as *Hm*-theromacin and destabilase, that cleave the cell wall components of Gram-positive bacteria digesting the β-1,4-glycosidic peptidoglycan links (Schikorski et al., 2008; Hildebrandt & Lemke, 2011), thus resulting more effective on Gram-positive than on Gram-negative bacteria. Therefore, *M. nishinomiyaensis* may

cause lower cell migration than that caused by Gram-negative.

This immune challenge give rise to an overview consistent with that obtained after wounding (de Eguileor et al., 2003; Tettamanti et al., 2003b; Grimaldi et al., 2004; Grimaldi et al., 2006), in agreement with the fact that the wound healing process, in *H. medicinalis,* is enhanced by a controlled bacterial infection. It suggests that, in leech, the immune response is based on the same molecules involved in wound healing and regenerative process (Schikorski et al., 2008).

Indeed, we found high level of *Hm*AIF-1 expression in the tissue of injured and grafted leeches. In particular we noted a significant increase of *Hm*AIF-1^{+} macrophage like cells migrating towards the injured area or forming a clot around the not-self tissue after 24-48 hours from surgery, while the expression level of this factor significantly decreases 7 days after injuries. Taken together, these findings suggest that besides cell-mediated defense reactions a cytokine-dependent immune response is also elicited during wound healing and graft recognition and rejection in leeches.

Our results are in perfect agreement with what previously seen both in vertebrates (Autieri et al., 2000) and in invertebrates (Kruse et al., 1999; Zhang et al., 2011),

where a steady increase of *Hm*AIF-1 was mainly detected in the initial stages of inflammation, 24-48 hours after surgery, and decline by 7-10 days after. Therefore, our data seem to confirm that, both in vertebrates and invertebrates, AIF-1 is involved in events that trigger inflammation more than in the progression of it (Autieri et al., 2000) and that this molecule has been highly evolutionarily conserved in both amino acid sequence and function.

***Hm*AIF-1 is involved in the recruitment and activation of leech macrophage-like cells**

The relationship between *Hm*AIF-1 and the functional responsiveness of macrophages was assessed by injecting the recombinant factor in the body wall of leeches.

We observed, 6 hours after r*Hm*AIF-1 administration, the formation of an extensive vessel network spanning through the entire avascular body wall of the animal and the accumulation of numerous migrating cells localized among the fields of muscle fibers. Indeed, the injection of the recombinant protein together with a function blocking anti-*Hm*AIF-1 antibody highly reduced angiogenesis and cells migration. Moreover, morphological analysis showed that macrophages recruitment induced by r*Hm*AIF-1, was

higher in the first 48 hours from injection and decreased starting from 72 hours to 7 days after treatment. These data are in accordance with the reports from Vertebrates, where AIF-1 is implicated in macrophages activation at the early stages, rather than in late steps of the inflammatory process (Autieri et al., 2000; Deininger et al., 2000; Deininger et al., 2002; Zhang et al., 2011). In particular, 24 hours after r*Hm*AIF-1 injection, a high positivity to ACP reaction, as well as immunoreactive signal, was detected in the cytoplasm of migrating cells, mostly of which were $CD68^+$ and $CD45^+$ macrophages. In addition, the r*Hm*AIF-1 injection not only promoted the migration of $CD45^+/CD68^+$ macrophages, but also induced an enhancement of *Hm*AIF-1 expression in the recruited cells. It suggests that macrophages, once chemoattracted in the stimulated area, express this factor to sustain the recruitment of further macrophages or to maintain their accumulation at the injured site. Despite of this positive feedback in *Hm*AIF-1 expression, its mechanisms of action on target cells remains largely unexplored.

The $CD68^+$ macrophages co-express also the leukocyte common antigen CD45, sharing 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 (Roach et al., 1997). Our data showed that the injection of r*Hm*AIF-1 pre-incubated with specific anti-*Hm*AIF-1 antibody reduced the number of migrating macrophages, most of which do not longer co-express the CD45 and CD68 markers, unlike what is observed with pre-immune serum. Since it has been reported that CD45 regulates chemokine receptor expression in myeloid leukocytes and that it can modulate leukocyte traffic by regulating responses to chemokines (Mitchell et al., 1999), we hypothesize that CD45 expression in leech might be implicated in maturation and functional responsiveness of macrophages to *Hm*AIF-1. Indeed, the injection of the r*Hm*AIF-1 together with the anti-CD45 polyclonal antibody reduced the macrophages migration. Although further experiments will be necessary, this result let hypothesize that the recruitment of macrophages is induced by a close interaction between *Hm*AIF-1 and CD45 protein expressed at the surface of responsive cells, as indicated by previous work on Vertebrates (Yamamoto et al., 2011).

Concluding remarks

Results here presented show that the expression of the *Hm*AIF-1 significantly increases during the early phases of the inflammatory response and it is mainly exerted by activated macrophages. We demonstrate for the first time that *Hm*AIF-1 is not only a potent angiogenic factor, but also a potent chemoattractant for macrophages. During bacterial infections, wound healing and grafts rejection *Hm*AIF-1 might be implicated in the activation of these migrating cells, which role is to clean up the area from bacteria and damaged tissue, and to isolate the not-self grafts from the surrounding tissues. These processes are probably linked to the interaction between *Hm*AIF-1 and CD45 to promote the integrin-mediated adhesion of macrophages to the extracellular matrix. Taken together these data indicate that leeches, sharing with vertebrates several morphofunctional and molecular mechanisms, can be considered a simple model useful to elucidate the role of AIF-1 in immune response, wound healing and graft rejection.

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Fig. 2: Acid phosphatase reaction and TEM analysis of cryosections and ultra-thin sections from *H. medicinalis* immune challenged by injection of *M. nishinomiyaensis* (**a, d, g**), LPS (**b, e, h**) and *A. hydrophila* (**c, f, i**). Numerous ACP positive macrophages cells (arrowheads) were visible among the muscular fibers (M) and underneath the epithelium (E) in *M. nishinomiyaensis* (**2a**) and LPS (**2b**) injected leeches. In *A. hydrophila* (**c**) injected animals a few ACP positive cells are detected. (**d-i**) Detail at TEM of macrophages migrating toward the epithelium (**d, e, g, h**) and in the extracellular matrix surrounding muscle fibers (**f, i**). Bar in a: 100 μm; bars in b, c: 50 μm; bars in d, g, h, i: 2 μm; bars in e, f: 5 μm

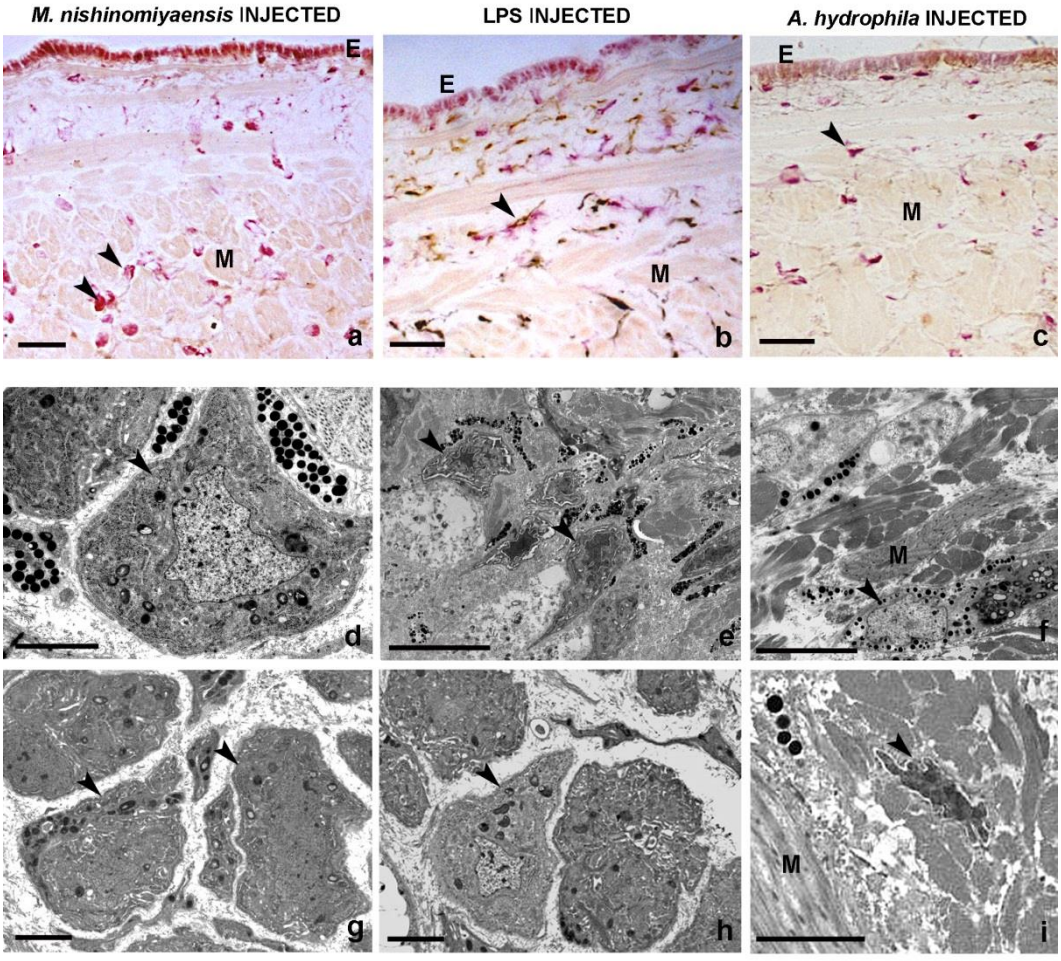

M. nishinomiyaensis INJECTED
LPS INJECTED
A. hydrophila INJECTED
E
M
a
b
c
d
e
f
g
h
i

Fig. 3: Morphological (optical microscopy) and immunohistochemical (fluorescence microscopy) analysis of cryosections from *H. medicinalis* body wall uninjured (**a-c**), PBS injected (**d-f**) or immune challenged by injection of *A. hydrophila* (**g-i**), *M. nishinomiyaensis* (**j-l**) and LPS (**m-o**). Arrowheads indicate the population of resident and migrating immune-responsive cells located under the epithelium (E) and among the muscular fibers (M). Immunohistochemistry was performed using a rabbit polyclonal anti-*Hm*AIF-1 antibody (red); nuclei were counterstained with 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 in a-i: 100 μm; bars in j-o: 50 μm

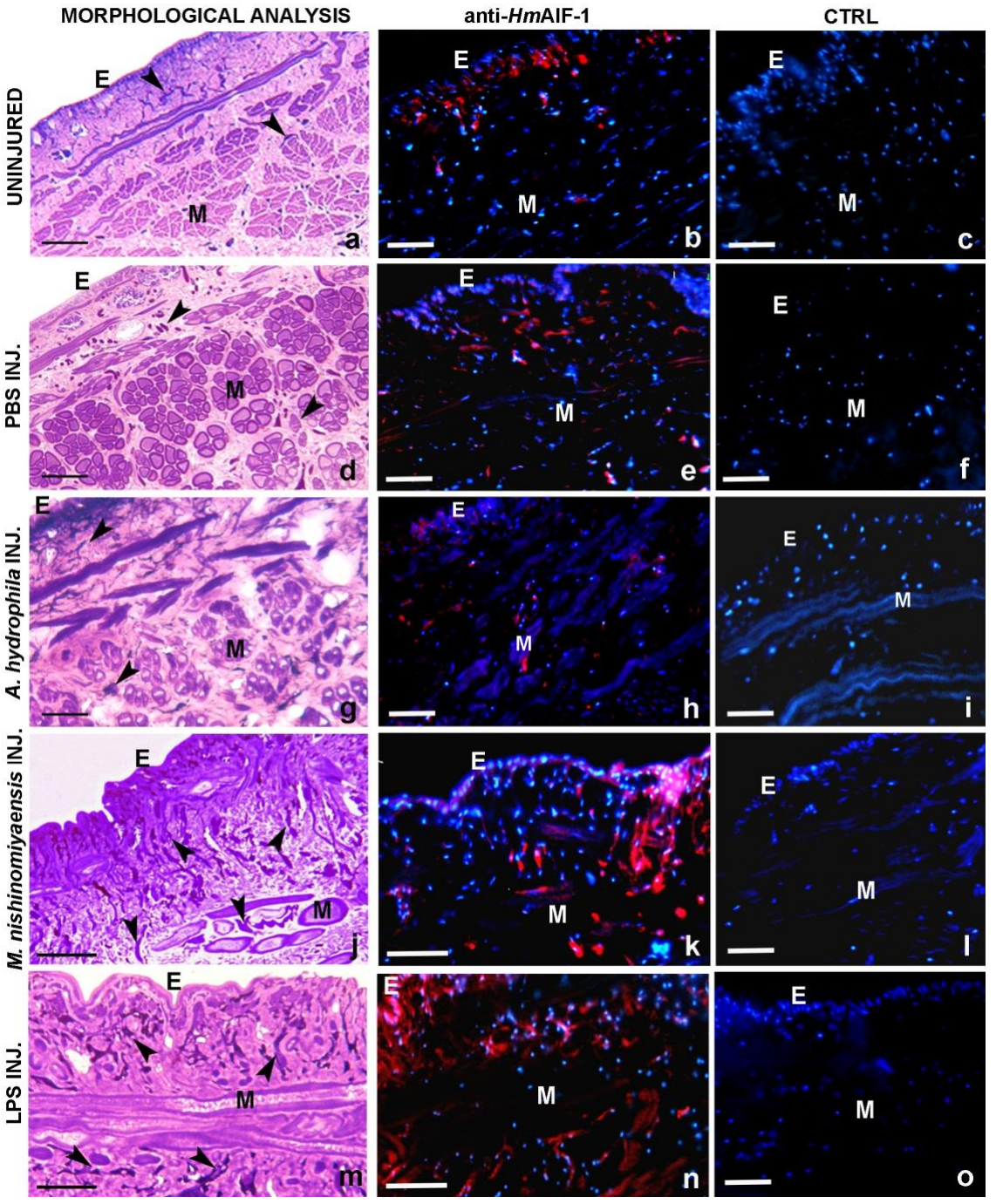
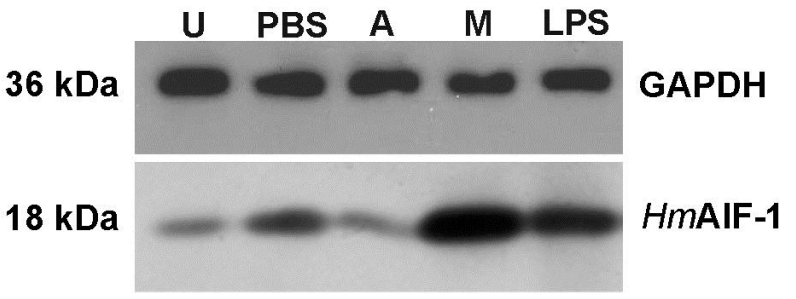
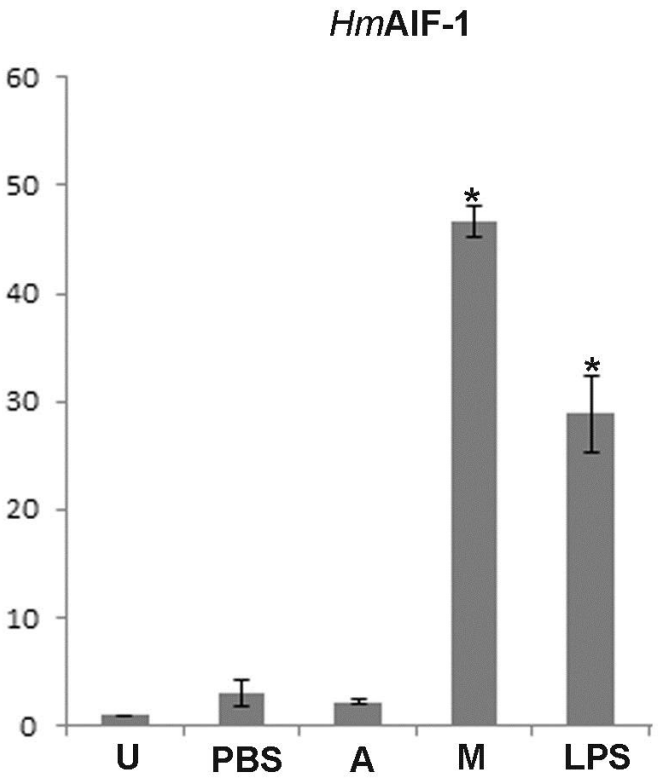

MORPHOLOGICAL ANALYSIS
anti-*Hm*AIF-1
CTRL
UNINJURED
PBS INJ.
A. hydrophila INJ.
M. nishinomiyaensis INJ.
LPS INJ.
E
M
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o

Fig. 4: Western blot analysis. (**a**) Protein extracts of uninjured (U), PBS, *A. hydrophila* (A), *M. nishinomiyaensis* (M) and LPS injected leeches body walls were probed with the anti-*Hm*AIF-1 antibody. The housekeeping protein GAPDH was used as a loading control. In all the samples, the anti-*Hm*AIF-1 detected specific immunoreactive bands of about 18 kDa, according to the molecular weight ladder. (**b**) *Hm*AIF-1 protein was quantified by densitometry from three experiments. *$p < 0.05$ compared with uninjured leeches.


U
PBS
A
M
LPS
36 kDa
GAPDH
18 kDa
HmAIF-1

a


HmAIF-1
60
50
40
30
20
10
0
*
*
U
PBS
A
M
LPS

b

Fig. 5: Double immunolocalization of *Hm*AIF-1 and macrophages markers on cryosections of control PBS injected (**a-c**) and bacterial challenged (**e-o**) leech body wall. After 6 h from *M. nishinomiyaensis* or LPS injection, numerous *Hm*AIF-1^+/$CD45^+$, *Hm*AIF-1^+/$CD68^+$, $CD68^+$/$CD45^+$ macrophages (in yellow), migrating towards the injected area, are detectable under the epithelium (E) and among the muscle fibers (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 were detected in negative control experiments where primary antibodies were omitted (**d, h**) or performed with primary anti-*Hm*AIF-1 pre-immune serum (**l, p**). Bars in a-d: 100 μm; bars in e-i, k-m, o, p: 50 μm; bars in j, n: 25 μm

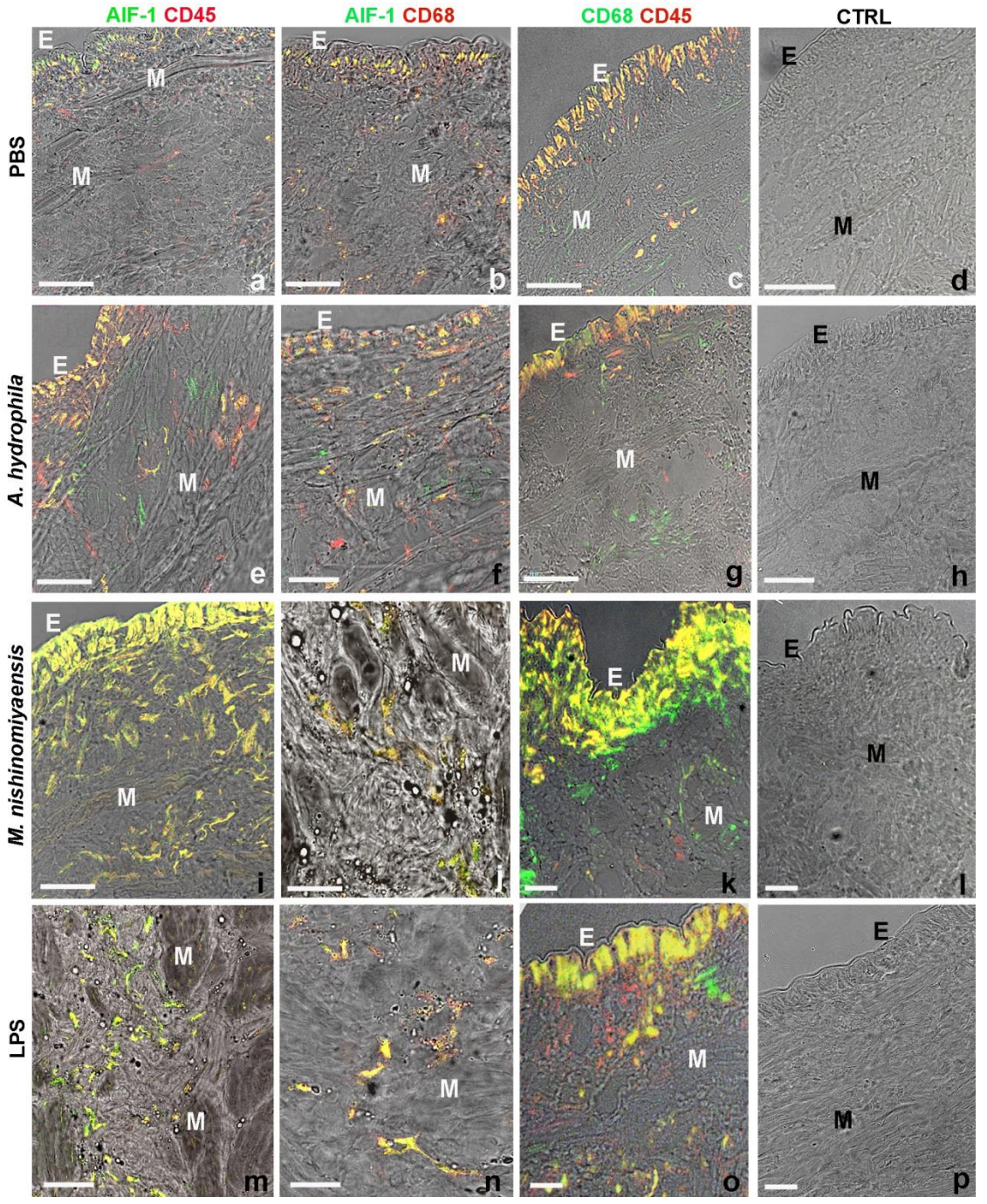
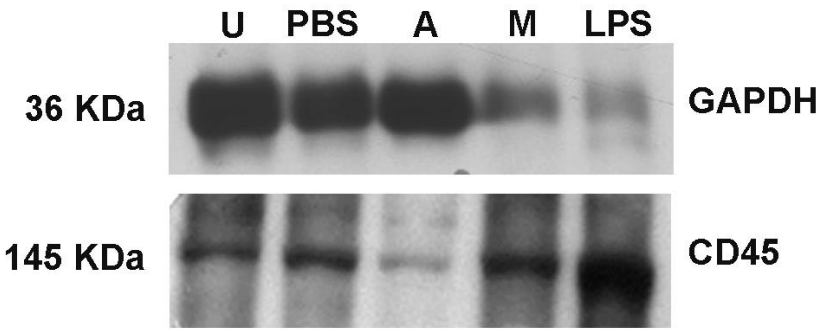
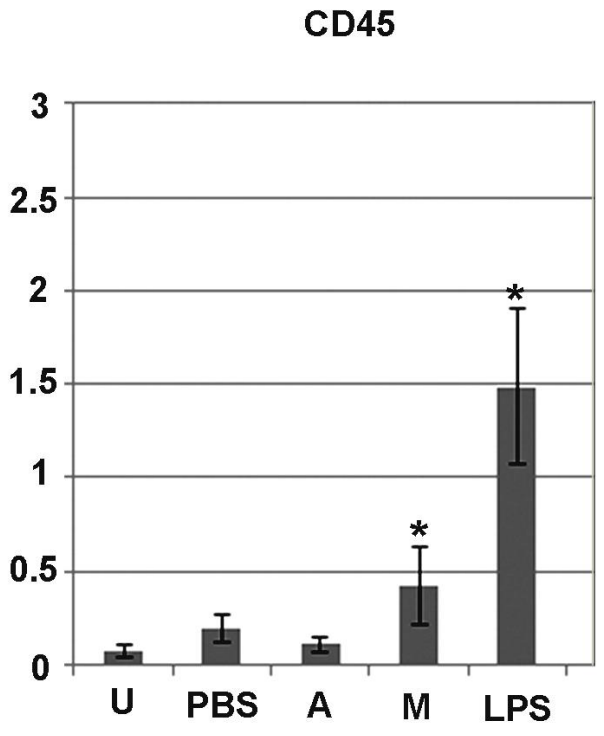

AIF-1 CD45
AIF-1 CD68
CD68 CD45
CTRL
PBS
A. hydrophila
M. nishinomiyaensis
LPS
E
M
a
b
c
d
e
f
g
h
i
l
k
l
m
n
o
p

Fig 6: Western blot analysis. (**a**) Protein extracts of uninjured (U), PBS, *A. hydrophila* (A), *M. nishinomiyaensis* (M) and LPS injected leeches body walls were probed with the anti-CD45 antibody. The housekeeping protein GAPDH was used as a loading control. In all the samples, the anti-CD45 detected specific immunoreactive bands of about 145 kDa, according to the molecular weight ladder. (**b**) CD45 protein was quantified by densitometry from three experiments. *$p<$ 0.05 compared with uninjured leeches.


U
PBS
A
M
LPS
36 KDa
GAPDH
145 KDa
CD45

a


CD45
3
2.5
2
1.5
1
0.5
0
*
*
U
PBS
A
M
LPS

b

Fig. 7: Acid phosphatase reaction and TEM analysis on cryosections and ultra-thin sections from *H. medicinalis* body wall surgically wounded and analyzed after at 24 h (**a, b**), 48 h (**c, d**), 72 h (**e, f**) and 7 days (**g, h**) from injury. Numerous macrophages ACP positive are visible migrating among muscles (M) under the epithelium (E) and close to pseudoblastema (P). Bars in a, c, e, g: 100 μm; bar in b: 4 μm; bars in d, f, h: 2 μm

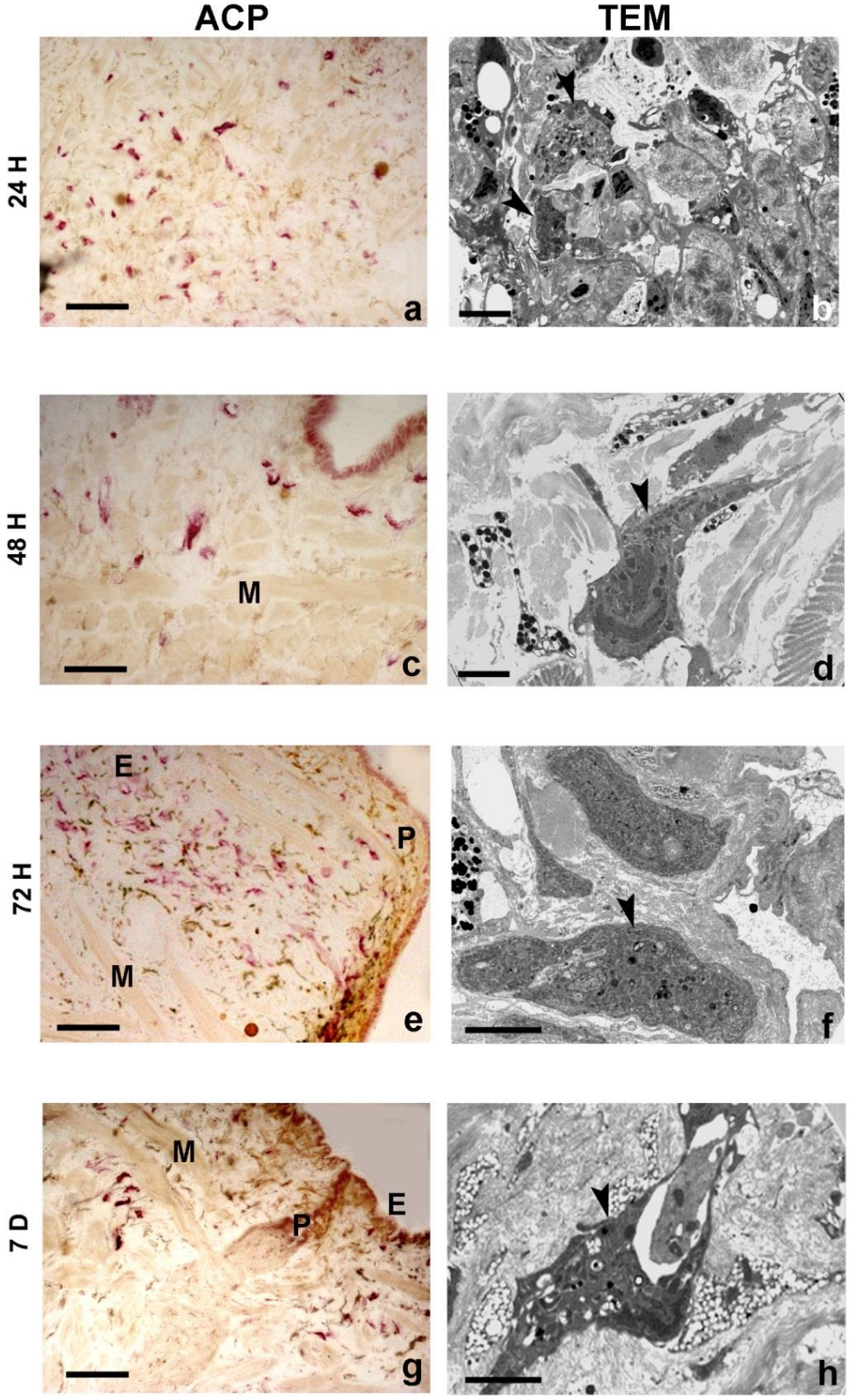

ACP
TEM
24 H
48 H
72 H
7 D
a
b
c
d
e
f
g
h
M
E
P

Fig. 8: Morphological (optical microscopy) and immunohistochemical (fluorescence microscopy) analysis of cryosections from *H. medicinalis* body wall surgically wounded and analyzed after 24 h (**a-c**), 48 h (**d-f**), 72 h (**g-i**) and 7 days (**j-l**) from injury. Numerous migrating cells (arrowheads) among muscle fibers (M) and close to the pseudoblastema (P) were visible. Immunohistochemistry using the rabbit polyclonal anti-*Hm*AIF-1 antibody marks the *Hm*AIF-1^{+} cells, in active migration towards the injured area (arrows). Nuclei were counterstained with DAPI (blue). No signal is detected in control experiment where the primary antibody was omitted (**c, f, i, l**). Bars in a-l: 100 μm

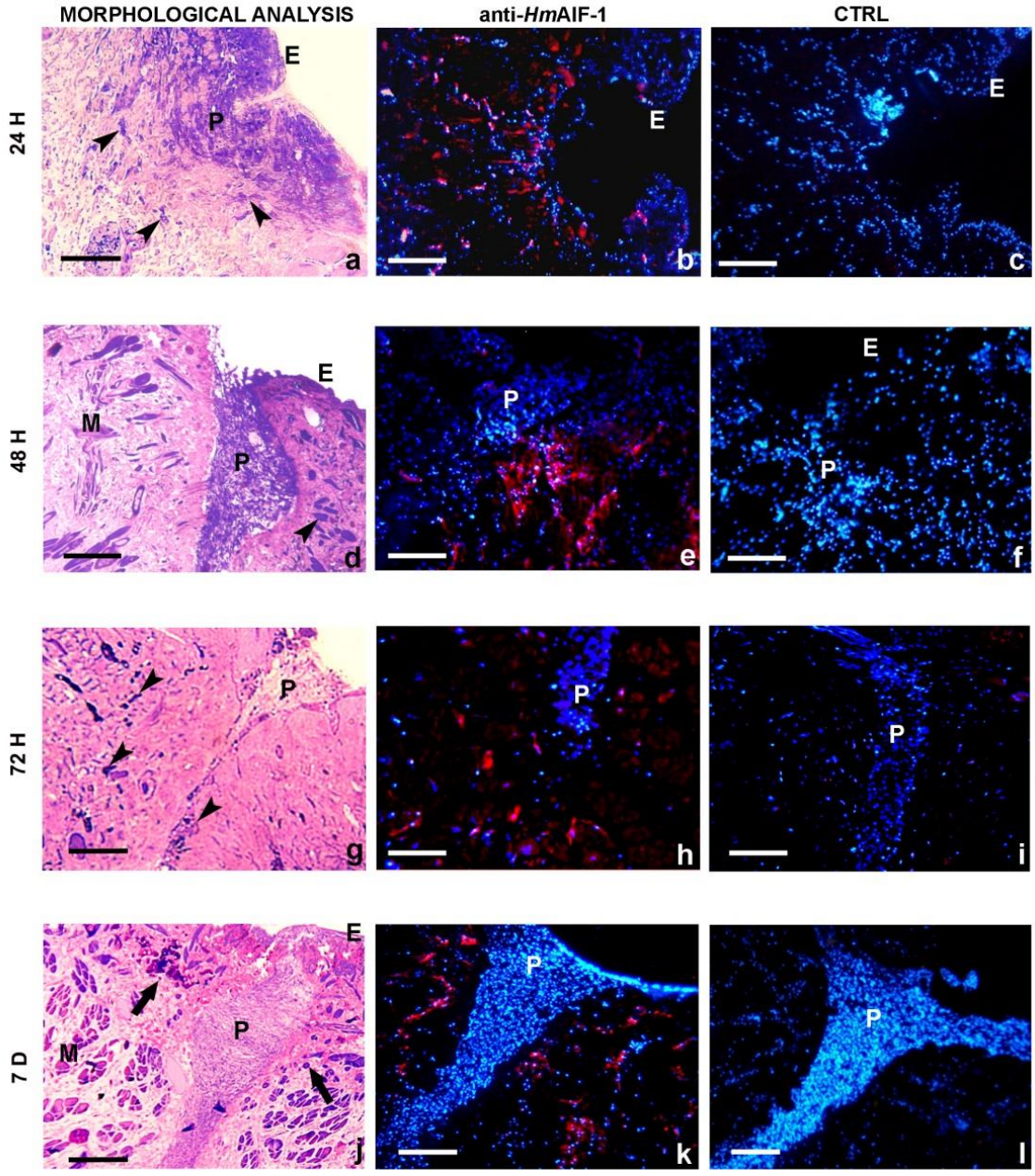
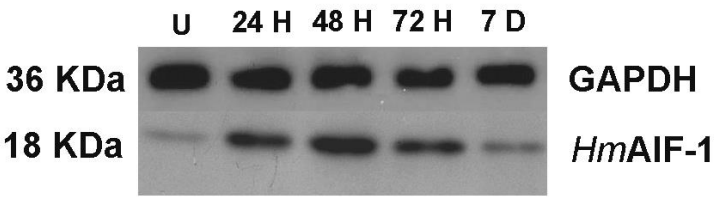
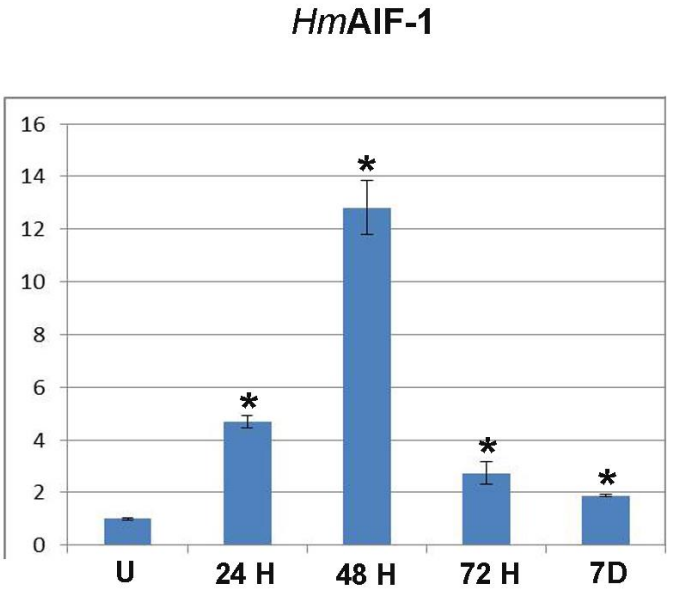

MORPHOLOGICAL ANALYSIS
anti-HmAIF-1
CTRL
24 H
48 H
72 H
7 D
E
P
M
a
b
c
d
e
f
g
h
i
j
k
l

Fig. 9: Western blot analysis. (**a**) Protein extracts of uninjured (U) and injured leeches after 24 h, 48 h, 72 h and 7 days from injury were probed with the anti-*Hm*AIF-1 antibody. The housekeeping protein GAPDH was used as a loading control. In all the samples, the anti-*Hm*AIF-1 detected specific immunoreactive bands of about 18 kDa, according to the molecular weight ladder. (**b**) *Hm*AIF-1 protein was quantified by densitometry from three experiments. *$p < 0.05$ compared with uninjured leeches.


U
24 H
48 H
72 H
7 D
36 KDa
18 KDa
GAPDH
HmAIF-1

a


HmAIF-1
16
14
12
10
8
6
4
2
0
*
*
*
*
U
24 H
48 H
72 H
7D

b

Fig. 10: After injury numerous migrating macrophages (in yellow) located among muscle fibers (**a, b**) and close to the pseudoblastema (P) region (**d, e**) are $CD45^+$/$AIF\text{-}1^+$ and $CD68^+$/$AIF\text{-}1^+$. (**c, f**): negative control experiments where the primary antibodies are omitted. (**g-i**): immunogold staining confirms the expression of AIF-1, CD45 and CD68 in macrophages cells. Bars in a-c: 20 μm; bars in d-f: 50 μm; bar in g: 100 nm; bars in h, i: 500 nm

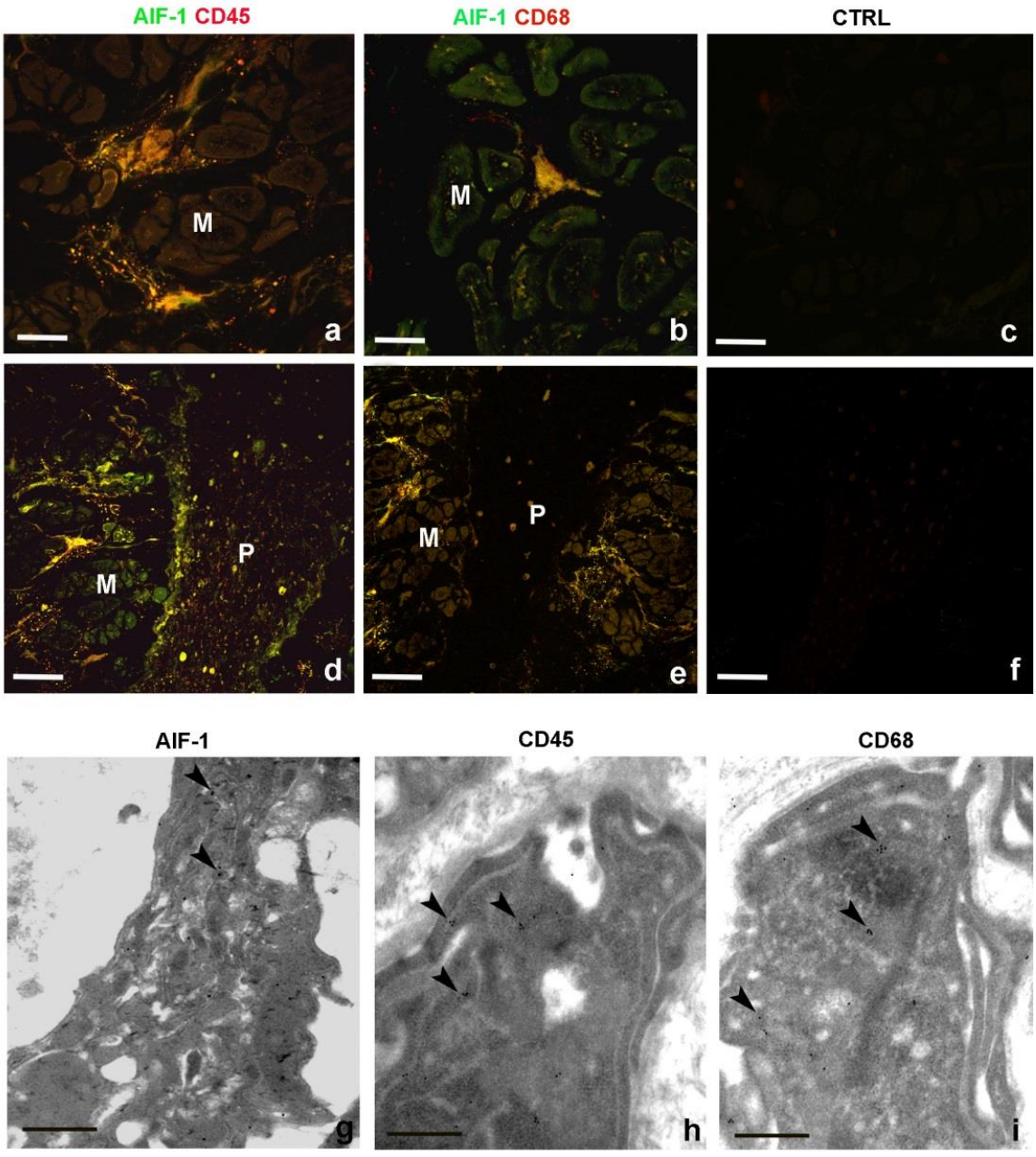
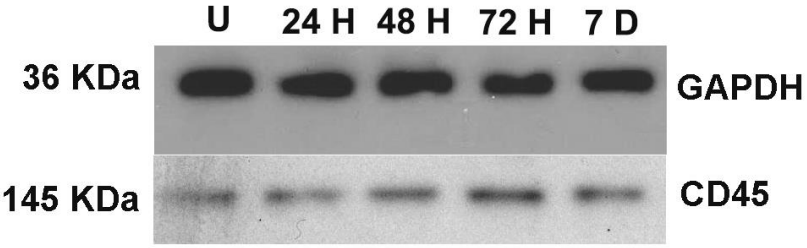
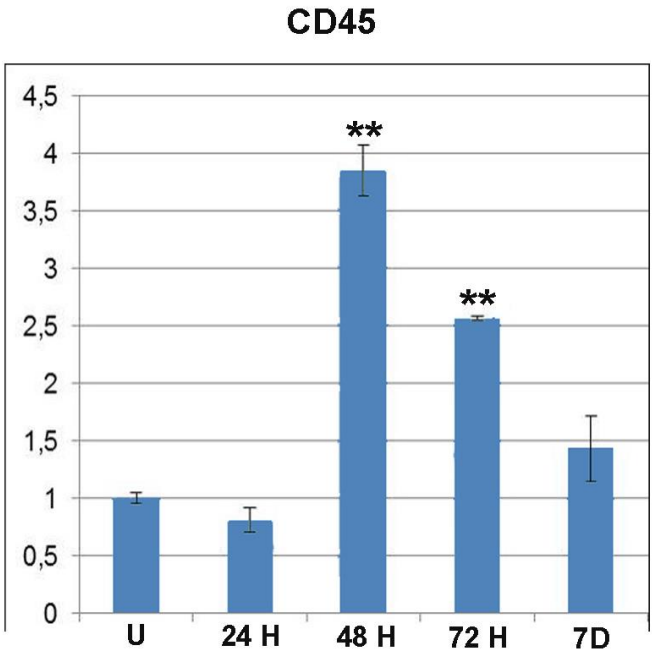

AIF-1 CD45
AIF-1 CD68
CTRL
M
a
M
b
c
M
P
d
M
P
e
f
AIF-1
CD45
CD68
g
h
i

Fig. 11: Western blot analysis. (**a**) Protein extracts of uninjured (U) and injured leeches at 24 h, 48 h, 72 h and 7 days were probed with the anti-human CD45 antibody. In all the samples, the anti-CD45 detected specific immunoreactive bands of about 145 kDa, according to the molecular weight ladder. The housekeeping protein GAPDH was used as a loading control. (**b**) CD45 protein was quantified by densitometry from three experiments. $^{**}p < 0.01$ compared with uninjured leeches.


U
24 H
48 H
72 H
7 D
36 KDa
GAPDH
145 KDa
CD45

a


CD45
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
**
**
U
24 H
48 H
72 H
7D

b

Fig. 12: Morphological (optical microscopy) histochemical enzymatic and immunohistochemical (fluorescence microscopy) analyses of cryosections from grafted *H. medicinalis*. Starting from 24h from autograft (**a-d**) a clot of ACP positive macrophages cells (**b**) surround graft (G). These cells weakly express AIF1 and CD45 (**c, d**). Starting from 24h after allograft (**e, i, m**), the clot of ACP positive macrophages (**f, j, n**) highly co-expressed *Hm*AIF-1 and CD45 (**g, k, o**). No signal is detected in control experiment where the primary antibodies are omitted (**h, l, p**). Bars in a-c: 50 μm; bars in d, e, g, m-p: 200 μm; bars in f, h-l: 100 μm

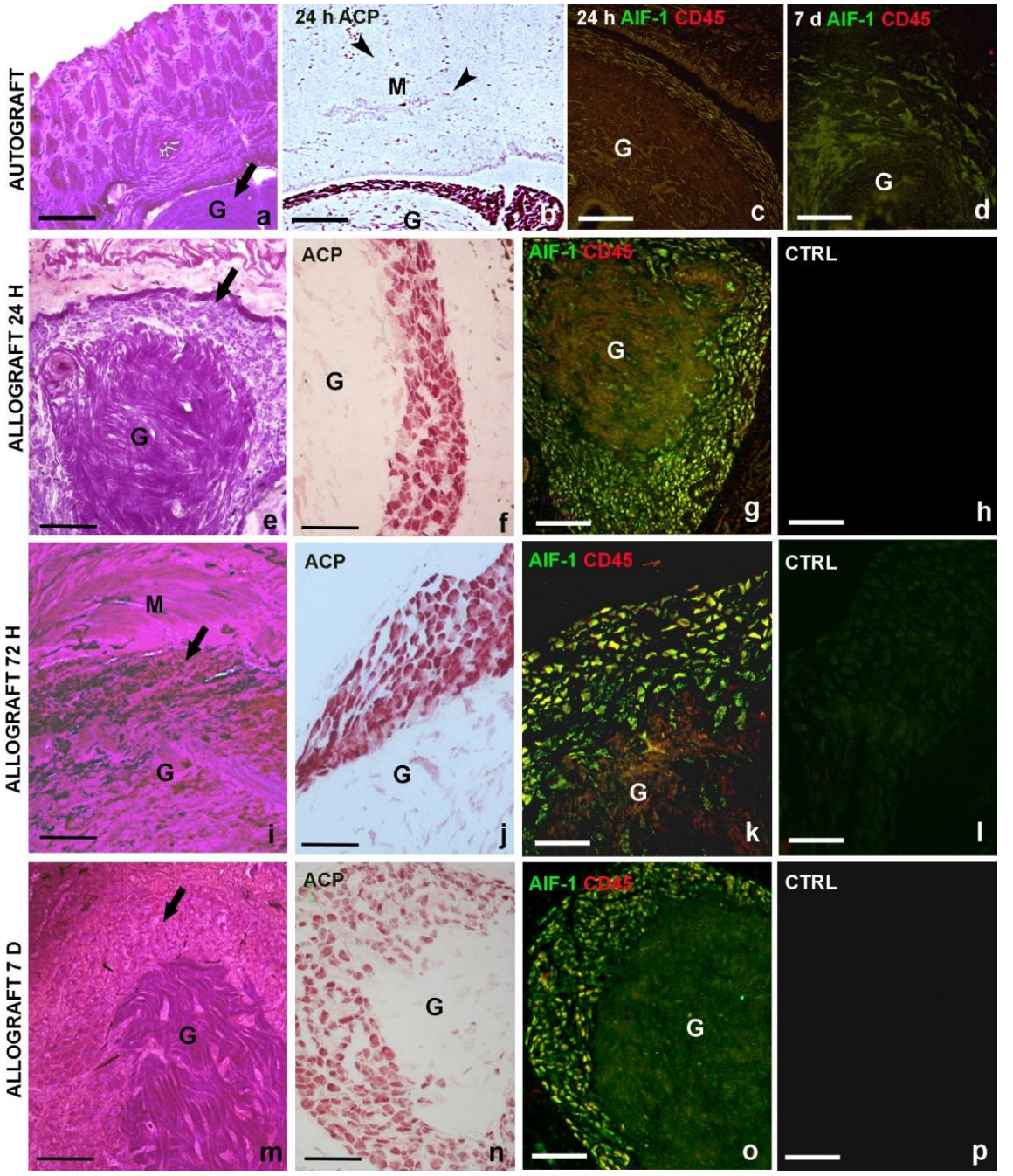

AUTOGRAFT
ALLOGRAFT 24 H
ALLOGRAFT 72 H
ALLOGRAFT 7 D
24 h ACP
M
24 h AIF-1 CD45
7 d AIF-1 CD45
ACP
AIF-1 CD45
CTRL
G
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

Fig. 13: Morphological analysis of leech body wall uninjured (**a**), injected with PBS (**b**), with both r*Hm*AIF-1 and function blocking anti-*Hm*AIF-1 antibody (**c**) or after injection of r*Hm*AIF-1 (**d-i**). After 24 h (**d**) and 48 h (**e**) numerous neo-vessels (arrows) and migrating cell (arrowheads) are visible among muscles (M) and under the epithelium (E). (**g**) After 7 days from r*Hm*AIF-I injection, vessels (arrows) are still visible in the leech body wall. (**h**) Detail at TEM of macrophages (arrowheads) and extravasated hematopoietic precursors cells (arrow) localized in the extracellular matrix (ECM) surrounding the muscle fibers (M). (**i**) Image at TEM of hematopoietic precursors cells (arrowhead) in the lumen of a neo-vessel (arrow). (**a-c**) In uninjured or after 24 h from PBS or r*Hm*AIF-1 + anti-*Hm*AIF-1 antibody injection, a reduced infiltration of cells and vessels among the groups of muscle fibers (M) and under the epithelium (E) is evident. Bars in a-g: 100 μm; bar in h: 2 μm; bar in i: 400 nm

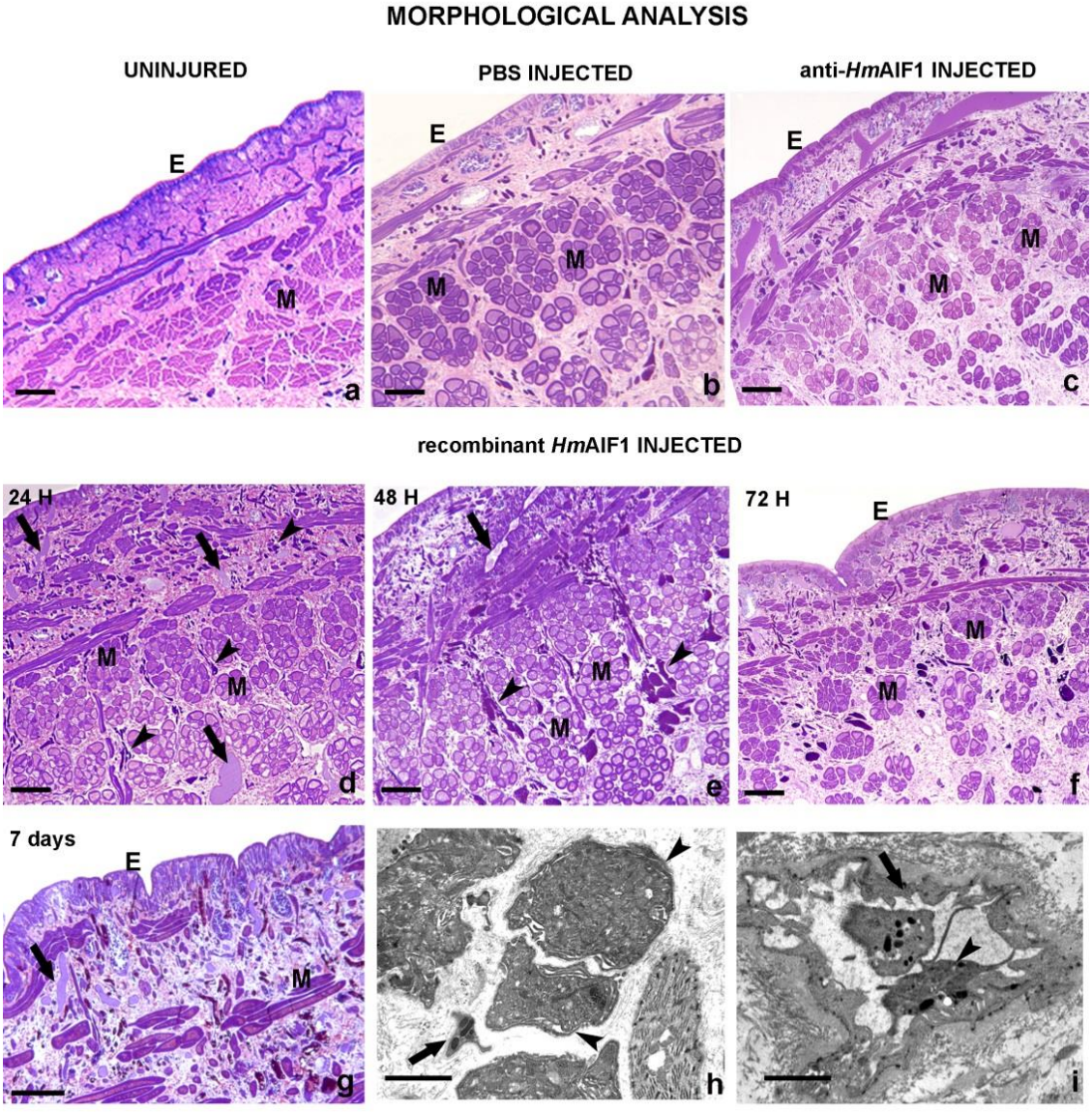

MORPHOLOGICAL ANALYSIS
UNINJURED
PBS INJECTED
anti-HmAIF1 INJECTED
recombinant HmAIF1 INJECTED
24 H
48 H
72 H
7 days
E
M
a
b
c
d
e
f
g
h
i

Fig. 14: ACP reaction on cryosections from uninjured (**a**), injected with PBS (**b**), with both r*Hm*AIF-1 and function blocking anti-*Hm*AIF-1 antibody (**c**), after injection of r*Hm*AIF-1 (**d, e**) or after injection of r*Hm*AIF-1 + pre-immune serum (**f, g**). After 24 h and 48 h from r*Hm*AIF-1 (**d, e**) or from r*Hm*AIF-1 + pre-immune serum mixture injection (**f, g**), numerous migrating cell (red) are visible among muscles (M) and under the epithelium (E). (**a-c**) In uninjured or after 24 h from PBS or r*Hm*AIF-1 + anti-*Hm*AIF-1 antibody injection, a reduced infiltration of ACP positive cells among the groups of muscle fibers (M) and under the epithelium (E) is evident. Bars in a-d, f: 100 μm; bars in e, g: 200 μm

ACID PHOSPHATASE REACTION

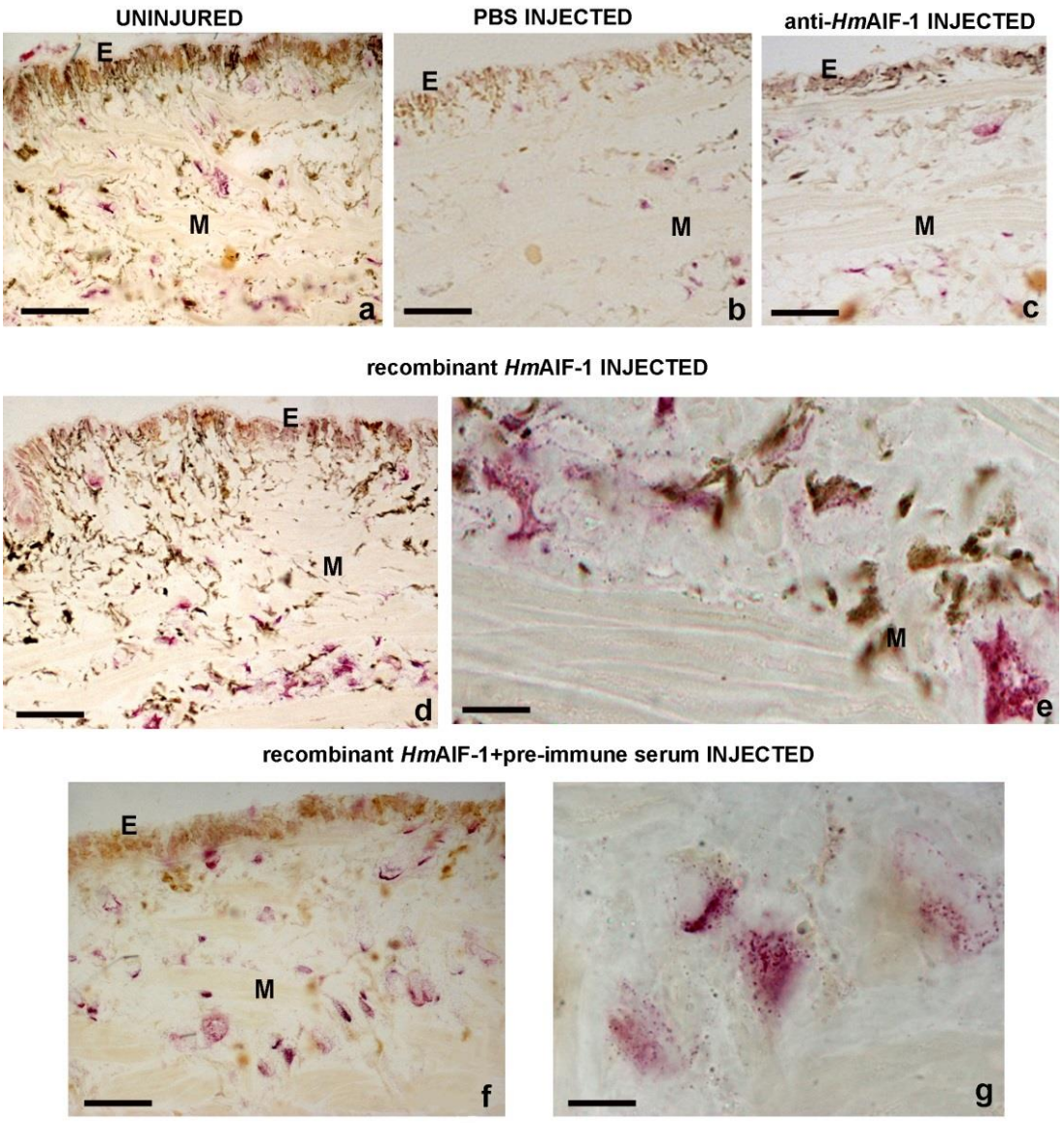
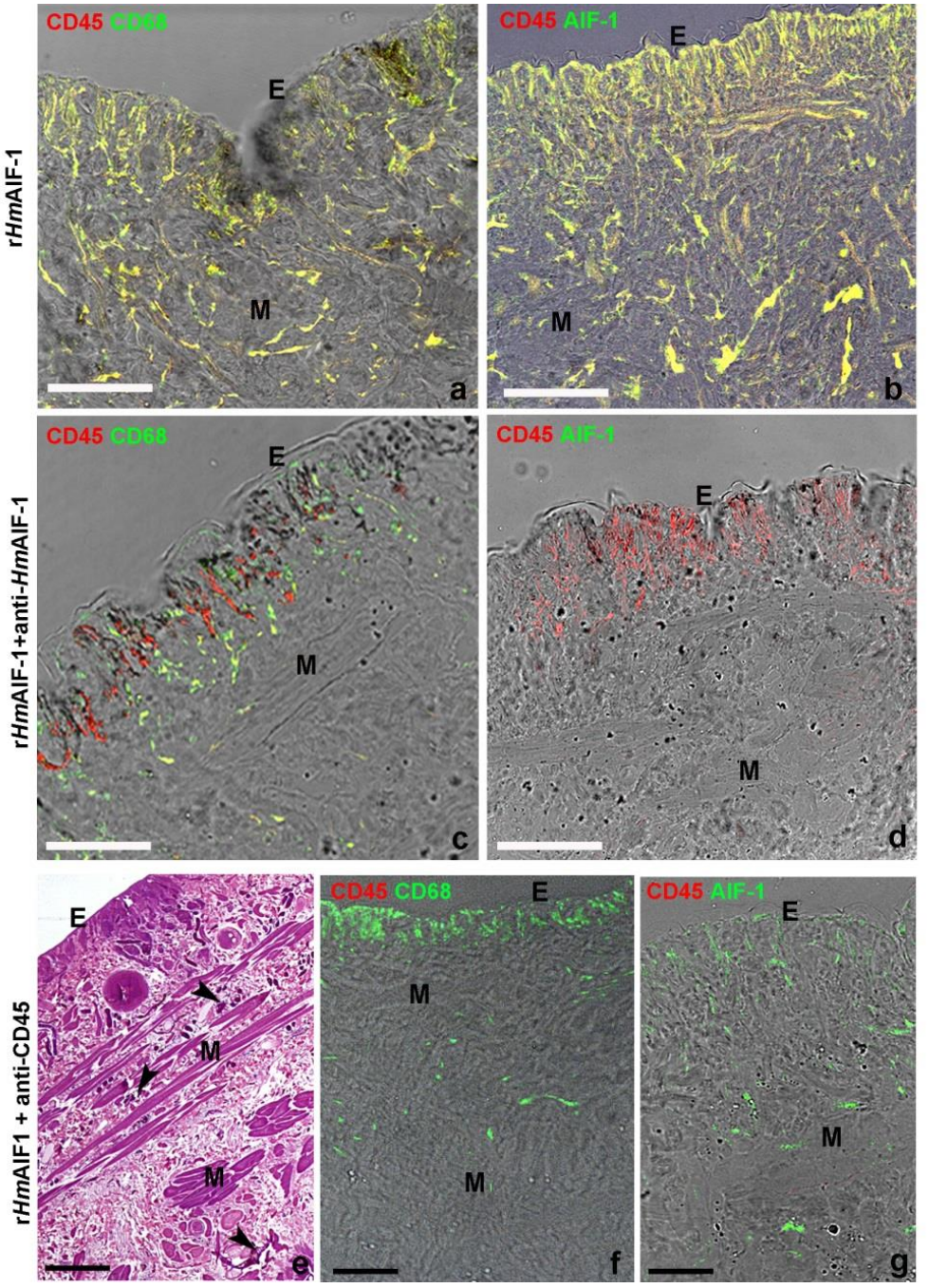


Fig. 15: (**a, b**) 24 h after r*Hm*AIF-1 injection numerous migrating macrophages (in yellow) are double stained by the antibodies CD45 and CD68 and are $CD45^{+}/AIF\text{-}1^{+}$. (**c, d**) After 24 h from the injection of the r*Hm*AIF-1 + anti-*Hm*AIF-1 mixture, a few activated macrophages co-expressing CD45 (red in **c**) and CD68 (green in **c**) or CD45 (red in **d**) and *Hm*AIF-1 (green in **d**) are visible. (**e-g**) After the injection of the antibody anti-CD45 together with the r*Hm*AIF-1 only a few macrophages (arrowheads in **e**) $CD45^{-}/CD68^{+}$ (**f**) are visible underneath the epithelium (E) and among muscles (M). (**g**) The antibody anti-CD45 injection inhibits the CD45 expression and the few resident macrophages are $CD45^{-}/AIF\text{-}1^{+}$. Bars in a-g: 50 μm


rHmAIF-1
rHmAIF-1+anti-HmAIF-1
rHmAIF1 + anti-CD45
CD45 CD68
CD45 AIF-1
E
M
a
b
c
d
e
f
g