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RESEARCH REPORT

The Allograft Inflammatory Factor-1 (AIF-1) homologous in *Hirudo medicinalis* (medicinal leech) is involved in immune response during wound healing and graft rejection processes

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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. Since its expression is mainly limited to the monocyte/macrophage lineage, it was recently suggested that it could play a key role during inflammatory responses, allograft rejection, as well as in the activation of macrophages. To clarify this point we have focused our research on the possible role of AIF-1 during the inflammatory response after injury in the leech *Hirudo medicinalis* (Annelida, Hirudinea). This invertebrate is an excellent animal model since the responses evoked during inflammation and tissue repair are clear and easily detectable and have a striking similarity with vertebrate responses. Moreover 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*. Our data show that the related protein, named *Hm*AIF-1, is constitutively expressed in unlesioned leeches and that dramatically increases 48 h after wounds and tissue transplants. Immunohistochemistry experiments, using a specific anti *Hm*AIF-1 polyclonal antibody, shows that this factor is present in spread, $CD68^+$ /$CD45^+$ macrophage-like cells. A few days after experimental wounding of the body wall, the amount of these immunopositive cells increases at the lesion site. In conclusion here we propose that in leech *Hm*AIF-1 factor is involved in inflammation events like its vertebrate counterparts.

Key Words: leech; CD45; AIF-1; wounds; grafts

Introduction

The Allograft Inflammatory Factor-1 (AIF-1), also called MRF-1, Iba1, and daintain, is an interferon-γ inducible cytoplasmic cytokine of 17 kDa, (Alkassab *et al*., 2007). It contains a Ca^{2+}-binding EF-hand domain and has been identified first in chronic rejection of rat cardiac allografts (Utans *et al*., 1995). AIF-1-like factors, have been described in other groups of Metazoans and share a similar aminoacid structure and a very well preserved functional role. AIF-1 expression increases significantly after transplantation, wounds or bacterial infections both in vertebrates (Utans *et al*., 1995; Watano *et al*., 2001; Deininger *et al*., 2000, 2002; Autieri and Chen, 2005; Alkassab *et al*., 2007) and in invertebrates, such as Sponges (Kruse *et al*., 1999), Molluscs (de Zoysa *et al*., 2010; Zhang *et al*., 2011, 2013; Li *et al*., 2012) and Echinoderms (Ovando *et al*., 2012). Since the release of AIF-1 is a Ca^{2+}-dependent mechanism, it seems that this protein may play a role in cell-cell interactions under inflammatory conditions (Tanaka and Koike, 2002). In particular, the ability to bind calcium allows developing 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).

Interestingly, 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;

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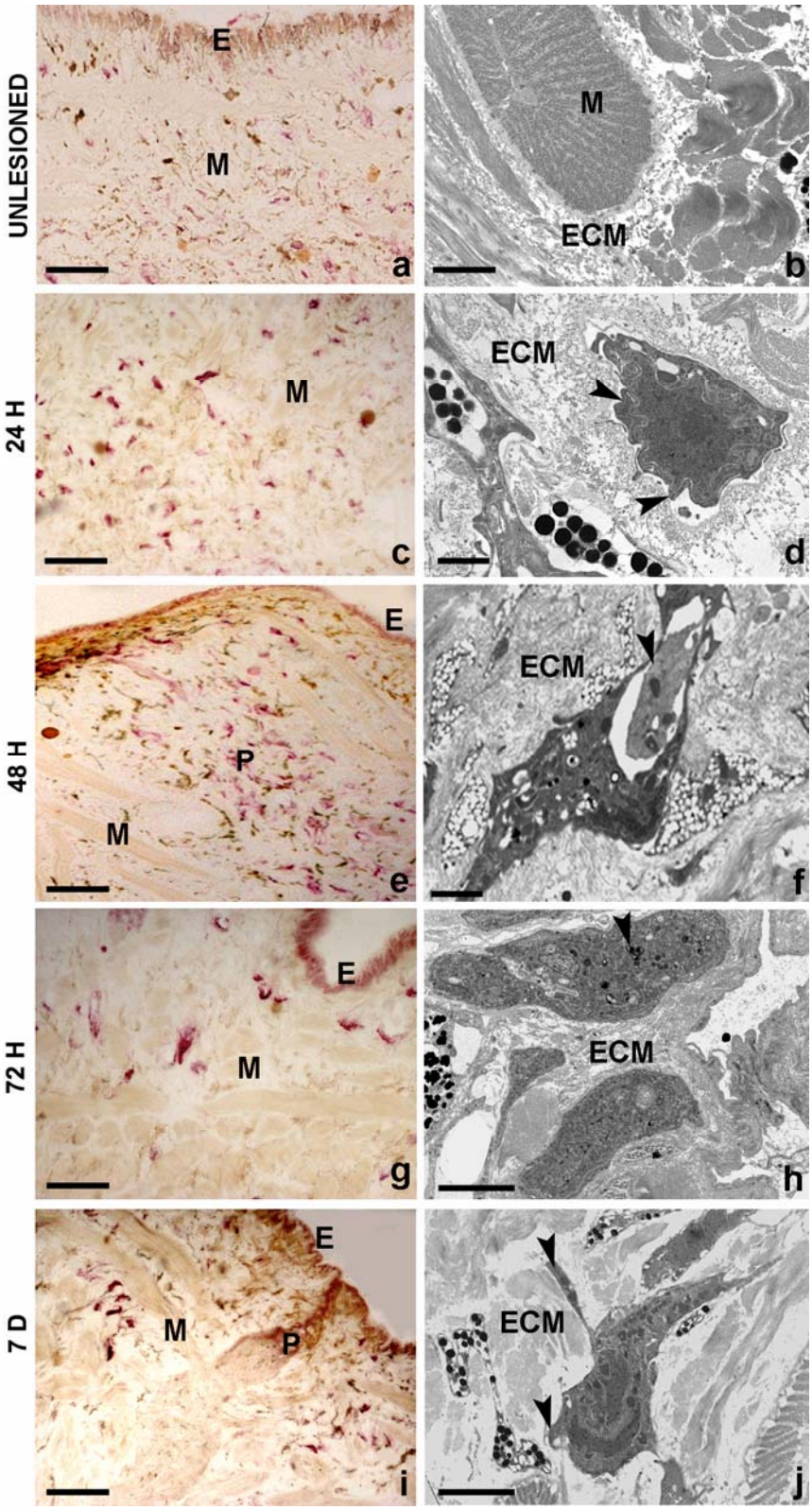

Fig. 1 Acid phosphatase (ACP) reaction on cryosections (a, c, e, g, i) and TEM analyses on ultra-thin sections (b, d, f, h, j) from *H. medicinalis* body wall unlesioned (a, b) and surgically wounded analyzed after at 24 h (c, d), 48 h (e, f), 72 h (g, h) and 7 days (i, j) from injury. Compared to control sections (a, b), after injury numerous macrophages ACP positive (red in c, e, g, i) are visible migrating among muscles (M), under the epithelium (E) and close to pseudoblastema (P). Detail at TEM of uninjured body wall of leeches (b) and wounded leeches (d, f, h, j). After injury macrophage-like cells moved in the connective tissue (ECM) with ruffled surfaces (arrowheads in d) and projections of variable thickness (arrowheads in j). In the cytoplasm phagocytized material (arrowhead in f) and phagolysosomes (arrowhead in h) are visible. Bars in a, c, e, g, i: 100 μm; bar in b, f, h, j: 2 μm; bars in d: 4 μm.

Li *et al*., 2013; Schorn *et al*. 2014). 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 and Ostergaard, 2013). It plays 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 (Jeong *et al*., 2013).

In vertebrates, despite the extensive investigation focused on both molecular characteristics and expression level of AIF-1 during the inflammatory response or wound healing, the direct relationship between AIF-1 and CD45 expression and macrophage activation/migration during the inflammation phase after injury or graft remains unclear. It is probably because the study of the immune response in Vertebrates appears to be a difficult challenge, primarily due to the complexity of these organisms.

We recently characterized in the central nervous system (CNS) of the leech a gene showing high similarity with vertebrate *aif-1*, named *Hmiba1*/alias *Hmaif-1* (GenBank accession number KF437461, Drago *et al.,* 2014). In peripheral tissues, the protein is mainly located in the macrophages and its production increases in body wall after bacterial injection (Schorn *et al*., 2014). We presently focused our research on the possible role of AIF-1 during the immune response after injury and grafts in the leech *Hirudo medicinalis* (Annelida, Hirudinea). This invertebrate, offering simpler anatomy and lacking complex feed-back control systems typical of vertebrates, represents a great alternative for studying basic steps of immune responses (de Eguileor *et al*., 2000b, 2001b, 2003, 2004; Grimaldi *et al*., 2006, 2009, 2011; Schikorski *et al*., 2009). *H. medicinalis* is characterized by the absence of a true vascular system within the muscular body wall and by the presence of a specific tissues, the botryoidal tissue, located close to the digestive system and involved in hematopoietic cells production and in the formation of new vessels (Grimaldi *et al*., 2006). The effects of lesion or grafts in leech body wall are rapidly induced and after 24 h the inflammatory phase is characterized by an influx of macrophages that are responsible of phagocytosis and immune cytotoxicity, clean the stimulated area and release various growth factors (de Eguileor *et al*., 1999, 2000a, b; Grimaldi *et al*., 2006; Tettamanti *et al*., 2006). In parallel, remodeling of the botryoidal tissue induces the formation of new vessels and inside the lumen of these growing vessel clusters of hematopoietic precursors develop. These cells, after transendothelial migration, diffuse in the wounded area and differentiate into mature leucocytes that mediate the inflammatory response (Grimaldi *et al*., 2006).

In order to better understand the role of *Hm*AIF-1 after wound healing and graft stimulations, immunohistochemistry and western blot studies have been performed to determine the localization and the modulation of this gene in leech body wall. The presence of *Hm*AIF-1 in uninjured, experimentally injured and grafted tissues was established using the specific rabbit anti-*H. medicinalis* 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 were performed to characterize the cells involved in the immune response and expressing *Hm*AIF-1.

Material and Methods

Animals and Treatments

Leeches (*Hirudo medicinalis*, Annelida, Hirudinea, from Ricarimpex, Eysines, France) measuring 10 cm were kept in tap 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 (wounds or tissue collection for grafting) was performed at the level of the 80th superficial metamere. Before each experiment, leeches were anaesthetized with a 10 % ethanol solution and then dissected. 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: leeches for each time points (24 h, 48 h, 72 h, 7 days) were injured at about the 80th superficial metamere with a razor blade, in order to assess the modulation of *Hm*AIF-1 during the wound healing.

Group 3: leeches for each time points (24 h, 48 h, 72 h, 7 days) were 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 80th superficial metamere): 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 80th superficial metamere 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 80th superficial metamere of a con-specific individual. Grafts were sutured with Dafilon® surgical synthetic monofilament as indicated above.

Electron Microscopy

Leech tissues, dissected from the area of 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). 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), 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 polyclonal anti-*Hm*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). In control sections, primary polyclonal anti-HmAIF-1 antibody was substituted with rabbit pre-immune serum (1:20,000) or primary antibody was omitted and sections were treated with BSA containing PBS and incubated only with the secondary antibodies. 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).

Acid phosphatase reaction (ACP)

Leech tissues, dissected from unlesioned animals and from area of wound or graft, 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 min, incubated with sodium acetate-acetic acid 0.1 M buffer for 5 min 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 $MnCl_2$ 0.5nM) for 90 min 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. Slides, 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 reacts with leech hematopoietic cells (de Eguileor *et al*., 2003) diluted 1:100, rabbit polyclonal anti-human CD68 (Santa Cruz Biotechnology) which reacts 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 (Grimaldi *et al*., 2004, 2006; de Eguileor *et al*., 2000a, b) and on animals phylogenetically related to Annelids (Cossarizza *et al*., 1996) and Sipunculids (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 *Hm*AIF-1, *Hm*AIF-1/CD45 or *Hm*AIF-1/CD68 the anti *Hm*AIF-1 was applied first, then sections were incubated with the secondary antibody goat anti-rabbit FITC conjugated. After washing the samples were incubated with the antibody anti CD45 or anti CD68. Subsequently, the sections were treated 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 samples were incubated with the antibody anti CD68 and subsequently with the secondary antibody goat anti-rabbit FITC conjugated. According to Würden and Homberg (1993), to inhibit binding of the primary antiserum of the second staining cycle to the goat anti-rabbit IgGs that were applied in the first sequence, the sections were incubated with rabbit IgG (Jackson ImmunoResearch) at 1:25 for 2 h. 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). In control samples, primary antibodies were omitted and sections, treated with BSA-containing PBS or with the rabbit pre-immune serum (1:20,000), were incubated only with the secondary antibodies. According to Schnell *et al*., 1999, after immunocytochemistry, the sections were treated with 1 mM $CuSO_4$ in 50 nM ammonium acetate buffer (pH 5.0) for 15 min and then washed in distilled water and PBS. Application of $CuSO_4$ for 10 minutes after immunohistochemistry substantially reduced tissue autofluorescence while preserving the specific fluorochrome signal.

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.).

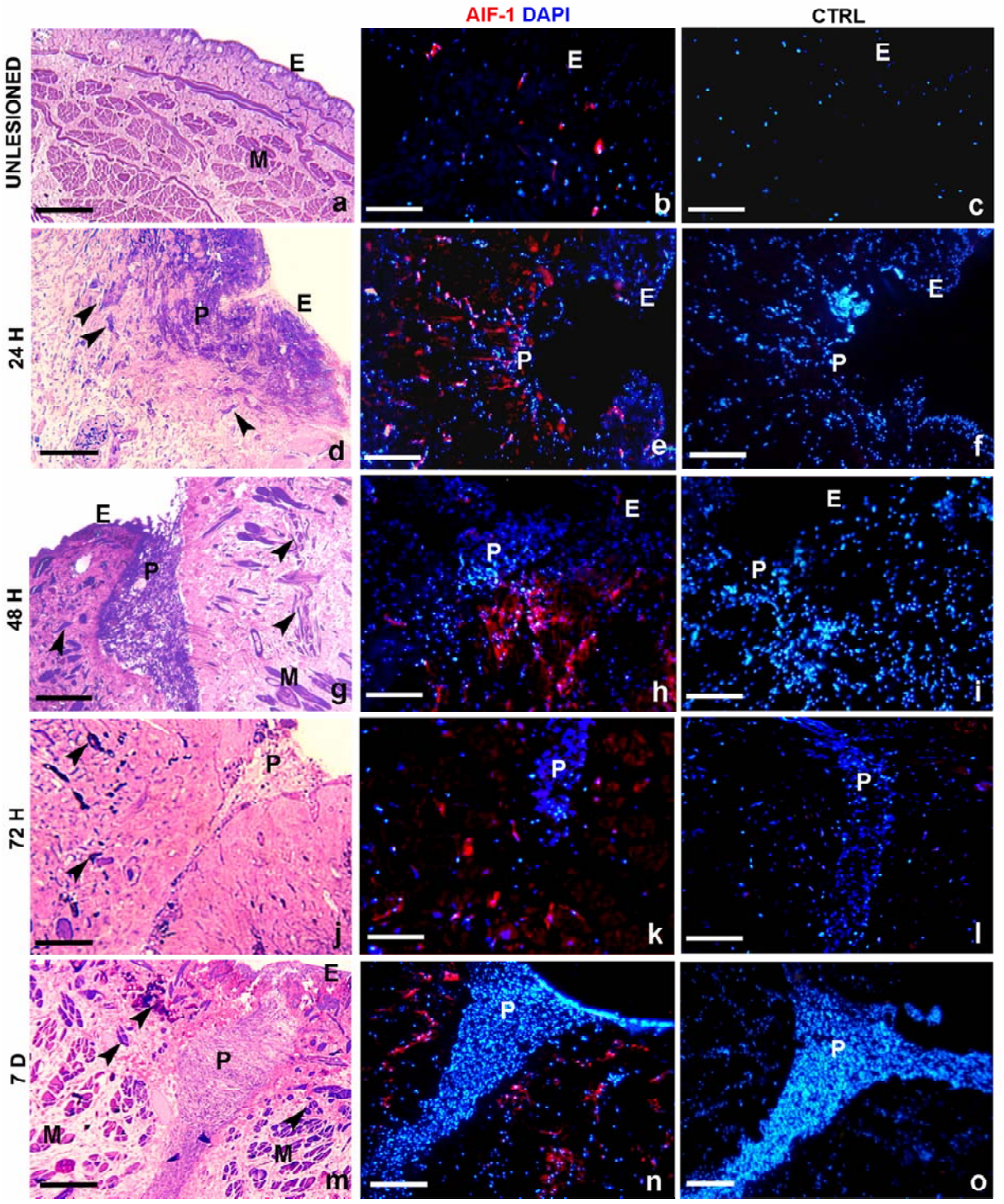

Fig. 2 Morphological (optical microscopy) and immunohistochemical (fluorescence microscopy) analysis of cryosections from *H. medicinalis* body wall unlesioned (a-c) and surgically wounded and analyzed after 24 h (d - f), 48 h (g - i), 72 h (j - l) and 7 days (m - o) from injury. Numerous migrating cells (arrowheads in d, g, j, m) among muscle fibers (M) and close to the pseudoblastema (P) were visible in injured leeches. Immunohistochemistry using the rabbit polyclonal anti-*Hm*AIF-1 antibody (red) marks the 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, o). Bars in a - l: 100 μm.

Biochemical procedures

H. medicinalis tissues from the unstimulated body wall or from 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 particulated material was removed by centrifugation at 13000 rpm for 10 min at 4 °C in a refrigerated Eppendorf Minispin microcentrifuge. Supernatants containing total protein extracts were denatured at 100 °C for 10 min and loaded on 10 % acrylamide minigels for SDS-PAGE analyses.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, 20 mM Tris-HCl buffer, 500 mM NaCl, pH 7.5) at room temperature for 2 h, and incubated for 90 min with a rabbit anti-*Hm*AIF-1 antibody (1:5000 dilution in 5 % TBS-milk) or rabbit polyclonal anti-human CD45 IgG (Twin Helix) diluted 1:1000. The membrane was washed three times with TBS-Tween 0.1 % and then incubated 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 Image J 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

Morphological, immunohistochemical and biochemical characterization of cells recruited in the wounded area

Ultrastructural and enzyme histochemical analyses

As already described in previous works (Grimaldi *et al*., 2004, 2006; Tettamanti *et al*., 2004), following tissue damage, wound healing initiates with an inflammatory phase characterized by a massive migration of immune cells, fibroblasts and myofibroblasts-like cells, towards the lesioned area. Wound size and retraction was then obtained by the formation of a pseudoblastema region formed by the myofibroblasts-like cells (Huguet and Molinas 1994, 1996; Grimaldi *et al*., 2009, 2011). Enzyme histochemical and ultrastructural analyses showed that in unlesioned leeches (Figs 1a, b) a few cells were located in the connective tissue, underlying the body wall epithelium and surrounding the fields of muscle fibers and were ACP positive. By contrast, after injury numerous migrating cells were highly positive for ACP reaction (Figs 1c, e, g, i), confirming their phagocytic activity. These migrating cells were clearly visible among muscle layers and reached the healing area at which they increased numerically in relation to the time elapsed after the lesions were inflicted. In particular, a significant increase of ACP positive cells was mainly observed in the area surrounding the pseudoblastema 48 h after the injury (Fig. 1e). Ultrastructural analysis of injured tissues at TEM highlighted the presence of numerous activated macrophages-like cells in the connective tissue close to the lesioned region (Figs 1d, f, h, j). These cells were characterized by a certain degree of surface ruffling, pseudopodia, and their phagocytic activity was mainly evident 48 h after the injury (Fig. 1f).

HmAIF-1 immunolocalization

As our previous data showed (Schorn *et al*., 2014), *Hm*AIF-1 was constitutively expressed in unlesioned animals (Figs 2a - c). This factor was mainly expressed in cells located in the connective tissue surrounding the fields of muscle fibres. Cryosections of injured leeches analyzed after 24 h, 48 h, 72 h and 7 days from lesion (Figs 2d - o), were immunostained with the antibody against *Hm*AIF-1. Cells expressing *Hm*AIF-1 were found dispersed in the extracellular matrix (ECM) surrounding the groups of muscle cells and close to the wound healing region of injured leeches (Figs 2d - l). Our data showed that *Hm*AIF-1 expression dramatically increased in 24/48 h injured leeches, when a massive migration of cells towards the lesioned area was detectable (Figs 2e, k, n). No signal was visible in negative controls experiments, in which primary antibody was omitted (Figs 2c, f, i, l, o).

Characterization of HmAIF-1$^+$ cells

In order to characterize the *Hm*AIF-1$^+$ migrating cells, double immunofluorescent stainings 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 *Hm*AIF-1$^+$ cells dispersed in the ECM surrounding the groups of muscle fibers (Figs 3a, b) and close to the wound healing region (Figs 3d, e) expressed also the common leukocyte marker CD45 and the macrophage cell marker CD68.

Control experiments performed in the absence of the primary antibodies were negative for all the samples (Figs 3c, f). Immunogold golds experiments confirmed the expression of *Hm*AIF-1 in CD45$^+$/CD68$^+$ macrophage-like cells (Figs 3g - i).

Biochemical analysis

Western blot assays were performed to assess the expression profile of *Hm*AIF-1 (Figs 4a, b) and CD45 (Figs 4c, d) in wounded leeches.

Compared to the basal expression level detected in unlesioned leeches, the amount of *Hm*AIF-1 protein significantly change in extracts of lesioned leech body wall, showing a pick of expression after 48 h from injury (Fig. 4 b). GAPDH

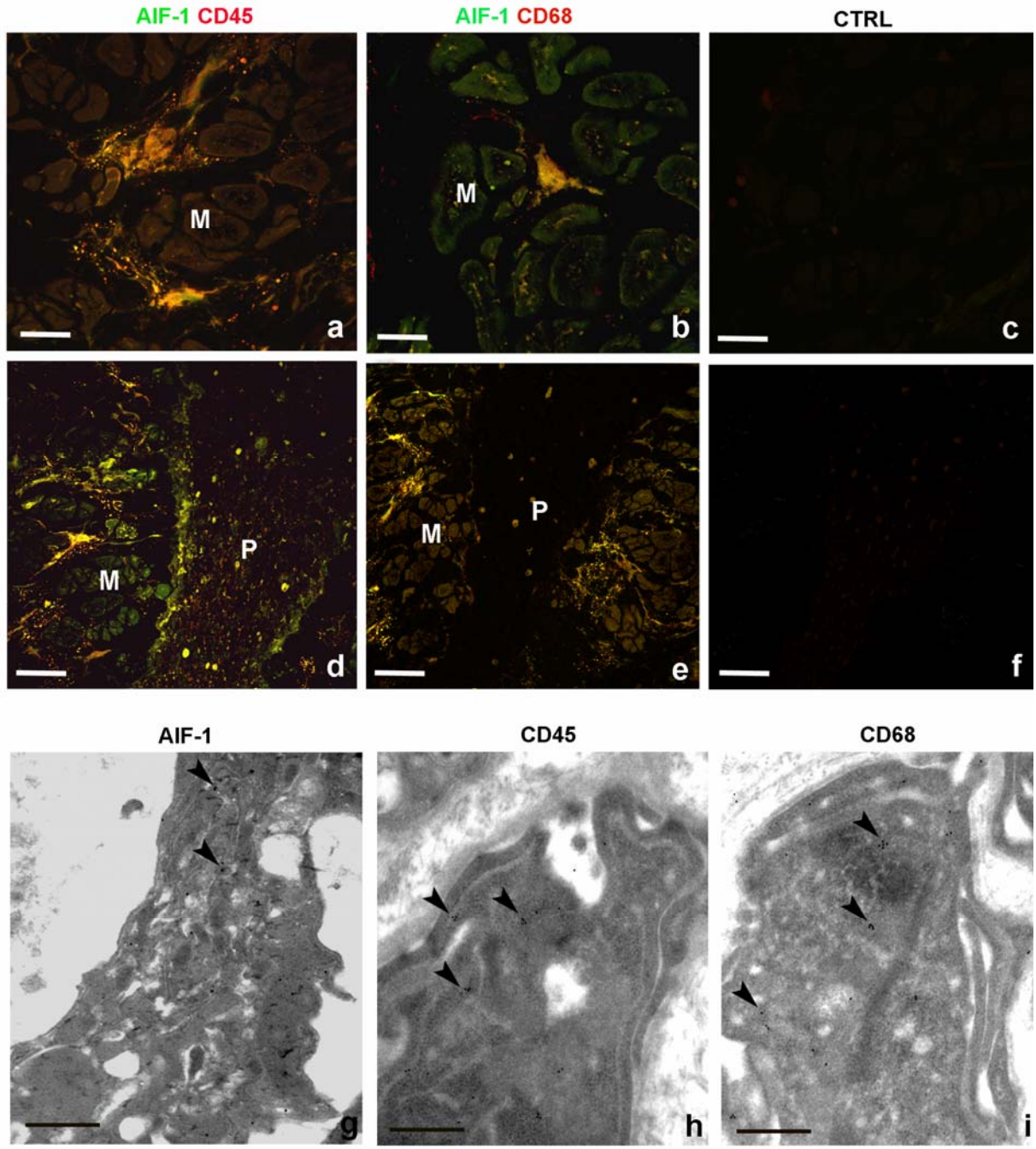

Fig. 3 After injury numerous migrating macrophages (in yellow) located among muscle fibers (a, b) and close to the pseudoblastema (P) region (d, e) are $CD45^{+}$/$AIF\text{-}1^{+}$ and $CD68^{+}$/$AIF\text{-}1^{+}$. (c, f) negative control experiments where the primary antibodies are omitted. (g-i) immunogold staining (arrowheads) 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

was used as internal reference and bands intensity appeared homogeneously distributed in the loaded samples (Fig. 4a).

A similar result was obtained analyzing the expression profile of CD45 (Figs 4c, d) Compared to the basal expression level detected in unlesioned leeches, the amount of CD45 protein significantly increased in body wall extracts of 48 h and 72 h lesioned leech (Fig. 4d). As described above, GAPDH was used as internal reference (Fig. 4c).

Morphological and immunohistochemical characterization of cells recruited in the grafted area

Our previous data demonstrated that self-transplantation caused no rejection but only an inflammatory response, whereas host *H. medicinalis*

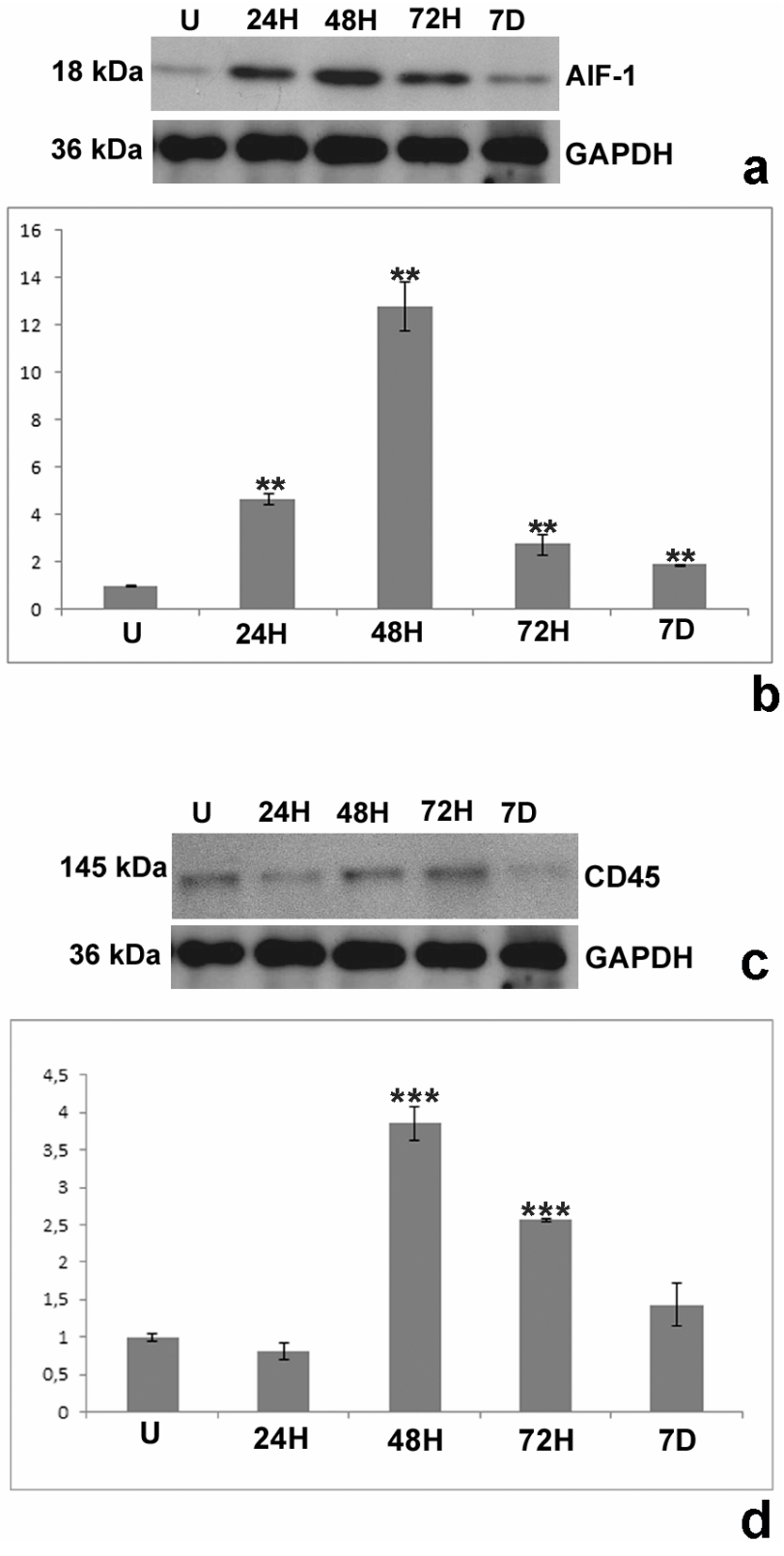

Fig. 4 Western blot analysis. Protein extracts of unlesioned (U) and injured leeches after 24 h, 48 h, 72 h and 7 days from injury were probed with the anti-*Hm*AIF-1 antibody (a) and CD45 (b). The housekeeping protein GAPDH was used as a loading control. In all the samples, the anti-*Hm*AIF-1 detected specific immunoreactive bands of about 18 kDa (a), 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. The anti-CD45 detected in all the samples an immunoreactive bands of about 145 kDa, according to the molecular weight ladder (c). The housekeeping protein GAPDH was used as a loading control. (d) CD45 protein was quantified by densitometry from three experiments. $^{***}p < 0.01$ compared with uninjured leeches.

leeches rejected both allo- and xenografts (Tettamanti *et al.,* 2003). 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. 5a). These migrating cells, morphologically and functionally already described as macrophage-like cells (Tettamanti *et al*., 2003) positively stained for ACP reaction (Fig. 5b). The ACP^+ cells, forming a clot surrounding the autograft, showed a low level of *Hm*AIF/CD45 expression (Figs 5c, d).

Leech responses to allograft

Starting from 24 h after allograft, an acute inflammatory phase started with migrating immunocompetent cells through the ECM. These cells were involved in clot formation and in graft isolation from neighboring 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 phagocytic activity directed to remove cell and matrix debris. They were positively stained for ACP reaction and highly co-expressed CD45 and *Hm*AIF-1 (Figs 5e - g; i - k; m - o). No signal was detected in control negative experiments of immunolocalization, were primary antibodies were omitted (Figs 5h, l, p).

Discussion

Both in 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 infecting microbes and a repair function to regenerate damaged tissues. This process is mediated by specific cells such as macrophages and neutrophils that infiltrate the damaged tissue removing 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 like angiogenesis and fibroplasia and are regulated by different cytokines and growth factors (Tettamanti *et al*., 2004; Grimaldi *et al*., 2006). Moreover, in our recent studies we demonstrated that in the leech *H. medicinalis* the inflammatory factor *Hm*AIF-1 is constitutively expressed in untreated animals but is dramatically enhanced after microbial infection. It particularly promotes macrophages and vessels migration towards the stimulated area (Schorn *et al*., 2014).

It has been demonstrated that in leech the immune response is based on the same molecules involved in wound healing and regenerative process (Schikorski *et al*., 2008). Here we were interested in understanding the possible involvement of *Hm*AIF-1 in the regulation of inflammatory response in both wounded and grafted leeches.

First we investigated the tissue-specific and temporal expression profile of *Hm*AIF-1 factor after different time points. Indeed, we found high level of *Hm*AIF-1 expression in the tissue of wounded and grafted leeches. In particular, we observed a significant increase of *Hm*AIF-1^+ cells migrating towards the wounded area or forming a clot around the non self-tissue after 24 - 48 h from surgery. On the other hand the level of *Hm*AIF-1 expression is very low in those cells forming a clot surrounding the autografts tissue.

Taken together, these findings suggest that, besides cell-mediated defense reactions, the cytokine *Hm*AIF-1 is also elicited during wound healing and graft recognition and rejection in leeches. A steady increase of *Hm*AIF-1 was mainly detected in the initial stages of inflammation, 24 - 48 h after surgery, and decline by 7-10 days after surgery. These data confirm that, in leech as well, *Hm*AIF-1 is involved in early events that trigger inflammation more than in the late ones (Autieri *et al*., 2000; Schorn *et al*., 2014).

Therefore AIF-1 not only has been highly evolutionarily conserved in amino acid sequence but also shows a similar function in both Vertebrates and invertebrates (Yamamoto *et al*., 2011).

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 (Schorn *et al*., 2014). The ultrastructural morphology and acid phosphatase reaction positivity confirmed that the cells migrating towards the injured or grafted areas have a strong phagocytic activity. This observation is in agreement with the fact that phagocytosis is an important process for the repair/regeneration of damaged tissue because it increases the clearance of tissue debris, limits further destruction and facilitates repair (Takahashi *et al.,* 2007). Moreover these results are consistent with previous data obtained after wounding and grafts (de Eguileor *et al*., 2003; Tettamanti *et al*., 2003; Grimaldi *et al*., 2004, 2006). Furthermore, double immunostainig experiments based on CD68 tissue expression confirmed the accumulation of macrophages both in the wound healing and grafted region but also highlighted that these cells co-expressed both CD45 and *Hm*AIF-1. As we recently demonstrated (Schorn *et al*., 2014), *Hm*AIF-1 in leeches is expressed by macrophages and it is involved in macrophage recruitment in the injured area. We speculated that macrophages may recruit and/or promote the proliferation of other macrophages suggesting a pivotal role in initial inflammatory response. Our results are consistent with what previous observed in Vertebrates, and highlight that AIF-1 plays an important role in linking injury, inflammatory and immune response in allograft tissue transplantation. Furthermore, these data suggest for the first time that the early activity of macrophages, involved in the initial phase of immune response during wound healing or allograft

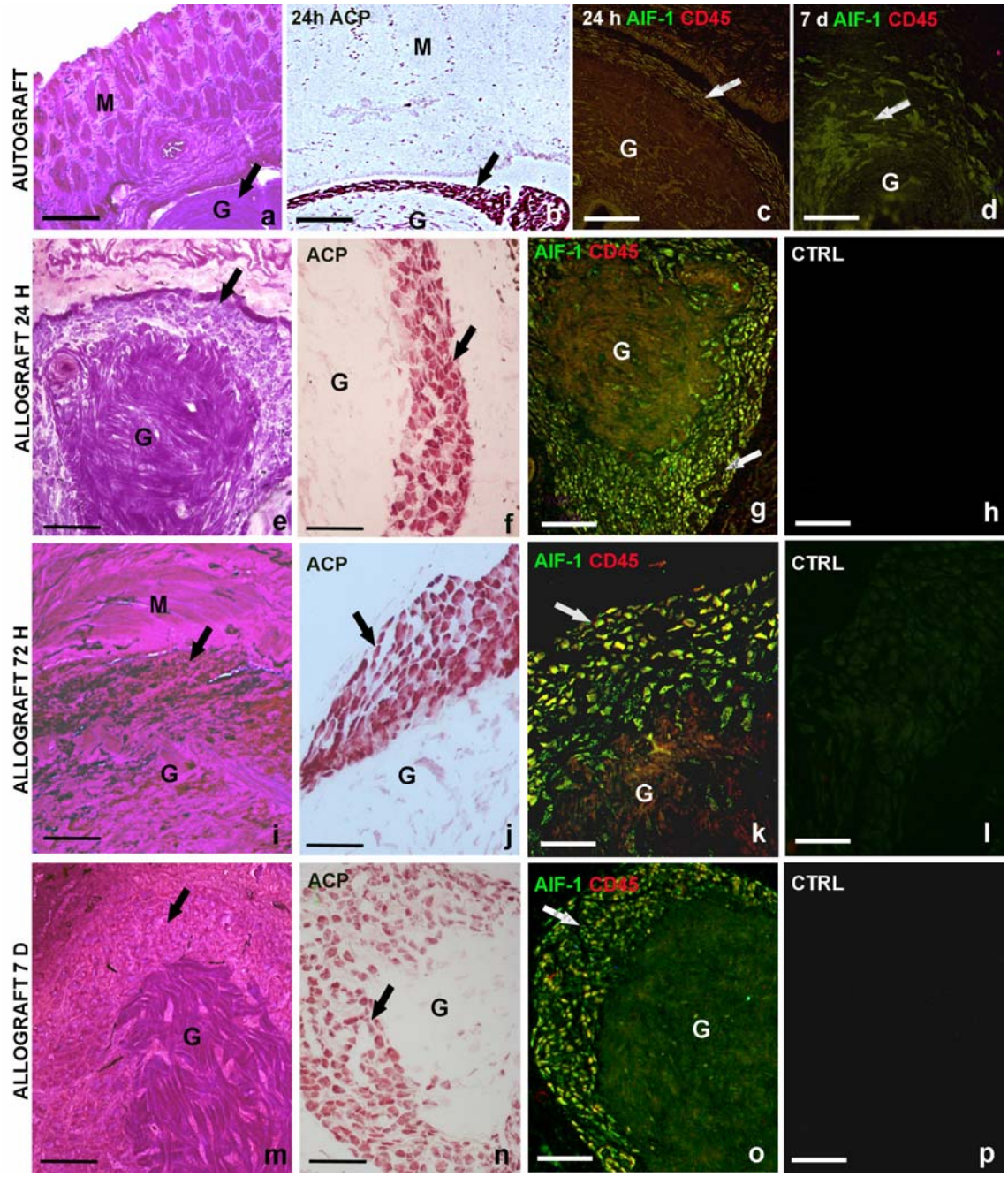

Fig. 5 Morphological at optical microscopy (a, e, i, m), Acid phosphatase reaction (b, f, j, n) and immunofluorescence (c, d, g, h, k, l, o, p) analyses of cryosections from grafted *H. medicinalis*. 24 h from autograft a clot of macrophages cells (arrow in a) surround the graft (G). These cells are ACP^+ (arrow in b) and weakly express AIF1 and CD45 (arrows in c, d). 24 h after allograft the clot of macrophages cells (arrows in e, i, m) surrounding the graft (G) are ACP^+ (arrows in f, j, n) and highly co-expressed *Hm*AIF-1 and CD45. No signal is detected in control experiments 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.

rejection, is mediated by both *Hm*AIF-1 and CD45 expression. Indeed, like in Vertebrates (Utans *et al*., 1995), *Hm*AIF-1 is highly expressed in allografts by 24 h and remained elevated through 72 h and 7 days. This early and sustained expression of *Hm*AIF-1 in allograft is consistent with an ongoing allogeneic inflammatory response. The high density of infiltrating *Hm*AIF-1/$CD45^+$ macrophages in injured and allografted areas could be sustained by cytokine-rich environment chemoactraction (de Eguileor *et al.*, 1999; Tettamanti *et al*., 2003). The pool of cytokine, in turn, may up-regulate *Hm*AIF-1 expression inducing an initial inflammatory response in transplanted host leeches. These macrophages,

co-expressing HmAIF-1/CD45 and CD68, co-operated to isolate and infiltrate the graft producing themselves a large amount of cytokines responsible of mitogenic and chemotactic events. This macrophages recruitment is a detrimental component of allograft rejection.

On the other hand, in autografts, macrophages show a low level of *Hm*AIF-1 and CD45 expression. We speculate that differences in *Hm*AIF-1 and CD45 expression level are linked to the different role played by macrophages in response to wound/allograft and to autograft. We suggest that like in Vertebrates (Mokarrama *et al.*, 2012), in leeches as well macrophages can have characteristics of anti-inflammatory or pro-inflammatory features. In allograft and wound healing, macrophages are mainly involved in inflammatory response and highly express *Hm*AIF-1 and CD45. In contrast, in autograft are mainly involved in regeneration of the body wall microenvironment and support tissue repair by producing anti-inflammatory cytokines which mediate angiogenesis, cell replacement and matrix remodeling while suppressing destructive immunity and low expressing *Hm*AIF-1 and CD45.

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. During wound healing and grafts rejection, *Hm*AIF-1 might be implicated in the activation of migrating cells, which role is to clean up the area from damaged tissue and also 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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