Role of RNASET2 in innate immune response regulation

Il ruolo di RNASET2 nella regolazione della risposta immunitaria innata

PhD Thesis of

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ABSTRACT

In the last decades, numerous studies have highlighted the existence of several proteins that, besides performing a specific and evolutionarily conserved function, are involved in the regulation of different and independent biological processes, thus acquiring new functional roles. Among them, the RNase T2 family enzymes represent one of the most known and investigated example. Indeed, T2 RNases are implicated not only in RNA maturation or degradation, but also perform additional functions in different organisms, often independently of their catalytic activity. Recently, it has been demonstrated that RNASET2, the only member of T2 family presents in human genome, regulates the tumorigenicity of ovarian cancer cells *in vivo*, establishing a functional cross-talk between the tumor mass and the surrounding microenvironment and stimulating an inflammatory response by inducing macrophages migration and activation. Although these evidences, the details on the biological mechanisms by which this evolutionarily conserved enzyme interacts with the innate immune system and promotes cells migration and extracellular remodeling are still poorly defined. To shed light on this aspect, here we propose the medicinal leech *Hirudo verbana* as a consolidated invertebrate model for studying these processes.

Our *in vivo* results demonstrate that injection of human recombinant hRNASET2 (or the *H. verbana* recombinant r*Hv*RNASET2) in the leech body wall caused fibroplasia, connective tissue remodeling and the recruitment of numerous $CD68^{+}$/*Hm*$AIF1^{+}$ macrophages. Moreover, LPS, LTA, Gram-negative and Gram-positive infection induces a release of this enzyme from granulocytes, reflecting a putative antibacterial role for this protein. Indeed, our *in vitro* results clearly confirm that that the medicinal leech recombinant *Hv*RNASET2 protein represents a promising candidate molecule for counteracting bacterial infections. In particular, *Hv*RNASET2 apparently plays a dual anti-microbial role: on the one hand, it induces bacterial clusters formation, likely to facilitate recognition by innate immune cellular effectors and, on the other hand, it stimulates macrophage recruitment and phagocytosis. In conclusion, *Hv*RNASET2 acts as an evolutionary conserved activator of the innate immune response, favoring an effective and rapid microbial pathogen removal or an effective oncosuppressive activity. Further investigations in *in vivo* experimental models will be crucial to evaluate the possible role of *Hv*RNASET2 as a potential agent to counteract microbial infection.

INTRODUCTION

Ribonucleases

The concept of pleiotropy was coined about one hundred years ago to define a condition in which a single gene affects the expression of different and seemingly unrelated phenotypic traits. Although this definition has originally been strictly applied to the research field of genetics, over the time it has been adopted and extended to other biological fields.
In fact, investigations on the cellular mechanisms underlying many biological processes revealed the existence of complex interactions, both intracellular and extracellular, in which a single molecule could generate a wide range of biological responses simply based on its biochemical structure. In the wake of these discoveries, it is currently common to define a protein or any type of molecule as pleiotropic when, in addition to its own defined and proper role, it shows the ability to perform independent biological roles.
The field of immunology has provided several examples on this topic, as many cytokines are today known to exert widely pleiotropic roles not only by activating different cellular pathways, but also by triggering multiple responses following their interaction with a particular receptor, based on the specific cellular and molecular context in which they act (**Katsutoshi and Warren 2002**) .

Within this frame, some enzymes also exhibit the ability to perform multifunctional roles and, among them, Ribonucleases (also called RNases) represented one of the most known and studied group. Their discovery dates back to 1938, when the first RNase has been isolated and purified from the bovine pancreas (**Kunitz 1940**), becoming an important model to investigate protein chemistry and leading the way that allowed the characterization of numerous other ribonucleases in other organisms, displaying a molecular weight that can vary between 26 to 188 kDa (**Wlodawer et al. 1982; Beintema and Kleineidam 1998; Dyer and Rosenberg 2006**). In all cases, a common nuclease activity has been observed, which proved necessary for the regulation of RNA metabolism, often with a preference for single-stranded RNA, double-stranded RNA or DNA/RNA hybrids (**Libonati and Sorrentino 1992; Beintema and Kleineidam 1998; Irie 1999**). Due to the huge heterogeneity presented by these enzymes, their classification has been much debated and initially they were divided in relation to their mechanisms of action (**Barnard 1969**). However, it was soon realized that early RNases

classifications efforts were oversimplified and more complex criteria were proposed based on different parameters. Indeed, although all RNases show a biochemically conserved catalytic role, they can be nevertheless split into several different families and classes, based on either their specificity for a particular base related to catabolism of RNA, the levels of pH at which they act, their cellular localization and their catalytic mechanism (**D'Alessio and Riordan 1997**).

In this context, a growing interest has been recently focused on an interesting group of ribonucleases, defined "transferase-type" for their mechanism of catalysis. These enzymes perform their ribonucleolytic role by hydrolyzing a single-stranded RNA through the transient creation of a 2'-3'-cyclic phosphate intermediate (trans-phosphorylation reaction) at the level of the 3'-end of RNA molecule, followed by the production of mononucleotides with a terminal 3'-phosphate (hydrolysis reaction) (**Ward et al. 1969; Wladkowski et al. 1995; Irie et al. 1997; MacIntosh et al. 2001**). Interestingly, it has been observed that, although the reaction was unique, it belonged to RNases endowed with a wide range of biological features (**D'Alessio 1993; Irie 1999; Deshpande and Shankar 2002**). For this reason, transferase-type RNases were further split into three large families. The RNases A family is composed by enzymes with a molecular mass ranging from 13 to 20 kDa that act preferentially in an alkaline environment to carry out RNA catalysis via pyrimidine-specific recognition. Of note, RNases A genes are present in vertebrates only and no evidences of these proteins were obtained from the analyses of genomes from deuterostomian organisms, such as *Ciona intestinalis*, or from lower invertebrates (**Pizzo and D'Alessio 2007**).

On the other hand, the alkaline T1 RNases family, with a molecular mass of about 12 kDa, was purified only in fungi and bacteria (**Sato and Egami 1957**) and shows a preference for guanylic acid residues.

The last important set is represented by the RNases T2 family, which differs from the previously described families in several aspects: the optimal pH range for the activity of these enzymes is between 4.0 and 5.0, they show little substrate cutting specificity at the base level and, most interesting, they are widely distributed throughout taxa, being found from viruses to eukaryotes, including vertebrates.

Moreover, unlike RNase A and RNase T1 family members, T2 RNases enzymes show a higher molecular masses,(**Horiuchi et al. 1988; Kurihara et al. 1992; D'Alessio and Riordan 1997; Irie**

1997; Deshpande and Shankar 2002) and are frequently endowed with highly pleiotropic roles, by performing different biological functions in the organisms.

For this reason, during the last thirty years, several research groups in the world focused their studies on the identification of the biological roles related to this particular family of enzymes.

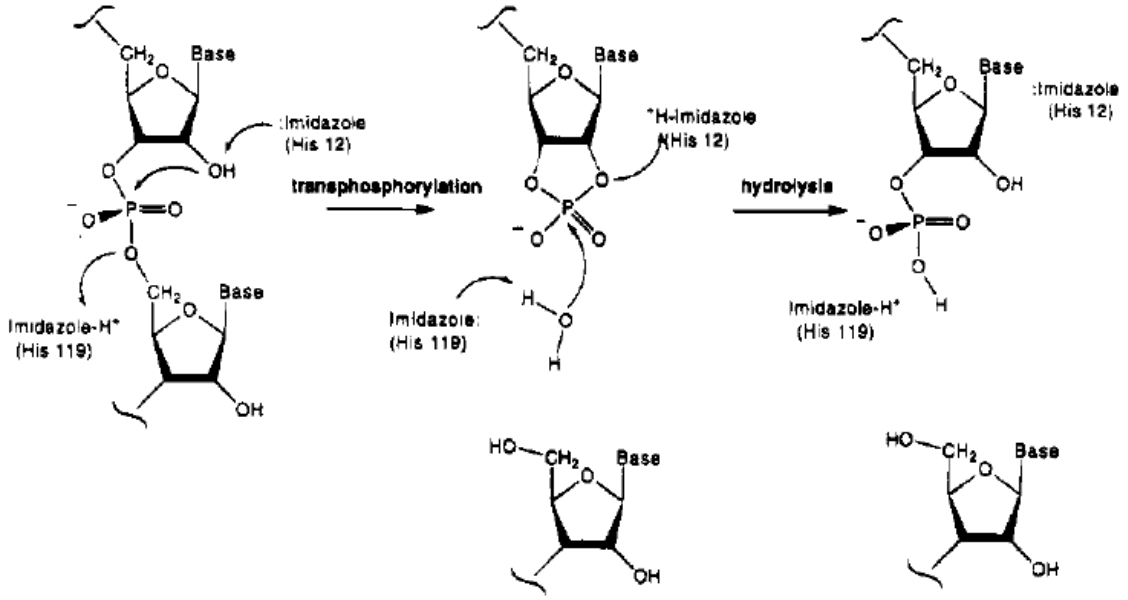

Figure 1. Schematic representation of the proposed overall phosphate ester hydrolysis mechanism catalyzed by RNase A. Reprinted (adapted) with permission from (**Wladkowski et al., 1995**). Copyright (1995) American Chemical Society.

The RNase T2 family

RNase T2 glycoproteins have been purified and isolated for the first time from an enzyme extract from the fungus *Aspergillus oryziae* (**Sato and Egami 1957**). Further studies defined the precise amino acid sequences of the T2 RNases extracted from *A. oryziae* and *Rhizopus niveus* (**Kawata et al. 1988; Horiuchi et al. 1988**). These results resulted crucial for understanding the biochemical mechanisms of action of these enzymes. In fact, two highly conserved active sites for catalysis (dubbed CAS I and CAS II), characterized by the presence of two specific histidine residues involved in RNA cleavage, were subsequently identified, (**Kawata et al. 1990**). Further investigations focused on the three-dimensional structure of T2 RNases and revealed a conserved α+β-type organization consisting in six α-helices and seven β-strands **(Kobayashi et al. 1992; De and Funatsu 1992; Inokuchi et al. 1993**). Interestingly, CAS I and CAS II domains are localized in the central β-strand and in the relative parallel α-helix, respectively (**Kurihara et al. 1992**).

A turning point in the studies of T2 RNases was attributed to McClure and colleagues, who demonstrated a high homology between the *A. oryziae* T2 RNase and T2 glycoprotein in plants (**McClure et al. 1989**). These investigations not only confirmed the presence and the conservation of the same CAS I and CAS II active sites also in plant T2 RNAse, but also identified a new function related to this class of enzymes in plants. Further studies demonstrated that, whereas the biochemical and structural characteristics have been preserved in a wide variety of T2 enzymes deriving from different organisms, some of them gained in addition alternative and unexpected biological roles, showing a pleiotropic nature (**Deshpande and Shankar 2002**).

The discovering of new additional characteristics and biological features of these enzymes further fuelled a great interest in the scientific community, mostly because the different functions were often associated with a particular subcellular localization.

Indeed, besides their canonical localization in the cytosol and nucleus, T2 RNases can be found into cytoplasmic vacuoles, lysosomes or, in many cases, they are secreted in the extracellular environment (**Morikawa 1967; Wiener and Ashworth 1970; Nakamura et al. 1989**), a tissue compartment in which RNA molecules were not usually found (**MacIntosh 2011**). For example, the T2 RNases from *Saccharomyces cerevisiae* (Rny1), was found to be released from the vacuole to the cytosol during oxidative stress in order to degrade tRNAs, independently from its catalytic activity (**Luhtala and Parker 2010**). This suggest the occurrence of a direct link between particular or specific roles of these proteins and their cellular localization, where the lost of the enzyme's catalytic activity is apparently associated with an atypical novel specialized biological role.

In this context, several studies carried out in the last decades have unveiled an impressive range of biological roles for T2 RNases both in both plants and animals, such as the regulation of self-pollination (**McClure et al. 1989; Huang et al. 1994**), a nutritional role in phosphate scavenging (**Nürnberger et al. 1990; Löffler et al. 1992**) and in nitrogen preservation (**Van Damme et al. 2000**), the ability to counteract harmful agents (**Irie 1999**), the control of cell senescence (**Acquati et al. 2001**), the recruitment of reactive oxygen species during stressful condition (**Caputa et al. 2016**), the control of the apoptotic process (**Wang et al. 2014**) or the triggering of cytotoxic events (**Huang et al. 1994**).

A single member of T2 RNase family, the *RNASET2* gene, was also found in human genome, encoding a protein with three different isoforms with a molecular weight of about 36, 31 and 27 kDa respectively, deriving from post-translational modifications. The 36 kDa isoform

represents the extracellular, secreted form, with a protein length of 256 amino acids, while the two smaller isoforms are localized in the cytoplasm (**Campomenosi et al. 2006**).

The role of human *RNASET2* as an oncosuppressor gene in different type of cancers has been well investigated and a marked anti-cancer action was recognized in ovarian carcinoma (**Acquati et al. 2001, 2005; Lualdi et al. 2015**), malignant melanoma (**Monti et al. 2008**), lymphoid neoplasm (**Steinemann et al. 2003**) and malignant glioma (**Kim et al. 2006**). Moreover, human RNASET2 has also been recently involved in other human pathologies, such as cystic leukoencephalopathy, vitiligo and possibly several autoimmune disorders (**Henneke et al. 2009; Wang et al. 2015**). Interestingly, *in vivo* studies carried out on human ovarian tumorigenic models revealed how, during tumor growth, cancer-cells-derived extracellular RNASET2 establishes a continuous cross-talk with the surrounding tumor microenvironment, by recruiting immunocompetent cells to the tumor area. In particular, M1-polarized macrophages were clearly detected in RNASET2-ovexpressing tumors, infiltrated among cancer cells, suggesting that macrophages recruitment was fundamental in RNASET2-mediated tumor suppression (**Acquati et al. 2011**).

Coupled to more recent data reporting RNASET2 as a stress response gene (**Wang et al. 2014; Lualdi et al. 2015; Caputa et el. 2016**) these data apparently place RNASET2 in the growing family of “alarmins”, molecules passively released by necrotic cells or actively secreted by epithelial or immune cells in order to signal to the innate immune system the occurrence of dangerous events, such as a growing tumor mass or a bacterial infection (**Oppenheim and Yang 2005; Coffelt and Scandurro 2008**).

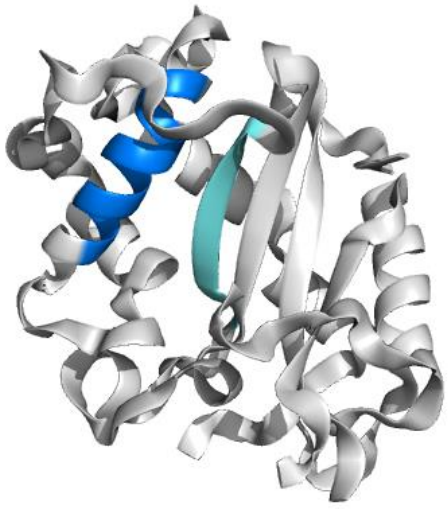

Figure 2. Crystal structure of the human RNASET2 protein (PDB code: 3t0o). In light blue and blue are represented the active sites CAS I and CAS II respectively.

Transferase-type RNases and host defence

In the last decades, a great attention has been focused on the antibacterial role of members of the RNase A superfamily in vertebrates and of T2 RNases in plants, in which the ability to counteract pathogens is displayed in different ways.

Indeed, several RNase A family members display a powerful antimicrobial activity and represent essential effectors involved in promoting host defenses. Among them, human RNase 3, also called eosinophil cationic protein (ECP), is a cationic arginine-rich protein released by white blood immune cells (**Ackerman et al. 1983; Boix et al. 2001**) displaying antiparasitic properties, evaluated and confirmed *in vitro* (**Lehrer et al. 1989; Hamann et al. 1990**). Of note, the canonical ribonucleases activity of this molecule has been lost and but it is not essential for the role played by this protein in the inflammatory processes mediated by eosinophils. In particular, ECP is released from the inner secondary cytoplasmatic granules to the extracellular environment during the activation of eosinophils and it makes the external membranes of infecting bacteria more permeable and susceptible of disruption (**Carreras et al. 2003; Torrent et al. 2007, 2008**). The direct effect of ECP on different bacterial species (**Torrent et al. 2010**) is due to its high affinity for lipopolysaccharides (LPS), the outer component of Gram-negative bacterial cell wall (**Pulido et al. 2012, 2016**). In addition, it has been observed that the N-terminal region of ECP triggers the agglutination and the aggregation of the microorganisms, thus promoting a systematic elimination by immune cells (**Pulido et al. 2012**).

Other human class A ribonucleases, such as RNase 2 and RNase 7, act as alarmins. As such, alarmins are known to promote inflammatory responses, often via Toll-like receptors family members (TLRs) (**Harder and Schroder 2002; Yang et al. 2008**). Among TLRs, TLR2 and TLR4 represent the most significant group of PRRs (Pattern Recognition Receptors), proteins expressed on the immune cell membranes which are evolutionary conserved in both vertebrate and invertebrate species (**Coscia et al. 2011; Molteni et al. 2016**). These receptors mediate the recognition of conserved bacterial biomolecules known as Pathogen-Associated Molecular Patterns (PAMPs), such as lipoteichoic acid (LTA) and lipopolysaccharide (LPS), which are normally displayed in the external membranes of Gram-positive and Gram-negative bacteria, respectively. Noteworthy, RNase 2 acts as an alarmin in order to activate the Toll-like receptor 2 (TLR2) in dendritic cells and to improve immune response mediated by T2

helper lymphocytes (**Yang et al. 2008**). Similarly, RNase 7 promotes inflammatory response mediated by TLR2 and TLR4 receptors after its activation due to different released inflammatory stimuli such as interferon-gamma (INF-γ), tumor necrosis factor-alpha (TNF-α) or interleukin-1beta (IL-1β) during bacterial infection (**Harder and Schroder 2002**). Moreover, it possesses also a high affinity to peptidoglycans (PNG) presented in bacterial cell-wall that promotes its antimicrobial activity (**Torrent et al. 2010**). Besides the most known human RNase 2 and RNase 3, defensive functions have been also identified for the cationic RNase A-2, presents in the leukocytes of the chicken *Gallus gallus* (**Nitto et al. 2006**) and for three RNase A enzymes in the zebrafish model *Danio rerio* (**Cho and Zhang 2007**). Furthermore, in the amphibian *Rana pipiens*, another RNase A member, known as Onconase, was shown to display an active role in immune response and tumor suppression. In fact, in bullfrogs this protein is normally involved in the oocytes defense even in the earlier embryonic stages, acting against possible external harmful invaders.

Strikingly, the antimicrobial role of some vertebrate RNase A proteins is very similar to that of T2 RNases of plants. For example, RNase MC1 in *Momordica charantia* (**Ide et al. 1991**) and the RNase LC1 and LC2 in *Luffa cylindrica* (**Irie 1999**) protect germinal seeds from different harmful agents and the RNase NE from tobacco, a S-like RNase, inhibits the growth of specific fungal hyphae (**Hugot et al. 2002**).

Of note, other S-like T2 RNase genes are activated during damages and mechanical wounds related to insect feeding, including RNS1 (discovered in the plant model *Arabidopsis thaliana*) or RNase NW and RNase Nk1, found in tobacco or other T2 enzymes proper of soybean and rice (**Ye and Droste 1996; Kariu et al. 1998; MacIntosh et al. 2010**).

All these evidences suggest that, besides the physiological RNA metabolism role, new and different biological activities have been acquired by T2 RNases in the evolutionary scale for each organism and, among them, a crucial role in immune modulation (**Acquati et al. 2011, 2013**) and likely in tissue regeneration has emerged.

Innate immunity modulation and tissue regeneration

The innate immune response serves not only to eliminate infections following injury but also to maintain homeostasis, functional integrity, and modulate the healing process (**Martin 1997; Saltzman 1999; Frantz et al. 2005**). Among the cellular effectors of innate immunity, recent data suggest that macrophages play a predominant role in the clearance of cellular debris, in

the synthesis of growth factors and cytokines and in promoting extracellular matrix (ECM) remodeling and tissue regeneration. Recently, different studies have highlighted the existence of two subtypes of macrophages, known as M1 and M2 respectively, which show different morphological and functional hallmarks that are specific in relation to the surrounding immunological microenviroment. In particular, the M1 macrophages present a pro-inflammatory phenotype, being activated by INF-γ, microbial stimuli or cytokines, such as TNF-α and GM-CSF, and producing pro-inflammatory effectors molecules. However, during the control and the retrieval of the correct tissues homeostasis after injuries, the dynamic transition to the M2 phenotype result extremely important. In fact, the anti-inflammatory M2 macropaghes predominate during the repair and regenration phases of damaged tissues and organs in order to reduce an inflammatory condition, which is harmful if it persists over time (**Sciorati et al. 2016**).

Thus, the successful and correct regeneration of tissues requires the specific coordination of several processes, which include a rapid immune response stimulated by cytokines produced by inflammatory cells (**Eming et al. 2009; Aurora and Olson 2014**).

In this context, TLRs play a key role not only in host defence by regulating both innate and adaptive immune responses (**Takeda and Akira 2005; Huang et al. 2008**), but are also involved in the different phases of wound healing, promoting regenerative responses. Indeed, their activation causes the recruitment of inflammatory cells and improves the fibrogenic response of fibroblasts in order to modify tissue lesions (**Serra et al. 2017**). Interestingly, recent studies revealed that macrophages and other immune cells, by activating TLR4 and TLR2 pathways, play a critical role in tissue regeneration as well (**Wynn and Barron 2010; Oishi and Manabe 2016**). They produce a variety of factors that stimulate the proliferation, differentiation and activation of fibroblasts, epithelial cells, endothelial cells, stem cells and progenitor cells that promote tissue repair (**Wynn and Vannella 2016**).

Although it is clear that T2 RNases, TLR4 and TLR2 play a crucial role in activating and/or in recruiting innate immune cells in relation to the inflammatory response, the relationship between these molecules in orchestrating immune response, wound healing and tissue regeneration has been poorly investigated. Therefore, innovative studies are necessary to provide new information on how these molecules are connected to each other and how they possibly cooperate in the regulation and modulation of these intimately connected processes.

It is well known that the use of animal models is crucial in wound healing and immune response researches, as they provide a means of studying the complex cellular and molecular interactions occurring in living tissues. In the last decades, several studies related to the administration of new drugs or molecules were essentially based on the use of vertebrates, which allowed assessing their effects both at the whole body and at the cellular level. However, ethical and economic conditions are actually leading to a consistent reduction of their use, with the aim of identifying alternative testing methods. As a result, the choice fell back on invertebrates and non-mammalian species, which are becoming more and more appreciated as alternative experimental models, mainly due to the underlying consistent biological analogies between all these species.

In this regard, invertebrates have found considerable space in medicine and in biology, taking place in the preliminary tests associated to administration of drugs related to human and animal diseases and in many other fields of research, including immunity, development, tissue remodeling, memory, learning, behavior, neurotoxicology and aging (**Wilson-Sanders 2011**).

Among these, the medicinal leech is a powerful model system for acquiring a new vision of the basic mechanisms of both innate immune response and regenerative processes, which are surprisingly similar to those of vertebrates. Moreover, the use of medicinal leech avoids ethical problems and is more cost-effective when compared to vertebrate models.

In addition, any biological response evoked in leeches by a wide range of different stimuli is easily interpretable due to its simple anatomy and can be detected within a short period of time (30 min–24 h). These features permit to analyze different morphological and molecular processes related to the various treatments (**Grimaldi 2016; Grimaldi et al. 2018**).

In order to better understand the molecules regulating the leech immune response and regenerative events, we first provide a short description of the animal model anatomy, of the cells and of the mechanisms involved in the related processes.

The leech body anatomy

The medicinal leech has a parenchymatous body with a reduced coelom, characterized by a relatively simple anatomy. The external surface is covered by a resistant cuticle, which is produced by the underling epithelial cells. This essential barrier not only protects the entire body wall from any potentially harmful agent, but also it prevents it from drying. Moreover, it also takes part in respiration, osmoregulation and excretion processes. Underneath the cuticle

and epithelium, there are three layers of helical muscle fibers (**Rohlich 1962; Forrester and Sawyer 1974**). In each layer, muscles are grouped in small bundles separated by scant extracellular matrix. Such tissue organization is known as musculocutaneous sac and it results separated from the inner digestive apparatus by a thick layer of loose connective tissue. In addition, in this collagenous matrix, gonads, nervous system and a reduced circulatory system are present, besides two characteristic tissues proper of leeches: the botryoidal and vasofibrous tissues, which are differently involved. The botryoidal tissue gives rise to new vessels and immunocompetent cells, specifically the leucocytes deriving from the myeloid lineage (**Grimaldi 2016; Grimaldi et al. 2018**). In fact, it possesses a myelo/erythroid and storage function (**Grimaldi et al. 2006**), besides playing a crucial role in vasculogenesis, angiogenesis (**de Eguileor et al. 2001a, b**). Of note, it is formed by two types of cells: botryoidal and endothelial cells. The former of the first is large, round or oval shaped with a cytoplasm containing numerous granules of different sizes. On the other hand, endothelial cells are small and flattened, with less granules in the cytoplasm. These two types of cells are linked by desmosome-like junctions and separated from the connective tissue by a basal lamina. After injuring, cytokine injection or bacterial challenging, the beginning of vasculogenesis is characterized by a remodelling of the botryoidal tissue. In fact, the cells change their shape, and from a solid cord a tubular pre-vascular structure is obtained. At the same time, hematopoietic stem precursor cells (HSPCs) become evident in the centre of the immature vessel lumen. The numerous circulating HSPCs are conveyed into the lesioned/stimulated area via new vessels and, after crawling between adjacent endothelial cells, they leave the bloodstream and disperse in the surrounding connective tissue where differentiate in mature leukocytes (i.e. macrophage-like, NK-like and granulocyte-like cells) that mediate the inflammatory response (**Grimaldi et al. 2006; Grimaldi 2016**).

Indeed, the vasofibrous tissue is involved in both tissues repair (**Huguet and Molinas 1994, 1996; Grimaldi 2016**) and immune response. It is constituted by the association of two different cell types: the vasocentral and the vasofibrous cells. The vasocentral cells show a few large granules inside the cytoplasm and they are normally surrounded by the vasofibrous cells, which instead are characterized by containing numerous small highly electron-dense granules. After an injury, numerous vasofibrous tissue cells migrate towards the lesioned area, crossing the muscle layers. At the lesion site, the vasocentral cells detach from the vasofibrous cells, differentiate into myofibroblasts (**Grimaldi et al. 2011**) and form the pseudoblastema (**LeGore**

and Sparks 1971; Huguet and Molinas 1994, 1996), whose retraction allows the wound closure. On the other hand, the vasofibrous cells give rise to granulocytes, involved in innate immune response (**Huguet and Molinas 1994, 1996; Grimaldi et al. 2011**).

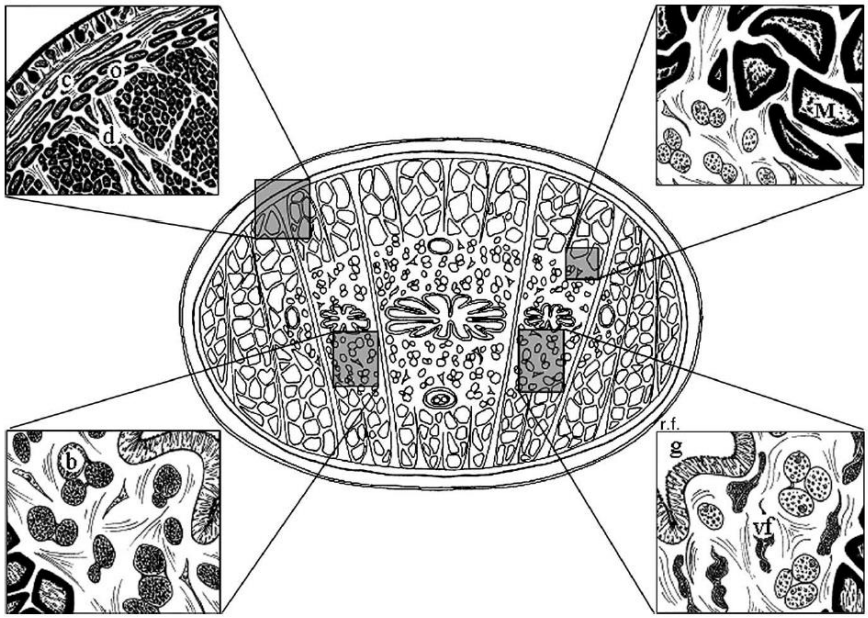

Figure 3. Drawing of the cross-sectioned body wall of *medicinal leech.* External cuticle and epithelium recover the muscular layers characterized by the presence of circular (c), oblique (o), longitudinal (l) and dorsoventral (d) fibres.
Under the musculocutaneous sac, the botryoidal (b) and the vasofibrous (vf) tissues are located within a scant connective tissue localized between the musculocutaneous sac and the inner gut (g).
Reprinted (adapted) with permission from (**Tettamanti et al., 2003**). Copyright (2003) Elsevier Science Ltd.

Molecules and cells and involved in leech immune response

In the medicinal leech, the immune response relies exclusively on innate immunity and the various processes related to hematopoiesis, neovascularization, inflammation are regulated by the same key molecules present in vertebrates, such as cytokines and growth factors. These molecules are produced by immune cells both in vertebrates (**Bacon et al. 2002**) and invertebrates (**Ottaviani et al. 2004; Tettamanti et al. 2006**) and they induce the production of other cytokines, chemokines and growth factors. Their functional conservation in leech has been widely demonstrated by previous investigations, in which the directly injection in the leech body wall of human cytokines and growth factors promoted hematopoiesis, vascular growth, immune cells migration and differentiation. Indeed, in stimulated leeches (wounded or injected with LPS or growth factors), the expression of VEGF (Vascular Endothelial Growth Factor), EGF (Epidermal Growth Factor), bFGF (basic Fibroblast Growth Factor), GM-CSF

(Granulocyte Macrophage-Colony Stimulating Factor) and AIF-1 (Allograft Inflammatory Factor 1) regulates the formation of new vessels and the recruitment of immune cells (**Grimaldi et al. 2006; Tettamanti et al. 2006; Schikorski et al. 2008; Schorn et al. 2015a, b**). These myeloid-derived cells, have been widely characterized, both morphologically and by the expression pattern on their membranes of specific cluster of differentiation (CD) (**de Eguileor et al. 2000a, b; Grimaldi et al. 2004; Macagno et al. 2010; Schorn et al. 2015a, b**). CD56 and CD57 positive NK-like migrating cells present a low ratio nucleus/cytoplasm, numerous lysosomal granules involved in exocytosis of toxic substances, abundant mitochondria and a developed endoplasmic reticulum. Macrophages, which derive from circulating monocytes, express CD61, CD68, CD14, AIF-1 and possess a high nucleus/cytoplasm ratio. Their cytoplasm contains numerous phagolysosomes, and the cell surface is irregular and characterized by the presence of pseudopodia, which are involved in phagocytosis and are useful during migration in the connective tissue. Two types of granulocytes (type I and type II) have also been founded, their differences being essentially based on the size and the shape of their cytoplasmic granules. Moreover, they express different markers and show diverse behavior during the innate immune response depending on the "non-self" characteristics. Type I granulocytes are $CD11b^{+}$ cells originating from the vasofibrous tissue and presenting small and round granules. In response to bacteria, such as *Escherichia coli*, the content of these granules is extruded from the cells (**de Eguileor et al. 2000b, a; Grimaldi et al. 2011**), suggesting that some antimicrobial peptides could be present inside them, as observed for those of oligochaete granulocytes (**Valembois et al. 1986; Dales and Kalaç 1992**). By contrast, type II granulocytes are positive for CD11c and derive from the botryoidal tissue. They are the first cells involved in the survey of large biotic agents, in terms of dimensions, such as a protozoan or a parasite nematode. The content of their large and irregular granules creates a melanin capsule for isolating the parasite from the host tissues (**de Eguileor et al. 1999a, 2000a**).

Following the introduction of foreign agents (bacteria, yeasts, spheres) or after injuries and transplantations, leech immune cells migrate towards the stimulated area through the loose connective tissue surrounding the muscle fibers and perform various and well-orchestrated functions depending on the recognized antigens (PAMPs or DAMPs). As in vertebrates, both PAMPs and DAMPs detection in leech implicate the specific sensing receptor TLRs. In particular, the TLR4-like molecule expressed by macrophages and granulocytes of leeches specifically recognizes LPS and its triggering induces intracellular signaling events

involving the cytoplasmic adaptor molecule myeloid differentiation factor 88 (MyD88) and leading to the production of the proinflammatory cytokines such as TNF-α-like (**Girardello et al. 2019**).

Molecules and cells and involved in leech regenerative events

In the medicinal leech, as in vertebrates, the ECM is a "reservoir" for signalling molecules modulating different processes such as angiogenesis, inflammation, cell proliferation and migration (**Tettamanti et al. 2005; Grimaldi et al. 2011**). Moreover, the ECM acts as a "scaffold" during tissue development and repair, providing structural support and attachment sites for cell surface receptors (**de Eguileor et al. 1999a**). Indeed, following a. lesion or a bacterial infection, several factors such as EGF (epithelial growth factor), bFGF (basic Fibroblast Growth Factor), Cathepsin B are by produced by fibroblasts and immune cells and regulate the reorganization of the connective tissue (**Grimaldi et al. 2004**). All these molecules promote new collagen production by fibroblasts, which are the main effectors of such remodelling (**Grimaldi et al. 2004; Tettamanti et al. 2005**). These cells, once activated, begin to proliferate and are characterized by projections of cytoplasmic laminae stretching towards the extracellular space (**Tettamanti et al. 2004**). These cytoplasmic folding form a microenvironment in which fibrillogenesis occurs and these projection are involved in the spatial organization of the collagen bundles and in the control of the orientation of the collagen fibrils in the ECM (**Tettamanti et al. 2005**). The resulting scaffold is then used for immune cells migration and for the growth of new vessels (**de Eguileor et al. 2004; Tettamanti et al. 2004**).

Since several data in literature suggest that the highly conserved T2 RNases are activated during host defense against different type of pathogens or after damages and mechanical wounds, we hypothesize that this RNase could be involved in a complex stress-response process triggered by a wide range of stimuli (such as bacterial infection) and involving tissue regeneration and the establishment of a functional cross-talk between the inflammatory response and the process of wound healing. However, the mechanisms by which these two processes are intimately connected and regulated by these enzymes are still far from being fully elucidated.

AIM

The aim of this project is to make a significant contribution to the current knowledge status concerning the ability of T2 RNases in orchestrating a functional cross-talk between the innate immune system and tissue regeneration, using as animal model the medicinal leech *Hirudo verbana*, an emerging experimental model which is cost-effective, easily manipulable and devoid of significant ethical considerations in relation to its use and regulatory restrictions.

Starting from these premises, my thesis project is divided according to the following schedule:

- *Evaluating the effects of the human recombinant RNASET2 in the medicinal leech*

  Detailed morphological and immunohistochemical analyses are carried out after the human recombinant protein hRNASET2 injection in the leech body wall, in order to investigate the effects of this enzyme on the leech tissues and its direct involvement immune response regulation.

- *In vitro matrigel assays: characterization of the cell types infiltrating rhRNASET2-supplemented Matrigel (MG)*

  To confirm the ability of hRNASET2 to induce macrophages migration *in vivo*, recombinant hRNASET2 protein is added to MG biopolymer held at a liquid state and the mixture will be subsequently inoculated in leeches. Vehicle-treated MG are used as a negative control. To characterize the cell types, infiltrating the polymerized biopolymer, the MG solid pellets formed following inoculation is explanted after 1 week and process for standard morphological and immunofluorescence analyses using the specific macrophage markers.

- *Evaluation of the endogenous leech RNASET2 expression after LPS treatment*

  To investigate the role of the endogenous RNASET2 enzyme in innate immune response, the leech T2 RNase expression level is investigated upon stimulation in LPS challenged leeches. The immunophenotype of cells involved in the inflammatory response and expressing the leech RNASET2 are evaluated by immunofluorescence and western blot assays. In particular, double-immunolocalization of RNASET2 and of macrophage markers *Hm*AIF-1 and CD68 will be carried out.

- *Investigation of the RNASET2 and AIF-1 immunomodulator role after LPS treatment*

  The temporal expression profiles of RNASET2 and AIF-1 are evaluated after LPS challenge performing morphological, immunofluorescence and western blot assays. Cell count based on double immunofluorescent analyses reveal that two different cells types are activated during the different phases of innate immune response. To better understand if these cells are involved in RNASET2 production, immunogold assays are performed. Moreover, to observe the direct role of RNASET2 against bacteria, in vitro and in vivo analyses are carried out using *P. aeruginosa* expressing GFP bacterial strain.

- *Cloning and characterization of the RNASET2 gene and protein from H. verbana*

  The coding sequence of RNASET2 from *H. verbana* is isolated as a cDNA recombinant clone by means of degenerate oligonucleotide primed RT-PCR. Interrogation of the leech genomic database is also evaluated as a potential source of DNA sequence information. Subsequently, a recombinant protein (r*Hv*RNASET2) is produced in the yeast *Pichia pastoris* expression system.

- *Evaluation of the rHvRNASET2 antibacterial role*

  For this purpose, leeches are treated with lipoteichoic acid (LTA) at different time points. Morphological analyses are performed to observe the ability of LTA to induce an inflammatory response. Subsequently, the expression of *Hv*RNASET2 is assessed by immunofluorescence and western blot assays. Moreover, the expression level of the cell markers CD11b and AIF-1, of the TLR2 receptor and of the pro-inflammatory cytokine TNF-α are evaluated by immunofluorescence and western blot.

  Finally, the antibacterial of the cloned recombinant *Hv*RNASET2 enzyme is investigated with *in vitro* and *in vivo* assays. The direct effect on the $Gram^+$ bacteria *S. aureus* is evaluated by optical, TEM and SEM images and after its direct injection in the leech body wall.

- *Connective tissue remodeling induced by HvRNASET2*

  Morphological analysis, Masson Trichrome and Sirius Red colorimetric assays are conducted to better understand the direct role of *Hv*RNASET2 in regulating connective tissue remodeling after the r*Hv*RNASET2 injection in leech. The expression of the bFGF-receptor,

involved in fibroblast activation, and of the collagen 1, which is produce during tissue remodeling are investigated by immunofluorescent analysis. To confirm the evidence that *Hv*RNASET2 induce extracellular matrix remodeling, the leech recombinant enzyme is also tested *in vivo* on human MRC-5 fibroblast cell culture.

Expected outcome

The data obtained in this project will provide new experimental data in support of a role for RNASET2 as a key component of the natural immune system not only in vertebrates, but also in invertebrates. These data would confirm a pleiotropic role for *Hv*RNASET2 in orchestrating an evolutionarily conserved strong innate immune response upon inflammatory stimuli, based on macrophage recruitment and activation, extracellular remodeling and antibacterial activity. Moreover, the consolidated biopolymer *in vivo* cell sorting method, which allows the isolation of specific cell populations in relation to the cytokines utilized and the possibility to subsequently culture these cell types, will provide an invaluable tool for studying *Hv*RNASET2 activity.

RESULTS

CHAPTER 1

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REGULAR ARTICLE

Human recombinant RNASET2-induced inflammatory response and connective tissue remodeling in the medicinal leech

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ABSTRACT In recent years, several studies have demonstrated that the *RNASET2* gene is involved in the control of tumorigenicity in ovarian cancer cells. Furthermore, a role in establishing a functional cross-talk between cancer cells and the surrounding tumor microenvironment has been unveiled for this gene, based on its ability to act as an inducer of the innate immune response. Although several studies have reported on the molecular features of RNASET2, the details on the mechanisms by which this evolutionarily conserved ribonuclease regulates the immune system are still poorly defined. In the effort to clarify this aspect, we report here the effect of recombinant human RNASET2 injection and its role in regulating the innate immune response after bacterial challenge in an invertebrate model, the medicinal leech. We found that recombinant RNASET2 injection induces fibroplasias, connective tissue remodeling and the recruitment of numerous infiltrating cells expressing the specific macrophage markers CD68 and *Hm*AIF-1. The RNASET2-mediated chemotactic activity for macrophages has been further confirmed by using a consolidated experimental approach based on injection of the Matrigel biomatrice (MG) supplemented with recombinant RNASET2 in the leech body wall. One week after injection, a large number of $CD68^+$ and *Hm*AIF-1^+ macrophages massively infiltrated MG sponges. Finally, in leeches challenged with lipopolysaccharides (LPS) or with the environmental bacteria pathogen *Micrococcus nishinomiyaensis*, numerous macrophages migrating to the site of inoculation expressed high levels of endogenous RNASET2. Taken together, these results suggest that RNASET2 is likely involved in the initial phase of the inflammatory response in leeches.

Keywords T2 RNases · *Hirudo* · Immune response · Macrophages · Inflammation

INTRODUCTION

Ribonucleases represent a wide family of enzymes whose common feature is the ability to process or degrade ribonucleic acids, yet our understanding of the roles played by these proteins in mammalian biology remains incomplete. Historically, investigations have focused on ribonucleases belonging to the RNase A family members, which are limited to vertebrate species (D'Alessio and Riordan 1997). However, the RNase superfamily also includes lineages of structurally related and evolutionarily conserved enzymes that have been shown to play a wide range of biological functions. In this regard, a growing interest has recently focused on ribonucleases belonging to the highly conserved T2/Sh/R family, due to their involvement in several key cellular and biological processes, such as nutritional stress response, neural development, modulation of the immune response, cell death, cancer growth control and stress response, to name a few (Luhtala and Parker 2010). T2 ribonucleases represent transferase-type RNases that catalyze the cleavage of single-stranded RNA through a 2',3'-cyclic phosphate intermediate. Of note, unlike the other known transferase-type ribonuclease families (i.e., RNase A and T1), T2 RNases represent the most widespread class, being found in viruses, bacteria, protozoans, plants and metazoans (Luhtala and Parker 2010). This feature, coupled to their rather unspecific substrate preference (they usually cleave RNAs at all four bases), suggest the occurrence of one or more ancient, evolutionarily relevant roles for T2 RNases. In this regard, a bulk of experimental evidence has recently been reported in support for a role of T2 ribonucleases in immune responses. Indeed, human RNASET2 has been reported to behave as a tumor suppressor gene in ovarian cancer xenograft models by means of its ability to recruit host macrophages endowed with oncosuppressive properties toward the tumor mass in vivo (Acquati et al. 2011, 2013).

Although several studies have recently reported the widespread tumor suppressive role for several members of the T2 RNase protein family (Smirnoff et al. 2006; Schwartz et al. 2007; Acquati et al. 2011, 2013), the relationship between these enzymes and their ability to activate and/or recruit innate immune cells in relation to the inflammatory response has not yet been thoroughly investigated. In order to improve our understanding of these functional features of T2 RNases and at the same time to evaluate their evolutionary conservation, in this work we focus our interest on the role of human recombinant RNASET2 in the activation and recruitment of immune cells using as an experimental model *Hirudo verbana*, a medicinal

leech species closely related to *H. medicinalis*. Furthermore, the endogenous expression pattern of leech T2 RNase following induction of a pro-inflammatory stimulus is also investigated. *Hirudo* is a consolidated experimental animal model for several reasons: first, it represents a suitable tool for experimental manipulation, being cost-effective, easily manipulable and devoid of significant ethical considerations in relation to its use and regulatory restrictions. Moreover, the main immune response processes in leeches have been shown to be strikingly similar to those reported in vertebrates, since they involve similar cellular mechanisms and a common equipment of key molecules playing pivotal roles for regulating hematopoietic cells activation and differentiation. In addition, any response evoked in leeches by a wide range of different stimuli is activated within a short period of time (6–24 h) and is clearly and easily detectable due to the small size and anatomical features of this experimental model (de Eguileor et al. 1999, 2003, 2004; Grimaldi et al. 2004, 2006; Tettamanti et al. 2004; Girardello et al. 2015a, b; Schorn et al. 2015b). Finally, we recently developed an innovative method to investigate several key aspects of the immune response in leeches, based on the use of the biopolymer Matrigel (MG) to which various cytokines can be added before polymerization. Once inoculated in leeches, the polymerized matrigel gradually releases the supplemented cytokine, resulting in the specific recruitment of defined cell populations from the surrounding tissues within the MG matrix. Indeed, in recent reports, injection into the leech body of MG supplemented with growth factors, cytokines or bacterial lipopolysaccharides (LPS) has allowed us to analyze the specific in vivo recruitment of several cell populations, which can subsequently be characterized at both the morphological and immunocytochemical levels (Grimaldi et al. 2008, 2009, 2011; Girardello et al. 2015a).

In this work, we confirm and extend previous results on the role of RNASET2 in the immune response by showing that human recombinant RNASET2 (rRNASET2) triggers a significant recruitment of host macrophages when injected in leeches. Moreover, we report for the first time that rRNASET2 injection is followed by a boost in endogenous T2 RNase expression in the same macrophage cell population. Finally, the role of endogenous leech T2 RNase in the immune response is further confirmed by the observed upregulation of its expression following induction of an inflammatory challenge represented by LPS injection.

MATERIALS AND METHODS

Animals and treatments

Medicinal leeches (Annelida, Hirudinea; from Ricarimpex, Eysines, France) measuring 10 cm were kept in water at 20°C in aerated tanks and fed weekly with calf blood. For each experimental group, treatment (PBS, LPS, recombinant protein or matrigel injection) was performed at the level of the 80th superficial metamere on leeches anaesthetized with a 10 % ethanol solution. Treated and control untreated animals were anesthetized and then dissected to remove body wall tissues or matrigel pellets at specific time points (T24 h, T7 days). Animals were randomly split into eight separate experimental groups (five individuals for each time point) and submitted to various protocols and treatments.

Group 1: control animals (uninjected)

Group 2: control animals injected with 100 µl sterilized PBS (138 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) and analyzed at T24 h and T7 days.

Group 3: animals injected with 100 µl sterilized PBS containing 20 ng of recombinant hRNASET2 protein (Lualdi et al. 2015) and analyzed at T24 h and T7 days to functionally characterize cells migrating under the influence of this factor. The best concentration of hRNASET2 required to induce significant cell migration was determined based on our previous work (Grimaldi et al. 2011; Schorn et al. 2015b).

Group 4: animals injected with 100 µl of sterilized PBS containing 50 ng of recombinant hRNASET2 previously incubated for 1 h at room temperature with 1 µg of anti-hRNASET2 specific polyclonal antibody (Campomenosi et al. 2011) to perform antibody-mediated neutralization experiments, analyzed at T24 h and T7 days.

Group 5: animals injected with 100 µl of PBS containing 100 ng/ml of bacterial lipopolysaccharides (LPS; the major outer surface membrane components present in almost all Gram-negative bacteria) from *Escherichia coli* serotype 0111: B4 (Sigma, St. Louis, MO, USA) for immune stimulation assays (Alexander and Rietschel 2001) in order to evaluate the expression of RNASET2 in cells involved in innate immune response.

Group 6: control animals injected with 300 µl of liquid matrigel MG (an extract of the murine Engelbreth–Holm–Swarm tumor produced as previously described) (Kleinman et al. 1986),

analyzed at T7 days.

Group 7: animals injected with 300 µl of liquid MG supplemented with 50 ng of hRNASET2 were used to selectively isolate the cells migrating under the influence of RNASET2 and were analyzed at T7 days.

Group 8: animals injected with 300 µl of liquid MG supplemented with 50 ng of hRNASET2 and 1 µg of the polyclonal antibody RNASET2, used for antibody-mediated neutralization experiments and analyzed at T7 days.

Optical and electron microscopy

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

Acid phosphatase reaction (ACP)

Leech tissues, dissected from unlesioned or injected areas, were embedded in Polyfreeze tissue freezing medium (OCT; Polysciences, Eppelheim, Germany) and immediately frozen in liquid nitrogen. Cryosections (7 µm in thickness) were obtained with a Leica CM 1850 cryotome, rehydrated with PBS for 5 min and incubated with sodium acetate-acetic acid 0.1 M buffer for 5 min, followed by incubation with 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 min at 37°C. After washings in PBS, slides were mounted with PBS/glycerol 2:1 and observed with a Nikon Eclipse Ni (Nikon) light microscope. Images were taken with a digital camera Nikon Digital Sight DS-SM (Nikon).

Immunohistochemistry

Samples (leech tissues or matrigel pellets) were embedded in Polyfreeze tissue freezing medium (OCT; Polysciences) and immediately frozen in liquid nitrogen. Cryosections were then obtained with a Leica CM 1850 cryotome. For morphological analyses, serial cryosections were stained with crystal violet and basic fuchsin. For immunofluorescence assays, sections were rehydrated with PBS for 5 min, pre-incubated for 30 min with PBS containing 2 % bovine serum albumin and later incubated (1 h at 37°C) with the following primary polyclonal antibodies, diluted in blocking solution: rabbit anti-human RNASET2 (Campomenosi et al. 2011), diluted 1:200; rabbit anti-human CD68 (Santa Cruz Biotechnology, CA, USA), which specifically stains leech macrophage-like cells (de Eguileor et al. 2003; Schorn et al. 2015b), diluted 1:100; rabbit anti-*Hm*AIF-1, specific marker for leech macrophages (Drago et al. 2014; Schorn et al. 2015b), kindly donated by Prof. Jacopo Vizioli, University of Lille 1, France, diluted 1:1000; rabbit anti-human phospho-histone H3 (H3P; Merck-Millipore, Temecula, CA, USA), a known marker of proliferating cells (Hans and Dimitrov 2001), diluted 1:100.

All specimens were washed before incubation with the secondary antibodies (Abcam, Cambridge, UK) for 1 h at room temperature. The secondary antibodies used were a goat Cy5-conjugated (excitation filter 650 nm, emission filter 672 nm) or a goat fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (excitation 493 nm, emission 518 nm), both diluted 1:200. Double-labeling experiments were performed as previously described (Grimaldi et al. 2009; Schorn et al. 2015a) to detect RNASET2/*Hm*AIF-1 or RNASET2/CD68 co-expressing cells. The anti-RNASET2 antibody was applied first, then sections were incubated with the secondary antibody, goat anti-rabbit (Cy5)-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 Laboratories, West Grove, USA) at 1:25 for 2 h. After washing the samples were incubated with the antibody anti-*Hm*AIF-1 or anti-CD68. Subsequently, the sections were treated with the secondary (FITC)-conjugated goat anti-rabbit antibody. 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 min after immunohistochemistry substantially reduced tissue autofluorescence while preserving the

specific fluorochrome signal. In all control experiments, primary antibodies were omitted and sections were incubated only with the secondary antibodies. 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, 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, San Jose, CA, USA).

Western blot analysis

Cultured human ovarian cancer-derived OVCAR3 cells were scraped in PBS/EDTA and resuspended in lysis buffer (0,5 % Igepal, 0,5 % Triton X-100 in PBS + 5 mM EDTA). *Hirudo* tissues extracted from the unstimulated body wall or from injected areas were immediately frozen in liquid nitrogen and then homogenized with a mortar. The homogenates were suspended (10 µl per mg of tissue), in RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % dodium deoxycholate, 0.1 % SDS, 50 mM Tris-Hcl, pH 8.0) in the presence of a protease inhibitor cocktail (Sigma, Milan, Italy). The particulate material was removed by centrifugation at 13,000 rpm for 10 min at 4°C in a refrigerated Eppendorf Minispin microcentrifuge (Hamburg, Germany). 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 (Richmond, MA, USA).

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 polyclonal anti-RNASET2 (Campomenosi et al. 2011) or an anti-*Hm*AIF-1 antibody (1:5000 dilution in 5 % TBS-milk). After the membrane was washed three times with TBS-Tween 0.1 %, antigens were revealed with a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories), diluted 1:5000. After a further washing step, immunocomplexes were revealed with luminol LiteAblot PLUS Enhanced Chemiluminescent Substrate (EuroClone, Pero, Italy). In control experiments, anti-RNASET2 and anti-*Hm*AIF-1 antibodies were substituted with rabbit pre-immune serum (1:20,000). Bands were normalized by using the ImageJ software package

(http://rsbweb.nih.gov/ij/download.html), with the housekeeping protein D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was detected with a rabbit polyclonal anti-human GAPDH IgG (Proteintech, Chicago, USA), diluted 1:2000.

RESULTS

In order to evaluate the putative role of RNASET2 in the regulation of innate immune response in *Hirudo*, in particular in the activation and migration of macrophages, we first assessed the effects of recombinant human RNASET2 (rRNASET2) injection in the leech body wall. To this aim, morphological and immunohistochemical analyses were carried out on leech tissue sections from uninjured, PBS-injected, rRNASET2-injected (with or without preincubation of the recombinant protein with an anti-RNASET2 antibody) and LPS-injected animals. The same analyses were also carried out on rRNASET2-soaked, polymerized matrigel pellets explanted from the leech body wall after implantation for 1 week.

Morphological analysis of the body wall in rRNASET2-injected leeches

The body wall of unlesioned (Fig. 1a) or PBS-injected (Fig. 1b) leeches showed a typical cutaneous muscle sac, formed by epithelium and muscle fibers arranged in groups and surrounded by a loose connective tissue. In the body wall of these control animals, a few non-muscle cells were immersed in the connective tissue surrounding the muscles. By contrast, at 24 h (Fig. 1c) and 1 week (Fig. 1d, e) following rRNASET2 injection, a large number of infiltrating cells was clearly detected underneath the epithelium and in the connective tissue surrounding the muscle fibers. A marked reorganization of the muscle layers in rRNASET2-treated animals was also evident at the injection site, since the distance between single muscle fields appeared to be greatly increased, due to the infiltration of several cells interposed between them. This cell infiltration process was closely associated with a massive reorganization of the connective tissue and the appearance of new blood vessels in the space between the muscle fields (Fig. 1c–e). By contrast, in uninjured (Fig. 1a) and PBS-injected control leeches (Fig. 1b) or in leeches injected with rRNASET2 previously pre-incubated with the anti-RNASET2 antibody (Fig. 1f–h), muscle fields were in close reciprocal contact and both the infiltration of cells and the appearance of vessels between the groups of muscle fibers or underneath the epithelium were negligible (Fig. 1f–h).

Ultrastructural TEM analysis of unlesioned (Fig. 2a) or PBS-injected (Fig. 2b) leeches confirmed that muscle fibers forming the muscular fields were very close to each other and the extracellular matrix surrounding them was scant and devoid of cells. By contrast, 24 h (Fig. 2c, d) and 1 week (Fig. 2e, f) following rRNASET2 injection, clearly recognizable neovessels were observed underneath the epithelium, with the vessels lumen containing circulating cells. The observed angiogenic process displayed the typical features of an inflammatory response-derived process (Grimaldi et al. 2006), with circulating cells extravasating and migrating into the nearby connective tissue and thereby differentiating into mature leukocytes, likely involved in the leech innate immune response. Indeed, a high prevalence of cells displaying the typical appearance of macrophages was detected in the extracellular matrix underlying the epithelium (Fig. 2c) and surrounding the muscle fibers of rRNASET2-treated animals (Fig. 2d, e). These cells were clearly distinguishable by an elongated shape, an irregular membrane (likely involved in the formation of pseudopodia, a typical feature of cells involved in active migration) (Fig. 2e) and a cytoplasm with a large nucleus and a few electrondense granules (Fig. 2d). Besides the infiltration of immunocompetent cells, rRNASET2-treated leeches were also characterized by a massive fibroplasia across the entire body wall. As in vertebrates, leech fibroblasts are responsible for the synthesis and remodeling of the extracellular matrix (Tettamanti et al. 2005). These cells were easily recognizable by TEM analysis in rRNASET2-injected animals due to their tapered shape and the presence of several lipid droplets in their cytoplasm. Moreover, the fibroblasts' cell membrane was characterized by the presence of laminar projections, known to be involved in the formation of a microenvironment that promotes fibrillogenesis (Fig. 2f). As already observed by optical microscope analysis, the number of vessels and immunocompetent cells underneath the epithelium and between the groups of muscle fibers was very scant in animals injected with rRNASET2 that was pre-incubated with anti-rRNASET2 neutralizing antibody, (Fig. 2g, h), suggesting that the observed tissue morphological changes represent a specific effect of rRNASET2 treatment.

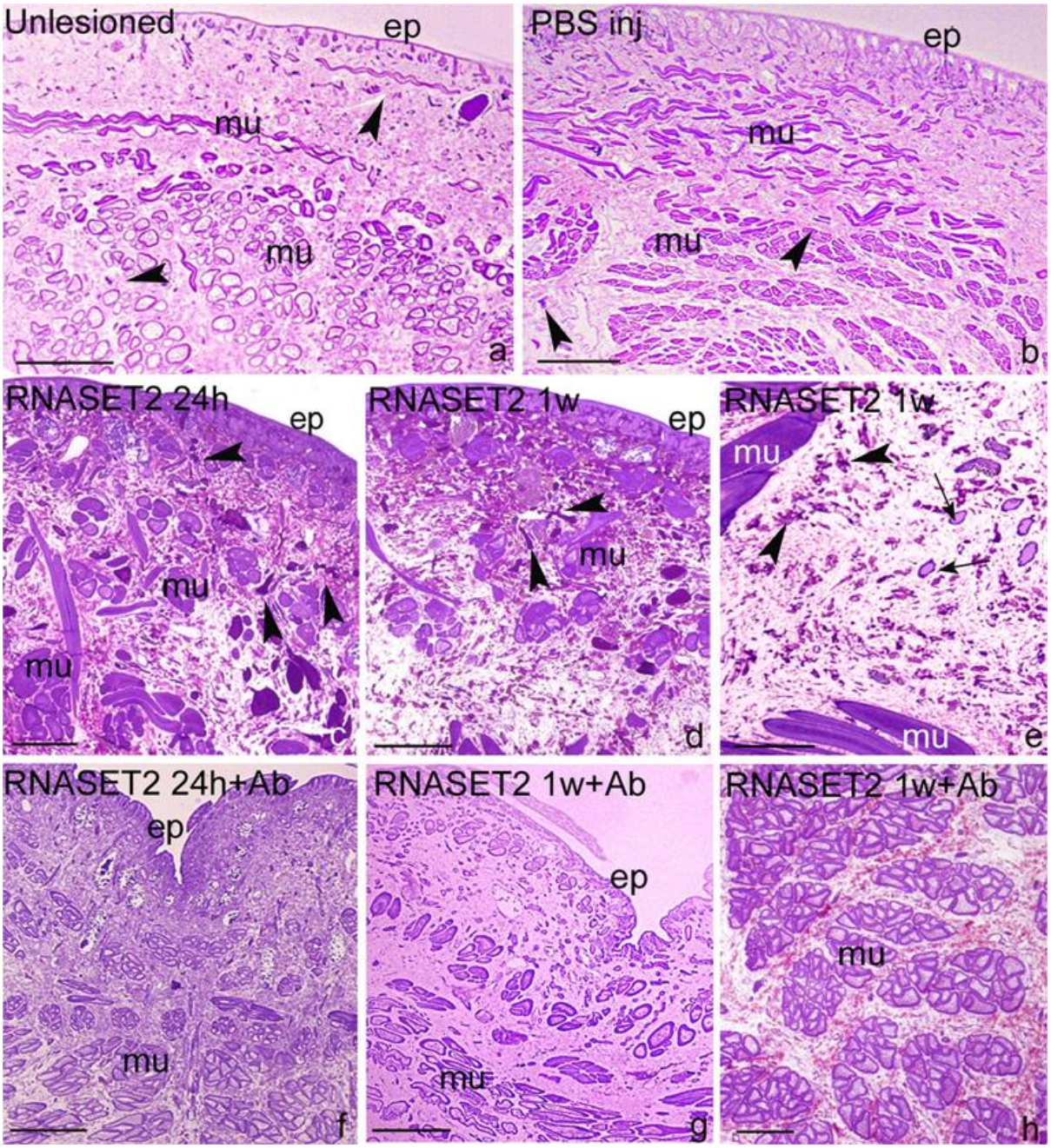

Fig. 1 Light microscope morphological analysis of leech body wall sections. In unlesioned(**a**) and PBS-injected (**b**) animals, a few cells (*arrowheads*) among muscle fibers are visible; 24 h (*24h*, **c**) and 1 week (*1w* **d, e**) following rRNASET2 injection, numerous migrating cells (*arrowheads*) and neovessels (*arrows*) are visible. Injection of rRNASET2 pre-incubated with the neutralizing anti-RNASET2 antibody (*Ab*) significantly reduces both cell migration and neo vessel formation (**f–h**). *mu* muscles; *ep* epithelium. *Bars* 100 µm

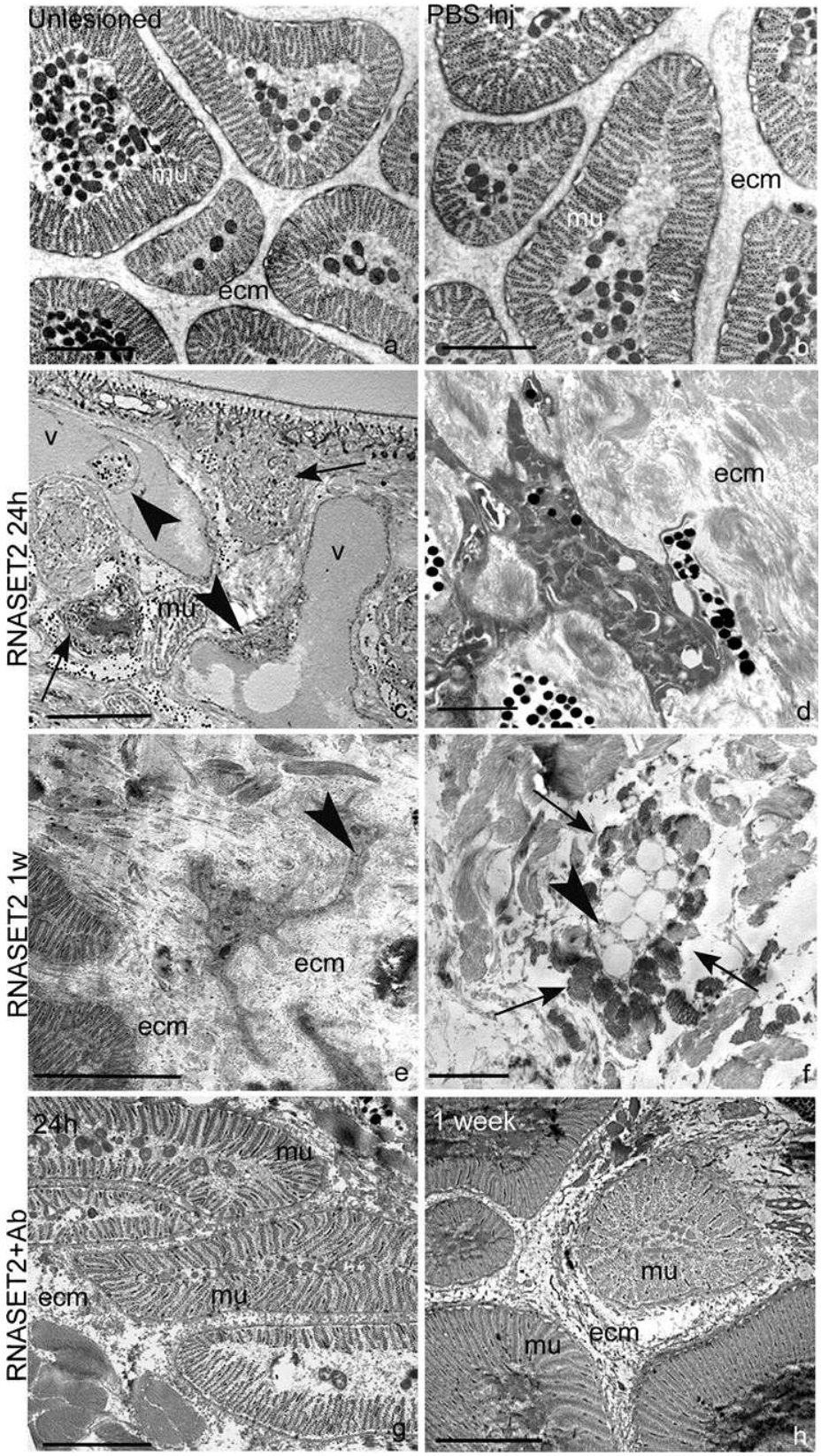

Fig. 2 Ultrastructural analysis at TEM of leech body wall: details of agroup of muscle fibers (*mu*) in unlesioned (**a**) and PBS-injected (**b**) leeches. Muscle fibers (*mu*) are close to each other and surrounded by scant extracellular matrix (*ecm*) devoid of cells. After 24 h from rRNASET2 injection (**c, d**), macrophages (*arrows*) and vessels (*v*) containing circulating cells (*arrowheads*) are visible underneath the epithelium. After 1 week from rRNASET2 injection, migrating macrophages (*arrowhead* in **e**) and fibroblasts (*arrowhead* in f) in active fibrillogenesis (arrows) are observable (**e–f**). The number of vessels and macrophages is highly reduced in rRNASET2 pre-incubated with anti-rRNASET2 Ab injected leeches (**g, h**). *Bars* (**a–c, e, g, h**) 5 μm, (**d, f**) 2 μm

Characterization of cell types involved in the inflammatory response induced by injection of rRNASET2

In order to better define the cell types recruited in leech tissues following rRNASET2 injection and thus confirm the occurrence of a RNASET2-driven inflammatory response, immunofluorescence experiments were performed on tissue cryosections from unlesioned, PBS-injected and rRNASET2-injected animals. Recombinant RNASET2-treated sections were investigated at both 24 h and 1 week, with and without neutralizing anti-RNASET2 antibody pretreatment (Figs. 3a– p; 4a–d). Immunohistochemical assays were carried out with an anti-*Hm*AIF-1 antibody, a known marker for leech macrophages (Drago et al. 2014; Schorn et al. 2015a, b) and an anti-human RNASET2 antibody, to evaluate the expression of endogenous RNASET2 by leech immunocompetent cells. By these assays, both *Hm*AIF-1 and RNASET2 turned out to be constitutively expressed at a low level in the body wall of uninjured animals. These proteins were mainly detected in a few cells located in the connective tissue underlying the epithelium and surrounding the muscle fibers fields (Fig. 3a–c). The same expression pattern was observed in the samples analyzed 24 h (Fig. 3e–g) after PBS injection, suggesting that the mechanical stress induced by injection of a saline solution did not affect significantly the expression of *Hm*AIF-1 and RNASET2 in the leech body wall. Of note, the presence of RNASET2-positive cells in uninjured and PBS-injected animals confirmed that the anti-human RNASET2 antibody used in immunofluorescence (IF) assays crossreacts with the endogenous leech T2 RNase protein, as further confirmed later by western blot analysis.

By contrast, a highly increased number of *Hm*AIF-1^+ and $RNASET2^+$ migrating cells were clearly recognizable 24 h (Fig. 3i–k) and 1 week (Fig. 4a, b) after rRNASET2 injection. These cells were mainly located under the epithelium and among muscle fibers. Strikingly, 24 h (Fig. 3m–o) and 1 week (Fig. 4c, d) from injection with rRNASET2 pre-incubated with the neutralizing anti-RNASET2 antibody, the number of migrating *Hm*AIF-1^+/$RNASET2^+$ cells was significantly reduced. Due to the limited amount of rRNASET2 injected, the widespread and intense staining observed by IF assay with the anti-RNASET2 antibody further suggests that the endogenous leech T2 RNase protein was detected by this antibody. No signal was detected in the negative control experiments in which the primary antibodies were omitted (Fig. 3d, h, l, p). Taken together, these data are indicative of a strong recruitment of cells belonging to the macrophage lineage at the rRNASET2 injection sites. Moreover, such recruitment seems to be associated by a concurrent increase in the expression of endogenous RNASET2 at the sites of

immune cells infiltration.

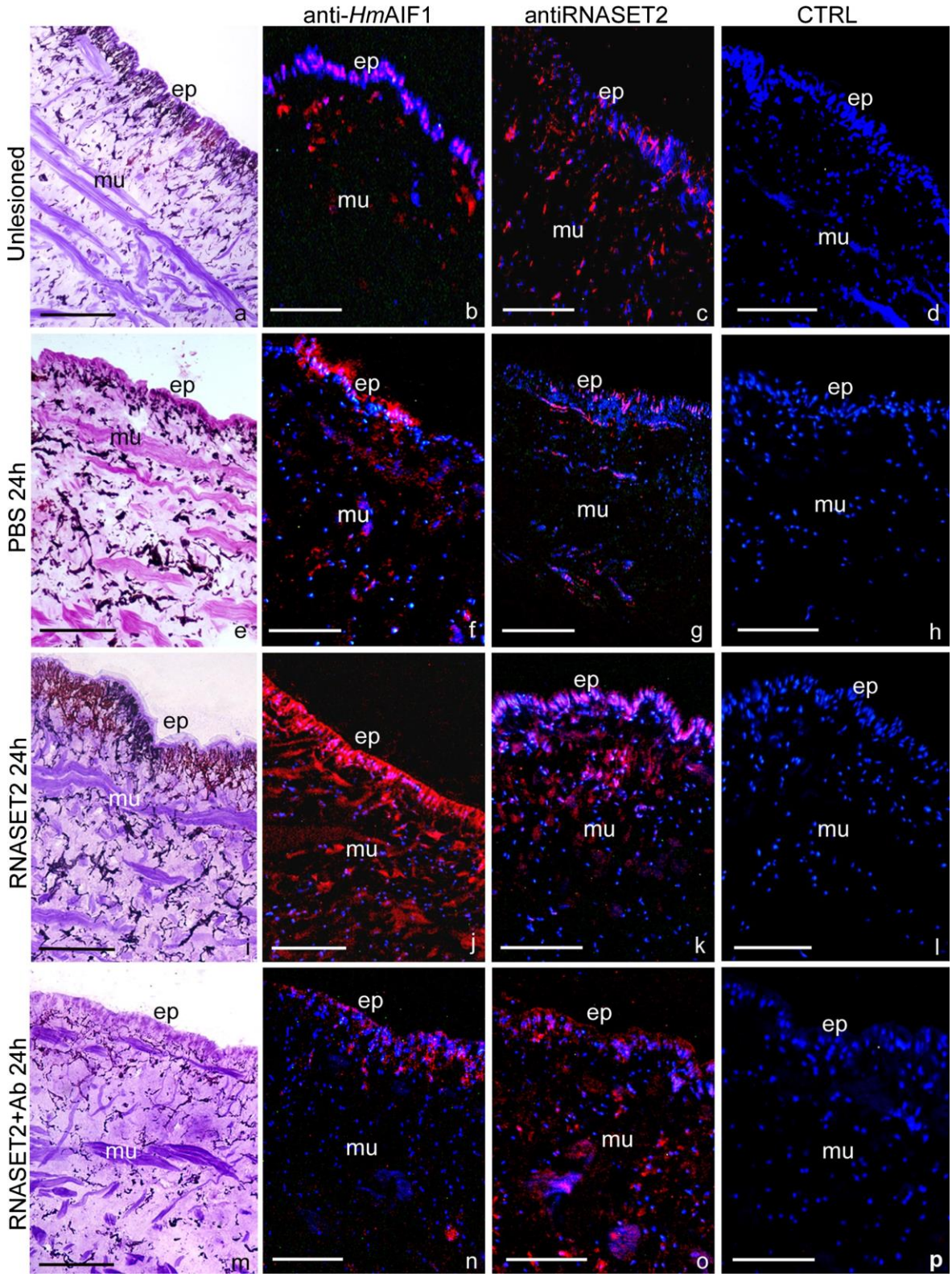

Fig. 3 Immunofluorescence analysis of cryosections from medicinal leech body wall in unlesioned (**a–c**), PBS-injected (**e–g**), rRNASET2-injected (**i–k**) and neutralizing antibody preincubated rRNASET2-injected animals (**m–o**). The anti-*Hm*AIF-1 and anti-RNASET2 antibodies stain in *red* a few cells among muscle fibers (*mu*) and underneath the epithelium (*ep*) in unlesioned, PBS-injected and anti-RNASET2-neutralized, rRNASET2-injected leeches, whereas several migrating immune-responsive cells located under the epithelium (*ep*) and among muscles (*mu*) are detected 24 h following RNASET2 injection. No signal is detected in negative control experiments in which the primary antibodies were omitted (**d, h, l, p**). Cell nuclei stained *blue* by treatment with DAPI. *Bars* 100 µm

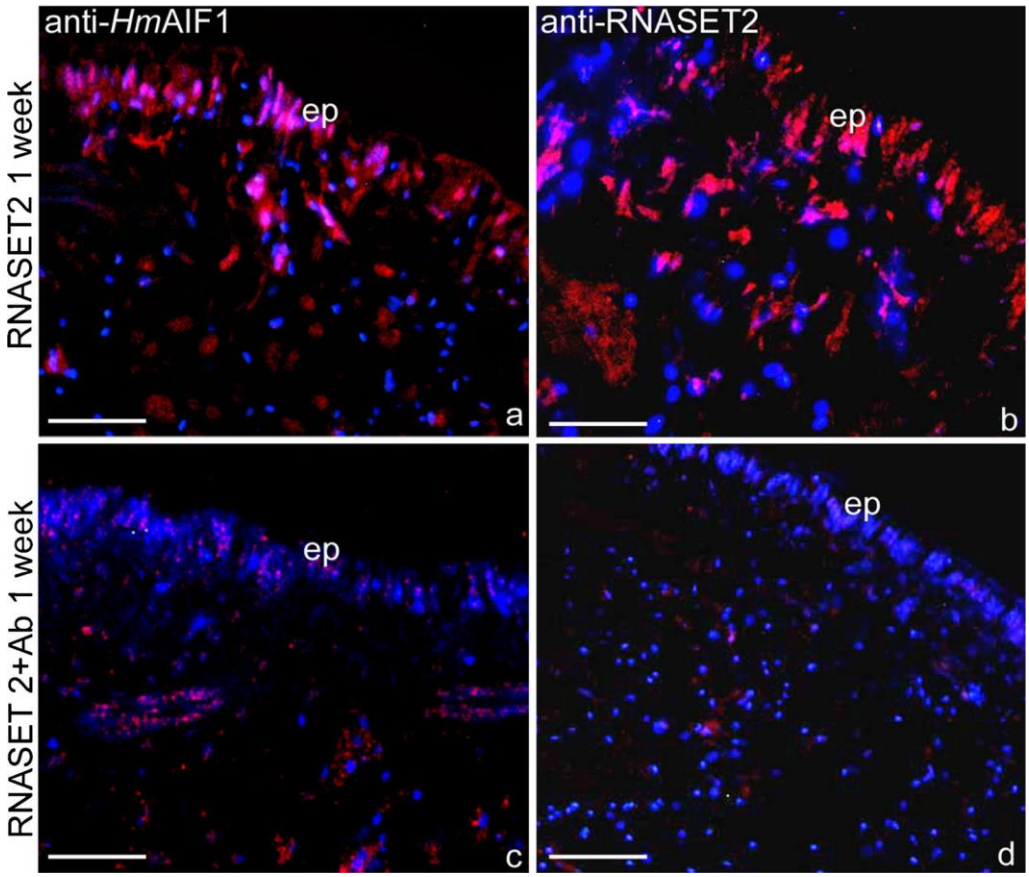

Fig. 4 Immunofluorescence analysis of cryosections from medicinal leech body wall injected with rRNASET2 (**a, b**) or rRNASET2 pre-incubated with the neutralizing anti-RNASET2 antibody (**c, d**). One week following injection of RNASET2 alone, several *Hm*AIF-1$^+$ and anti RNASET2$^+$ cells (in *red*) are mainly located underneath the epithelium (*ep*). Cell nuclei are stained in *blue* with DAPI. *Bars* 50 µm

Proliferative and enzyme ACP assays

To further investigate the nature of the putative inflammatory process triggered by hRNASET2 injection, leeches were stained with antibodies targeting known markers of cell proliferation and macrophage activation. Of note, unlike unlesioned leeches (Fig. 5a–c), a significant increase in the number of anti-Histone H3P-positive cells was observed 24 h (Fig. 5d–f) and 1 week (Fig. 5g–i) after rRNASET2 injection, suggesting that infiltrating cells were undergoing cell division. Furthermore, the ACP assay showed a marked increase in cells endowed with phagocytic activity (de Eguileor et al. 1999; Girardello et al. 2015b; Schorn et al. 2015a) among muscle layers and next to the rRNASET2-injected area.

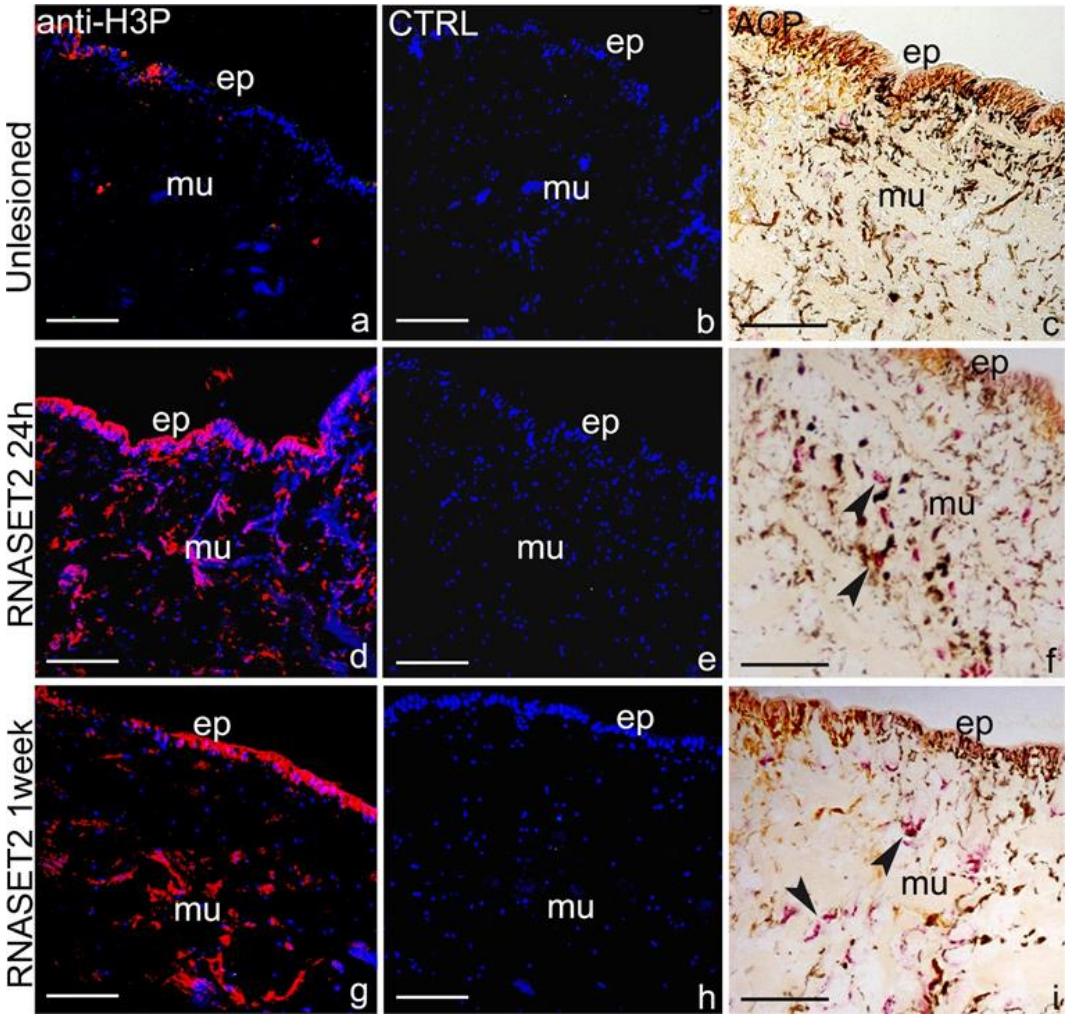

Fig. 5 HP3 immunostaining and ACP reaction on cryosections from unlesioned or rRNASET2 injected leech. Compared to control sections (**a-c**), after 24 h (**d–f**) and 1 week (g–i) from rRNASET2 injection, numerous cells with mitotic (*red* in **a, d, g**) and lysosomal activities (red in **c, f, i**) are visible underneath the epithelium (*ep*) and among muscle fibers (*mu*). Cell nuclei are stained in blue with DAPI. No signal is present in negative control experiments in which the primary antibodies were omitted (**b, e, h**). *Bars* 100 µm

Characterization of cell types infiltrating MG pellets supplemented with rRNASET2

To further confirm the ability of rRNASET2 to induce macrophages migration *in vivo*, recombinant RNASET2 protein was added to MG biopolymer held at a liquid state and the mixture was subsequently inoculated in leeches. In order to characterize the cell types infiltrating the polymerized biopolymer, MG solid pellets formed following inoculation were explanted after 1 week and processed for standard histological and immunofluorescence analyses using anti-CD68 and anti-*Hm*AIF-1 antibodies (both specific for leech macrophages) (Schorn et al. 2015a, b) and an anti-RNASET2 antibody (Fig. 6a–o). In keeping with our previous results, MG samples containing rRNASET2 protein appeared massively infiltrated by elongated and irregularly-shaped cells characterized by numerous cytoplasmic expansions (Fig. 6g).

These cells were *Hm*AIF-1^{+} (Fig. 6h), CD68^{+} (Fig. 6j) and RNASET2^{+} (Fig. 6k). A much lower number of infiltrating cells was observed in control MG specimens lacking rRNASET2 (Fig. 6a, b), or added with PBS (Fig. 6d, e) or rRNASET2 pre-incubated with anti-RNASET2 antibody (Fig. 6m, n). No signal was detected in negative control experiments in which the primary antibodies were omitted (Fig. 6c, f, i, l, o).

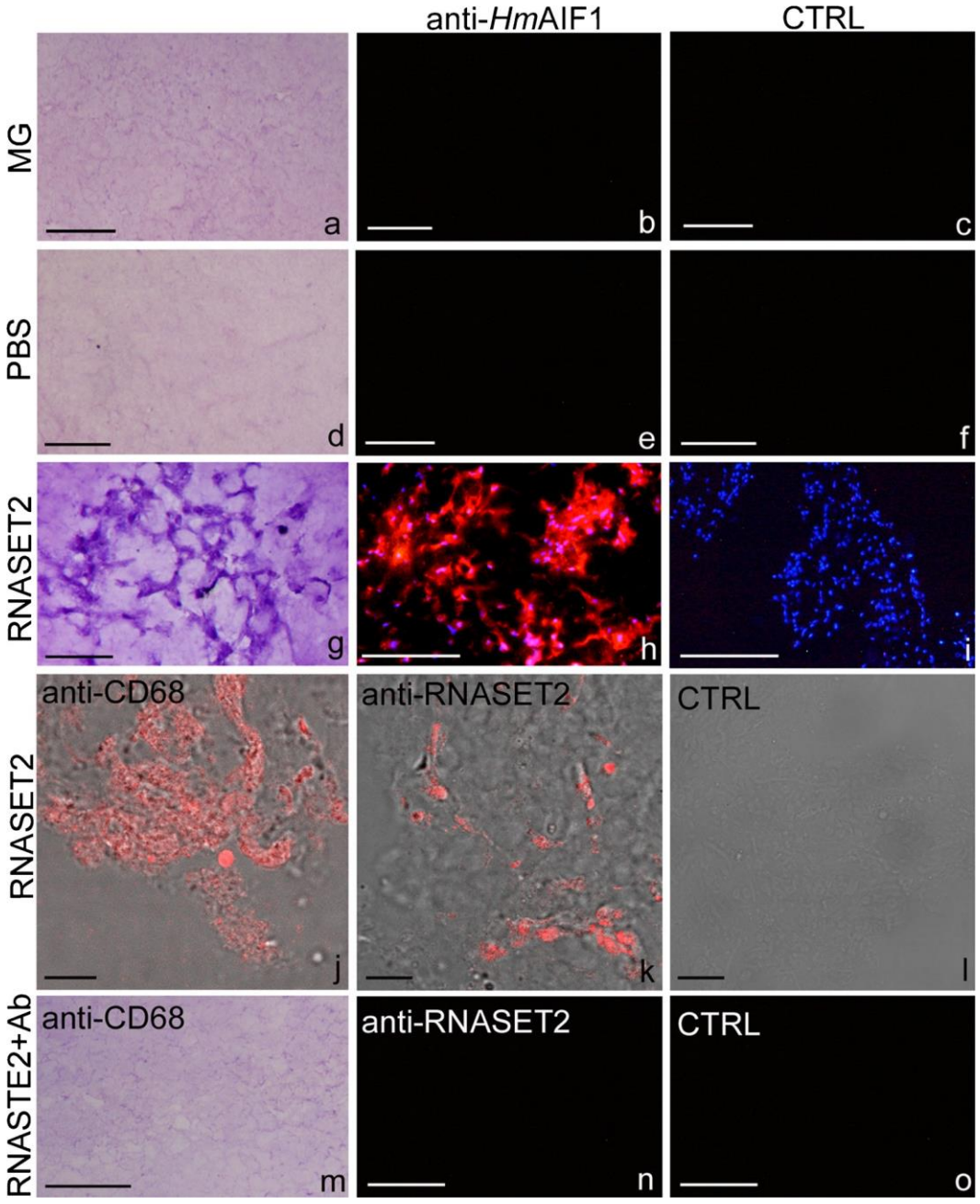

Fig. 6 Morphological and immunohistochemical analyses of untreated control matrigel sponges (**a–c**) and MG sponges treated with PBS (**d–f**), rRNASET2 (**g–l**) and neutralizing antibody-pretreated rRNASET2 (**m–o**). After 1 week, only MG plugs supplemented with rRNASET2 are highly infiltrated by elongated and irregularly-shaped characterized cells and by numerous cytoplasmic expansions (**g**). These cells are *Hm*AIF-1^{+} (**h**), CD68^{+} (**j**) and RNASET2^{+} (**k**). Cell nuclei are stained in *blue* with DAPI (h, i). No signal is detected in negative control experiments in which the primary antibodies are omitted (**c, f, i, l, o**). *Bars* (**a–i**, **m–o**) 100 µm, (**j–l**) 10 µm

LPS injection induces RNASET2 expression in leech macrophages

We next addressed the putative involvement of rRNASET2 in regulating the inflammatory response following injection with LPS, a well-known strong stimulator of innate immune response in almost all eukaryotic species, ranging from insects to humans. We previously reported that LPS injection promotes a massive migration of macrophages in the body wall of *Hirudo* (de Eguileor et al. 1999; Schorn et al. 2015a). The immunophenotype of cells involved in LPS-mediated inflammatory response was evaluated by double-labeling with an anti-RNASET2 antibody and anti-*Hm*AIF-1 or anti-CD68 antibodies. As expected, the assays showed that, unlike unlesioned animals (Fig. 7a, b), LPS-injected animals (Fig. 7c, d) displayed a marked migration of macrophages that were mainly localized underneath the epithelium and in the connective tissue surrounding the muscle fibers. Strikingly, these infiltrating macrophages were expressing both RNASET2 and *Hm*AIF-1 or RNASET2 and CD68. Thus, the massive recruitment of macrophages coupled to upregulation of endogenous RNASET2 that we previously observed following rRNASET2 treatment was recapitulated when leeches were challenged with a strong inflammatory stimulus. Moreover, the cellular source of leech endogenous RNASET2 turned out to be the macrophage cell itself.

In order to quantify the changes in expression for the markers of interest, the RNASET2 and *Hm*AIF-1 expression pattern of uninjured, PBS-treated, rRNASET2-treated (with and without pre-incubation with the neutralizing anti-RNASET2 antibody) and LPS-challenged animals was also evaluated by means of western blot analysis (Fig. 7e–g). Injection of the anti-RNASET2 antibody alone was also performed as a further control. The analysis was carried out on protein extracts taken from the leech body wall at the site of injection. Two main RNASET2-immunoreactive bands of approximately 34 and 29 kDa (the latter being visible only in a subset of the tested samples) were detected in leech tissues (Fig. 7e). Of note, a control sample from the human OVCAR3 ovarian cancer cell line (known to express high endogenous RNASET2 levels) also showed two main anti-RNASET2-reactive bands, although their molecular mass were slightly different from those observed in leech tissues. As RNASET2 is a highly glycosylated protein, these differences are likely attributable to a different glycosylation pattern for this protein in leech as compared to human cells.

By using an anti-AIF-1 antibody, a single band of 18 kDa corresponding to the expected molecular weight for *Hm*AIF-1 (Schorn et al. 2015a) was detected (Fig. 7f).

Strikingly, the expression pattern of RNASET2 and AIF-1 turned out to be remarkably similar in most if not all the samples tested and was in keeping with the pattern previously reported by immunofluorescence assays. Indeed, both RNASET2 and *Hm*AIF-1 turned out to be constitutively expressed at a low baseline level in unlesioned, PBS- and rRNASET2 plus neutralizing anti-RNASET2 antibody-injected animals (Fig. 7e,f). By contrast, expression of both RNASET2 and *Hm*AIF-1 was significantly increased in the samples from LPS- and RNASET2-injected animals. Moreover, a very weak or undetectable signal was observed in the proteins extract of leech injected with the anti-RNASET2 antibody alone (Fig. 7e), again suggesting the occurrence of a RNASET2-specific effect.

Thus, rRNASET2 injection was shown to induce a change in the expression pattern of both proteins that is very similar to that induced by injection with a highly pro-inflammatory agent such as LPS.

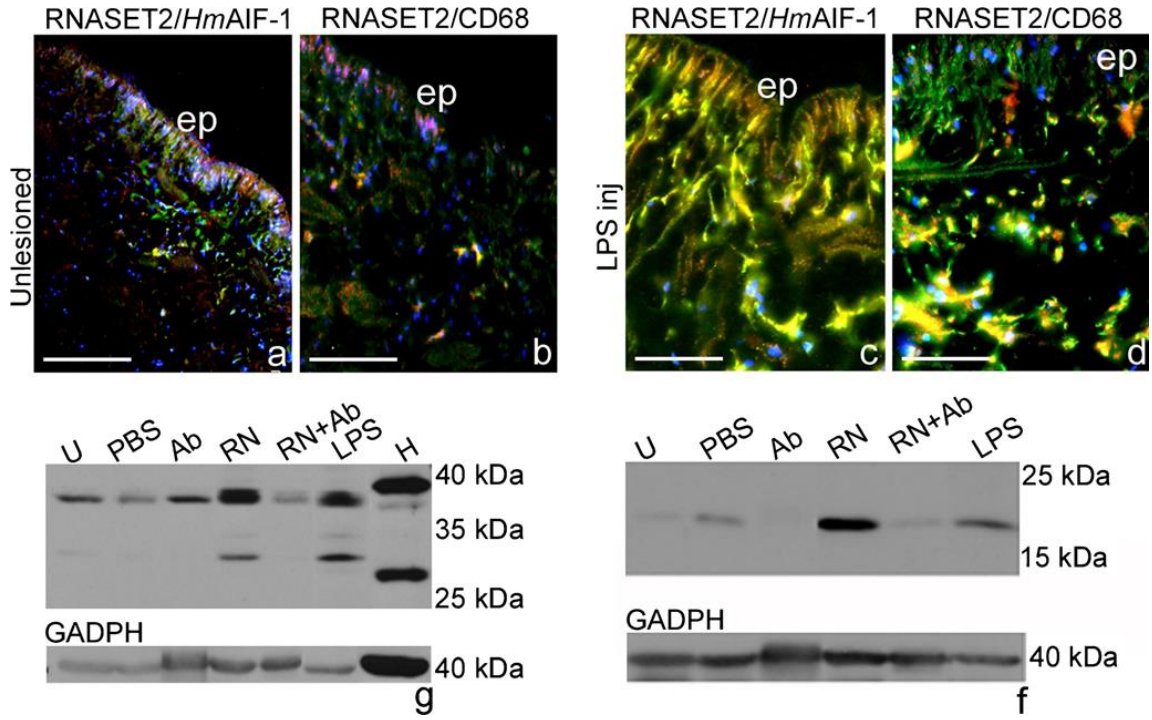

Fig. 7 Double immunolocalization of RNASET2 and *Hm*AIF-1 or RNASET2 and CD68 of unlesioned (**a, b**), LPS-injected (**c, d**) leech body wall and western blot analysis (**e, f**). At 24 h following LPS injection, numerous RNASET2$^+$/*Hm*AIF-1$^+$ and RNASET2$^+$/CD68$^+$ macrophages (*yellow*) migrating towards the injected area are visible under the epithelium (*ep*) and among the muscle fibers (*mu*). Double-immunostaining is performed with anti-RNASET2 (*red*) and anti-*Hm*AIF-1 or anti-CD68 (*green*). No signal is detected in negative control experiments in which primary antibodies are omitted. Nuclei are stained in *blue* with DAPI. *Bars* (**a–d**) 50 µm. **e, f** Western blot analysis. A protein extract of human OVCAR3 ovarian cancer cell line (*H*), used as positive control and of body wall biopsies at the injection sites from unlesioned (*U*), PBS-treated, anti-RNASET2 alone-treated (*Ab*), rRNASET2-treated (*RN*), rRNASET2 plus neutralizing anti-RNASET2 antibody (*RN+Ab*) or LPS-treated leeches were probed with the anti- RNASET2 (**e**) or anti-*Hm*AIF-1 (**f**) antibodies. The antibody anti-RNASET2 recognizes two RNASET2-immunoreactive bands of approximately 34 and 29 kDa in leech tissues and of 36 and 27 kDa in OVCAR3 cell line extract, while the anti-*Hm*AIF-1 detects in leech tissues a specific immunoreactive band of about 18 kDa, according to the molecular weight ladder (*kDa*). The housekeeping protein D-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is used as a loading control. No signal was observed in the proteins extract of leech injected with the Ab alone

DISCUSSION

In the present study, we report that RNASET2, the only human member of the highly conserved T2 ribonuclease protein family, is involved in the regulation of the inflammatory response in the medicinal leech, an excellent model for investigating innate immune responses, since the effects of different types of stimuli, such as bacterial infection or injection of cytokines or chemokines (Tettamanti et al. 2006; Schorn et al. 2015a) can be easily assessed and interpreted in this animal model. Indeed, rRNASET2 was shown to induce both angiogenesis and the migration and functional activation of immunocompetent cells in *H. verbana*. In particular, our study suggests that human recombinant RNASET2 protein provides a marked chemotactic activity in leeches by promoting the massive migration of macrophages displaying high lysosomal activity towards the stimulated area. Furthermore, we show that, although the endogenous RNASET2 protein is constitutively expressed in untreated leeches, its level of expression significantly increases following LPS injection.

rRNASET2 is involved in recruitment of leech macrophages

We have demonstrated a direct effect of human recombinant RNASET2 on macrophage migration by injecting rRNASET2 in the body wall of the medicinal leech. Such treatment was shown to induce a massive migration of cells belonging to the macrophages lineage within 24 h, coupled to the formation of new blood vessels. Indeed, following rRNASET2 injection, a marked increase in cells expressing specific macrophage markers (*Hm*AIF-1 and CD68) (Schorn et al. 2015a, b) and characterized by phagocytic or proliferative activity (as demonstrated by positivity to ACP reaction and H3P expression, respectively) was detected underneath the epithelium and among the muscle fiber fields. Of note, the observed inflammatory response was specifically dependent of recombinant RNASET2 injection, since infiltrating macrophages and neovessel formation were not observed following injection of either PBS or rRNASET2 protein that was pre-incubated with a neutralizing anti-RNASET2 antibody. Of note, the fact that animals injected with human rRNASET2 pre-incubated with the anti-RNASET2 antibody did not show the marked infiltration of immune cells strongly suggests that the observed phenomena did not represent a specific inflammatory response against foreign host proteins. This conclusion is further strengthened by the observation that injection of the anti-RNASET2 antibody alone did not elicit any inflammatory response as well, as assessed in our western blot analysis. Noteworthy, the number of macrophages recruited in the body wall remained

very high nearly a week after rRNASET2 injection. This observation suggests that the observed recruitment of macrophages might be sustained by a positive feedback mechanism by which the infiltrating cells, once migrated in the stimulated area, produce and secrete one or more factors to further promote the recruitment of inflammatory cells. Taken together, these data strongly suggest that rRNASET2 injection in leeches induces a marked inflammatory response characterized by macrophage recruitment and functional activation coupled to cell proliferation. In this regard, the observation that recombinant RNASET2-mediated infiltration of leech macrophages is associated with a marked increase in the expression of endogenous RNASET2 by the same macrophage cells suggests that such an endogenous RNASET2 boost might induce the recruitment and accumulation of more macrophages in the stimulated body region. Despite the occurrence of such RNASET2-based positive feedback mechanisms on target infiltrating cells being largely speculative, our results are in agreement with data previously reported in vertebrate organisms. Indeed, proteins belonging to the Rh/T2/S ribonuclease family (including RNASET2) are known to be actively secreted in the extracellular medium, where they carry out several biological functions (Luhtala and Parker 2010). In particular, the human RNASET2 protein has recently been shown to suppress the growth of ovarian cancer-derived cells in vivo in murine xenograft models by inducing the recruitment of a tumor-suppressive (M1) macrophage cell population within the tumor mass (Acquati et al. 2011). Moreover, a direct chemotactic effect of recombinant RNASET2 on cells belonging to the human monocyte-derived U937 cell line has also been reported (Acquati et al. 2013) and recently confirmed on human peripheral blood-derived monocytes. Finally, a survey by immunohistochemical assays has recently shown that RNASET2 is endogenously expressed by tissue macrophages in most human tissues investigated (manuscript in preparation). Taken together, these observations are in keeping with the role of RNASET2 as an inducer of a strong inflammatory response, as reported in this work.

Of note, RNASET2-mediated activation and recruitment of macrophages seems to represent an evolutionarily conserved process, since in this work we were able to recapitulate it in an invertebrate experimental model. In keeping with this hypothesis, recent work on the trematode *Schistosoma mansoni* has demonstrated a role for a T2 RNase protein encoded by the parasite's genome (omega-1) in the modulation of the innate immune response in infected hosts (Ferguson et al. 2015).

We investigated the marked RNASET2-mediated infiltration of leech macrophages

observed in vivo by further assessing the chemotactic activity of recombinant RNASET2, using a consolidated experimental approach based on injection of MG biopolymers supplemented with soluble factors in the leech body wall. Our previous studies have shown that MG supplemented with specific cytokines, such as interleukin IL-8, VEGF (vascular endothelial factor) or *Hm*AIF-1, were able to form a microenvironment suitable to induce the migration of specific cell populations when injected in vivo in the body wall of the leech (Grimaldi et al. 2008, 2009, 2011; Girardello et al. 2015a). This experimental approach is therefore a key tool to define the cell types chemoattracted by specific factors. In this work, histological and immunocytochemical assays on explanted rRNASET2-supplemented MG pellets showed a massive infiltration of host cells that stained positive for CD68 or *Hm*AIF-1 markers, thus providing further evidence in support of the chemotactic activity of RNASET2 on macrophage cells.

rRNASET2 injection causes rearrangement of the extracellular matrix

After rRNASET2 injection, a significant increase in the production of collagen fibrils and a consequent remodeling of the muscle layers were also observed. As previously demonstrated in animals injected with soluble factors such as cytokines (de Eguileor et al. 2001; Tettamanti et al. 2005; Schorn et al. 2015a), a massive production of a new matrix also leads to a reorganization of the muscle cutaneous sac. The muscle fibers, which in control animals are adjacent to each other and arranged to form groups surrounded by scarce extracellular matrix, move away due to the overproduction of collagen. The main effectors of such remodeling turned out to be secreting fibroblasts characterized by projections of cytoplasmic laminae stretching towards the extracellular space (Tettamanti et al. 2004). The cytoplasmic folding was in turn shown to form a microenvironment in which fibrillogenesis occurs and was involved in the spatial organization of collagen bundles, governing the orientation of the collagen fibrils with respect to the cell axis (Tettamanti et al. 2005). The resulting massive production of connective tissues is then used as a scaffold for immune cells migration and for proper orientation of growth of new vessels (de Eguileor et al. 2004; Tettamanti et al. 2004). In this regard, it is worth noting that human RNASET2 was recently shown to affect both the cytoskeletal organization and the migratory pattern of ovarian cancer cell lines (Lualdi et al. 2015).

RNASET2 is expressed by leech macrophage-like cells

Previous studies carried out in vertebrates have shown that bacterial infections can induce the expression of ribonucleases such as RNase 7, RNase and angiogenin 8 (Harder and Schroder 2002), simultaneously to the release of inflammatory cytokines such as interferon gamma, TNF-alpha and IL-1beta. The data presented in this work suggest for the first time that ribonucleases belonging to the T2 family can play a key role in the recruitment of activated macrophages in the context of Gram-negative bacteria cell wall components injection. Indeed, in this work we show that injection of LPS in the body wall of leech not only causes a massive migration of $CD68^{+}$/*Hm*$AIF\text{-}1^{+}$ macrophages as previously described (de Eguileor et al. 1999; Schorn et al. 2015a, b) but also induces a strongly increased expression of endogenous RNASET2 in these migrating macrophages, as assessed by both immunohistochemical and western blot analysis. It is well known that, in response to infections, leech macrophages produce antimicrobial proteins and peptides that specifically disrupt several components of Gram-positive bacterial cell walls (Schikorski et al. 2008; Hildebrandt and Lemke 2011). The observed increase of RNASET2 expression by leech macrophages could therefore be directly correlated to its putative ability to act as a stress response protein by priming host macrophages for mounting an anti-bacterial response. In this regard, it is worth noting that a marked stress response role for human RNASET2 has also been recently reported in human cancer cells (Lualdi et al. 2015), again supporting the occurrence of an ancient and evolutionarily conserved role for this gene. Moreover, since some ribonucleases have been described to display a marked antimicrobial activity (Boix et al. 2012), a direct anti-bacterial role for the RNASET2 enzyme cannot be ruled out, although this hypothesis remains largely speculative at present.

The data reported in this work thus provide compelling evidence in support of a role for RNASET2 as a key component of the natural immune system, not only in vertebrates but also in invertebrates. If confirmed by further investigations, this scenario would suggest a complex, pleiotropic role for RNASET2 in orchestrating an evolutionarily conserved inflammatory response, based on macrophage recruitment and activation coupled to a massive extracellular remodeling.

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AIF-1 and RNASET2 Play Complementary Roles in the Innate Immune Response of Medicinal Leech

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Abstract

Recent studies demonstrated that allograft inflammatory factor-1 (AIF-1) and RNASET2 act as chemoattractants for macrophages and modulate the inflammatory processes in both vertebrates and invertebrates. The expression of these proteins significantly increases after bacterial infection; however, the mechanisms by which they regulate the innate immune response are still poorly defined. Here, we evaluate the effect of bacterial lipopolysaccharide injection on the expression pattern of these genes and the interrelation between them during innate immune response in the medicinal leech, an invertebrate model with a simple anatomy and a marked similarity with vertebrates in inflammatory processes. Collectively, prokaryotic-eukaryotic co-cultures and *in vivo* infection assays suggest that RNASET2 and AIF-1 play a crucial role in orchestrating a functional cross-talk between granulocytes and macrophages in leeches, resulting in the activation of an effective response against pathogen infection. RNASET2, firstly released by granulocytes, likely plays an early antibacterial role. Subsequently, AIF-1$^+$ RNASET2-recruited macrophages further recruit other macrophages to potentiate the antibacterial inflammatory response. These experimental data are in keeping with the notion of RNASET2 acting as an alarmin-like molecule whose role is to locally transmit a “danger” signal (such as a bacterial infection) to the innate immune system in order to trigger an appropriate host response.

Keywords
Leech innate immunity · RNASET2 · Allograft inflammatory factor-1 · Macrophages · Granulocytes

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Introduction

The innate immune response serves not only to counteract infections (either naturally occurring or following injury) but also to maintain tissue homeostasis and functional integrity, thus restoring the architectural structure to damaged organs and tissues (Frantz et al. 2005). In this context, efficient clearance of cellular debris by macrophages is known to prevent the persistence of potentially toxic or immunogenic material in the tissue environment and at the same time promotes tissue regeneration. A wide range of cytokines produced by inflammatory cells, including macrophages themselves, mainly orchestrates these processes. Thus, the generation of a rapid inflammatory response plays key roles in both host defense and tissue repair.

Several studies have demonstrated the importance of macrophages in the production and secretion of different molecules such as growth factors and cytokines, which induce vessels and mesenchymal cell recruitment to injured/grafted or bacteria-infected tissues. Among these cytokines, two macrophage-derived interesting molecules have been recently demonstrated to be involved in inflammatory responses and tissue regeneration: allograft inflammatory factor-1 (AIF-1) and the RNASET2, a member of ribonuclease T2 family (Schorn et al. 2015b, a; Baranzini et al. 2017).

AIF-1 is a 17-kDa calcium-binding protein originally identified in rat cardiac transplant subject to chronic rejection and later found to be selectively expressed in macrophages and neutrophils (Utans et al. 1995). Subsequently, several AIF- 1-like factors showing high amino acid sequence conservation have been identified in other metazoans (both vertebrates (Deininger et al. 2000; Watano et al. 2001) and invertebrates (Kruse et al. 1999; Ovando et al. 2012; Li et al. 2013; Drago et al. 2014)). AIF-1 expression was shown to increase significantly after transplantation, wound healing, or bacterial infections, strongly suggesting its involvement in the inflammatory response and in immune system regulation by attracting macrophages to the challenged area.

Ribonucleases (RNases) are hydrolytic enzymes that cut phosphodiester bonds within RNA molecules and represent one of the most versatile enzyme families, involved in an impressively wide range of biological processes (Luhtala and Parker 2010). Among the different ribonuclease families, the transferase-type RNase subfamily is split into three main groups: A, T1, and T2 RNases, among which only T2 RNases have been reported in all phyla

examined so far, suggesting a very ancient and evolutionary crucial role for this subclass of ribonucleases (Luhtala and Parker 2010). Moreover, a growing interest has been recently focused on T2 RNase family members, due their key role in several critical biological processes such as angiogenesis, biogenesis of ribosomes, apoptosis, cell proliferation control and, most interestingly, regulation of immune response (Acquati et al. 2011). For instance, the human RNASET2 protein, which acts as a tumor suppressor in different types of cancer, has been reported to trigger the innate immune response by recruiting host macrophages endowed with oncosuppressive properties toward the tumor mass *in vivo* (Acquati et al. 2011, 2013). Moreover, other members of the T2 RNase family have recently been shown to carry out a modulatory role in the immune response as well (Everts et al. 2012; Xu et al. 2013).

Although it is now acknowledged that both AIF-1 and RNASET2 play a crucial role in activating and modulating the innate immune response, the mechanism(s) by which they act are largely unexplored. A better understanding of the interplay between these two molecules may thus provide valuable insights into how the innate immune system regulates inflammation, disease development, or wound healing and at the same time yield valuable new therapeutics and interventional strategies to control immune and systemic responses to disease, injury, and bacterial infection.

Here, we propose the medicinal leech *Hirudo verbana* as a well-established experimental model to investigate the above-mentioned processes related to the innate immune response. Indeed, this invertebrate represents a cost-effective and easily manipulable model and without significant ethical considerations in relation to its use. In addition, its simple anatomy, physiological characteristics, and the less varied repertoire of cell types involved in immune response and wound healing allow to easily define the cellular and molecular mechanisms linked to these biological processes (Tettamanti et al. 2003b; Grimaldi et al. 2006).

Differently from other Annelids (i.e., oligochaetes and polychaetes), leeches have a parenchymatous body and a reduced coelom (Mann and Kerkut 1962; Sawyer 1986). As previously mentioned, the anatomy of leech is relatively simple (Fig. 1): underneath the epithelium, muscle fibers are organized in fields, surrounded by a scant extracellular matrix. The muscular sac is separated from the inner digestive tube by a loose connective tissue containing the botryoidal and the vasofibrous tissues (Mann and Kerkut 1962; Sawyer 1986; de Eguileor et al. 2001b), from which most of the myeloid lineages-derived leucocytes arise

(Grimaldi et al. 2006).

In healthy medicinal leech, a few resident immunocompetent cells, such as macrophages, type I and II granulocytes, and natural killer cells are poorly represented in the extracellular matrix surrounding muscle (Grimaldi 2016). These immunocytes display features and behaviors typical of those found in vertebrates (de Eguileor et al. 1999a, 2000b, a). Moreover, a plethora of cytokines, growth factors, and cluster of differentiation proteins (CDs) have been reported in leeches, where they act as modulators of these processes in a very similar way when compared to vertebrates (de Eguileor et al. 2000b; Grimaldi et al. 2004; Tettamanti et al. 2006; Macagno et al. 2010b). In fact, our previous investigations showed that injection in leeches of human cytokines and growth factors promote hematopoiesis, vascular growth, immune cell migration, and myofibroblast differentiation (Tettamanti et al. 2003b). Among these factors, AIF-1 and RNASET2 have recently been shown to play a pivotal role in the leech immune response and tissue repair (Drago et al. 2014; Schorn et al. 2015b; Baranzini et al. 2017). Indeed, expression of both factors is significantly increased following lipopolysaccharides (LPS) or bacterial infection in leeches. We have previously demonstrated that the injection in the leech body wall of LPS induces a massive migration of macrophages towards the stimulated area. These immune cells in turn express and produce AIF-1 and RNASET2, both involved in other macrophage recruitment (Schorn et al. 2015a; Baranzini et al. 2017).

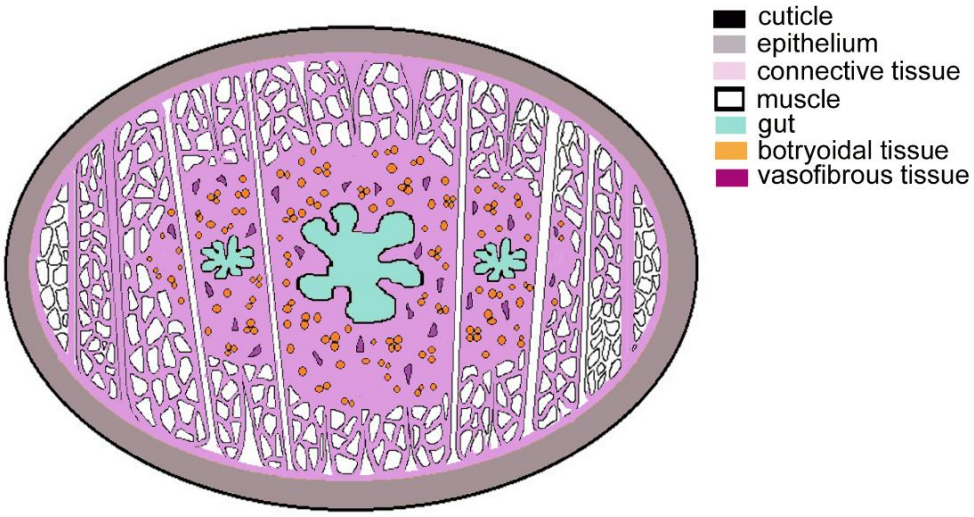

Fig. 1. Drawing representing a general view of leech body in cross section. Under the cuticle and epithelium, the muscle fibers and gut are visible, and between them a loose connective tissue containing the botryoidal and the vasofibrous tissue can be seen. Modified from Grimaldi et al. [55].

Based on these previous studies, we demonstrate for the first time an interrelation between these two molecules, which orchestrate a functional cross talk between granulocytes and macrophages. In particular, we show that RNASET2 is firstly released by granulocytes to play an early antibacterial role. Subsequently, RNASET2 recruits AIF-1^{+} macrophages with phagocytic activities in order to potentiate the antibacterial inflammatory response.

Materials and Methods

Animals and Treatment

Leeches (*H. verbana*, Annelida, Hirudinea, from Ricarimpex, Eysines, France) were kept in lightly salted water (NaCl 1.5 g/L) at 19–20°C in aerated tanks. Animals were randomly divided into separate experimental groups (5 individuals for each time point). Each injection was performed in the body wall at the level of the 20th metamere on leeches anesthetized by immersing them in a 10% ethanol solution. Treated and untreated (control) animals were anesthetized and then dissected to remove body wall tissues at specific time points. Samples were processed for optical and electron microscopy and immunofluorescence protocols. Animals were randomly split into three groups (5 individuals from each time point) and submitted to various protocols and treatments.

Group 1. Unstimulated control or iso-osmotic PBS solution (138 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4)-injected leeches, to verify the normal and correct morphological information about the body organization and to demonstrate that both the saline solution and the injection did not induce any immune response.

Group 2. T 30 min, T 1 h, T 3 h, T 6 h, T 24 h: samples injected with 100 µL sterilized PBS containing, respectively, 100 ng/mL LPS from *Escherichia coli* (Serotype 0111: B4, Sigma, St. Louis, MO, USA).

Group 3. T 30 min: samples injected with a solution of PBS containing *Pseudomonas aeruginosa* PAO1 (expressing the green fluorescent protein [GFP]) alone or added with an anti-RNASET2 antibody for functional blocking experiments.

Optical and Electron Microscopy

Tissues were fixed for 2 h in 0.1 M cacodylate buffer at pH 7.4, containing 2% glutaraldehyde, washed in the same buffer and post-fixed for 1 h with 1% osmium tetroxide in cacodylate buffer, pH 7.4. After standard ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture (Sigma-Aldrich, Milan, Italy). Sections were obtained with a Reichert Ultracut S ultratome (Leica, Wien, Austria). Semi-thin sections (0.75 µm in thickness) were stained by crystal violet and basic fuchsin (Moore et al. 1960) and observed under the light microscope Nikon Eclipse Ni (Nikon, Tokyo, Japan). Data were recorded with a DS-5 M-L1 digital camera system (Nikon). Ultrathin sections (80 nm) 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).

Western Blot Assays

Tissues from injected and uninjected animals were immediately frozen in liquid nitrogen and then homogenized with a mortar. The homogenates were suspended in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0), at a concentration of 10 µL per mg of tissue, in the presence of a protease inhibitor cocktail (Sigma-Aldrich). The particulate material was removed by centrifugation at 15,100 g for 10 min at 4°C in a refrigerated Eppendorf Minispin microcentrifuge (Hamburg, Germany). 30 µL of supernatants (40 µg/mL) containing total protein extract of 1 leech (in total 3 different animals were used for each treatment), were denatured at 100°C for 10 min and were loaded on 12% acrylamide minigels for SDS-PAGE analyses. Molecular weights were determined by concurrently running broad-range standards from Bio-Rad (Richmond, MA, USA). 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 polyclonal anti-RNASET2 (Campomenosi et al. 2011) or an anti-*Hm*AIF-1 (Drago et al. 2014) antibody (1:5,000 dilution in 5% TBS-milk). After three washes with TBS-Tween 0.1%, antigens were revealed with a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories), diluted at 1:5,000. Immunocomplexes were revealed with luminol LiteAblot PLUS Enhanced Chemiluminescent Substrate (Euro-

Clone, Pero, Italy). In control experiments, anti-RNASET2 (Acquati et al. 2011, 2013) and anti-*Hm*AIF-1 (specific for medicinal leech macrophage (Drago et al. 2014; Schorn et al. 2015a)) antibodies were substituted with rabbit preimmune serum (1:20,000). Bands were normalized by using the ImageJ software package (http://rsbweb.nih.gov/ij/download.html), and the housekeeping protein D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as an internal reference, was detected with a rabbit polyclonal anti-human GAPDH IgG (Proteintech, Chicago, IL, USA), diluted at 1:2,000.

Immunohistochemistry

Tissue samples were embedded in Polyfreeze tissue-freezing medium (OCT, Tebu-Bio, Le Perray-en-Yvelines, France/Poly- sciences, Eppelheim, Germany), immediately frozen in liquid nitrogen, and then kept at –80°C. Cryosections (7 µm) obtained with a cryotome (Leica CM1850) were collected on gelatinous slides and kept at 20°C.

For double-labelling immunofluorescence assays, sections were rehydrated with PBS 1× for 10 min and then preincubated for 30 min with blocking solution (2% bovine serum albumin and 0.1% Tween20 in PBS) that block the nonspecific sites. The same blocking solution was used to dilute the following primary antibodies: rabbit anti-RNASET2 (1:100); goat anti-CD11b (Santa Cruz Biotechnology, CA, USA), known to react with leech granulocytes (de Eguileor et al. 2000b) (1:100); rabbit anti-*Hm*AIF-1, (1:1,000). After several washes, specimens were incubated for 45 min with the following secondary antibodies (Abcam, Cambridge, UK): goat anti-rabbit Cy5 conjugated (excitation filter 650 nm, emission filter 672 nm), donkey anti-goat Cy5 conjugated, or donkey anti-rabbit fluorescein isocyanate (FITC)/conjugated (excitation filter 493 nm, emission filter 518 nm), all diluted at 1:200. Sections were treated with 1 mM CuSO4 in 50 nM ammonium acetate buffer (pH 5.0) for 15 min and then washed in distilled water and PBS for reducing tissue autofluorescence while preserving the specific fluorochrome signal (Schnell et al. 1999). In all control experiments, primary antibodies were substituted with rabbit preimmune serum (1:100) or were omitted, and sections were incubated only with the secondary antibodies. Nuclei were stained by incubating for 3 min with 4',6-diamidino-2-phenylindole (DAPI; 0.1 mg/mL in PBS, excitation 340 nm, emission 488 nm). The slides were examined with a Nikon fluorescence microscope. Images were combined with Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Immunogold

Samples were fixed for 2 h at 4°C with 4% paraformaldehyde and 0.5% glutaraldehyde in PBS, dehydrated in ethanol series and embedded in an Epon-Araldite 812 mixture (Sigma-Aldrich). Ultrathin sections, obtained as above, were collected on gold grids (300 mesh). After etching with NaOH 3% in absolute ethanol (Causton 1984), they were incubated for 30 min in blocking solution containing PBS, 1% bovine serum albumin, and 0.1% Tween and then with the polyclonal primary antibody rabbit anti-human RNASET2 diluted at 1:20 in blocking solution. After several washings with PBS, the primary antibody was visualized by immunostaining with the secondary goat anti-rabbit IgG (H+L)-gold conjugate antibody (GE Healthcare, Amersham, UK; particle size, 10 nm) diluted at 1: 100 in blocking solution for 1 h. In control experiments, the primary antibody was omitted or was substituted with the rabbit pre-immune serum (1:100), and sections were incubated with secondary antibody alone. After several washings with PBS, samples were treated for 5 min with PBS containing 0.5% glutaraldehyde, counterstained with uranyl acetate and observed under a Jeol 1010 EX transmission electron microscope (Jeol).

Cell Culture Lines and Bacterial Strain Growth Conditions

The human promonocytic THP-1 cell line (Auwerx 1991) was cultured in RPMI-1640 media, 10% FBS, 1% glutamine. The silence RNASET2 THP-1 SH cell line was cultured in the same medium supplemented with 0.75 µg/mL puromycin. Both cell lines were maintained in a humidified incubator (37°C, 5% CO_2). THP-1 cells were differentiated into macrophages by addition of 5 ng/mL phorbol 12-myristate 13-acetate for 48 h in MT12 wells at a concentration of 0.65×10^6 cells/mL.

P. aeruginosa PAO1 (Stover et al. 2000) was transformed using the pivo (GFP) expression vector; a pJB3 KmD derivative, in which the GFP coding sequence was cloned under an arabinose inducible promoter (pAra). Both strains were grown overnight in LB medium (Sigma-Aldrich) at 37°C under 200 rpm shaking.

THP-1 and P. aeruginosa PAO1 Co-Cultures

The medium of THP-1 cell cultures treated for 48 h with phorbol was changed and three washes were performed before the bacterial inoculum. *P. aeruginosa* PAO1 expressing GFP was induced for 30 min with arabinose 1 mM inoculum to THP-1 cell cultures. A sample of

PAO1 cells suspended in 50 mM phosphate buffer, pH 7.4 has been dispensed to THP-1 cells (500,000 cells/well) to reach a concentration equal to ~10^7 CFU/mL. Upon 2 h co-culture at 37°C, GFP expression was checked through fluorescence microscopy (488/525 nm, for FITC signals). Bacterial viability was checked through a plate count technique. A volume (0.1 or 0.01 mL) of undiluted or serially diluted samples was plated on LB agar plates and incubated for 24 h at 37°C. Viable counts were expressed as colony-forming units (CFU mL^{-1}).

Leech Infection

Leeches were injected in the body wall, at the level of the 20th metamere, with 100 µL of PBS containing *P. aeruginosa* PAO1 expressing GFP at a concentration equal to 10^7 CFU/mL. For functional blocking experiments, animals were injected with a sample of bacterial culture treated with 1 µL of antibody anti-RNASET2 to inhibit the possible bactericidal activity of this enzyme. After 30 min, tissue samples were collected, embedded in Polyfreeze tissue-freezing medium (OCT) and immediately frozen in liquid nitrogen. Cryosections (7 µm) were counterstained with crystal violet and basic fuchsin for morphological analysis and with DAPI (0.1 mg/mL in PBS) to highlight bacterial chromosomal DNA. Specimens were then observed under a light optical microscope and fluorescence Nikon Eclipse Ni (Nikon, Japan). DAPI was visualized with excitation and emission filter 360/420 nm, and to evaluate bacterial GFP, excitation and emission filter 488/525 nm was used.

Statistical Analysis

The percentages of $CD11b^+/RNASET2^+$ cells were assessed by analyzing 5 different slides (random fields of 45,000 μm^2 for each slide) for each experimental time point using the Image J software package. Cells in the chosen fields were counted by hand as macrophages if they were $RNASET2^+$ FITC labelled (Baranzini et al. 2017) or as granulocytes if they were $CD11b^+/RNASET2^+$ (yellow labelled as a result of CY5/FITC double staining). Statistical analyses were performed using Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA), and differences were calculated by one-way ANOVA followed by Fisher's post hoc test, and $p < 0.05$ was considered statistically significant.

RESULTS

We have previously reported that AIF-1 and RNASET2 are significantly upregulated at 6 and 24 h following LPS injection, and that this change in their expression is involved in leech macrophage recruitment (Schorn et al. 2015b; Baranzini et al. 2017). However, the underlying mechanisms by which these two factors operate are still poorly characterized. For instance, it is still not clear whether they display a different temporal expression profile during the earliest phases of the inflammatory response (30 min to 24 h) and what their specific role in the innate immune cell activation is. To address these issues, here we first carried out a morphological analysis on leech tissue sections from unlesioned and PBS- and LPS-injected leeches to better characterize the phenotype of the immune cells activated at different time points following LPS injection. Further, the temporal expression profile of AIF-1 and RNASET2 was evaluated by Western blot and immunohistochemistry.

Morphological Analysis of Leech Tissues Injected with LPS

The immune cell population recruited following LPS injection was characterized by both optical and ultrastructural analysis. Morphological examination of the body wall showed that, in both unlesioned (Fig. 2a) and control PBS-injected leeches (Fig. 2b), macrophages (Fig. 2c) and vasofibrous tissue (Fig. 2d) were poorly represented underneath the epithelium and among muscle fibers. Of note, the vasofibrous tissue was formed by vasocentral cells showing an electron-dense cytoplasm containing a few large granules and by vasofibrous cells characterized by a cytoplasm filled with several small highly electron-dense granules (Fig. 2d). As previously reported, following LPS stimulation the vasocentral and vasofibrous cells dissociated from each other (Huguet and Molinas 1994, 1996), and the vasofibrous cells gave rise to type I granulocytes (Grimaldi et al. 2011). Indeed, 30 min after LPS injection, several vasofibrous tissue cells crossing the thick muscle layers were readily recognizable by light microscopy due to their dark pigmentation (Fig. 2e). By transmission electron microscopy (TEM) analysis, vasofibrous cell-derived type I granulocytes (Grimaldi et al. 2011) were clearly recognizable underneath the epithelium and characterized by small round electron-dense granules (Fig. 2f). At 6 h following LPS injection (Fig. 3a– c), numerous type I granulocytes and macrophages were readily detected in the injected area in both connective tissue surrounding the muscle fibers and underneath the epithelial region. Macrophages were clearly

recognizable by their irregular membrane border, involved in the formation of pseudopodia (Fig. 3a–b1), a typical feature of cells engaged in active migration and phagocytosis. Significantly, ultrastructural TEM analysis also showed that recruited macrophages in LPS-challenged areas were strictly associated with type I granulocytes (Fig. 3b1). Indeed, at 6 h following LPS stimulation, numerous macrophages were detected also underneath the epithelium close to type I granulocytes, characterized by a large nucleus and small electron-dense granule-filled cytoplasm (Fig. 3d, c). Interestingly, some granulocytes apparently emptied of their granules were also present (Fig. 3e), suggesting that these cells were releasing antimicrobial and/or cytotoxic molecules from their granules in the extracellular environment, as already described for other invertebrates (Destoumieux et al. 2000; Otero-Gonzalez et al. 2010).

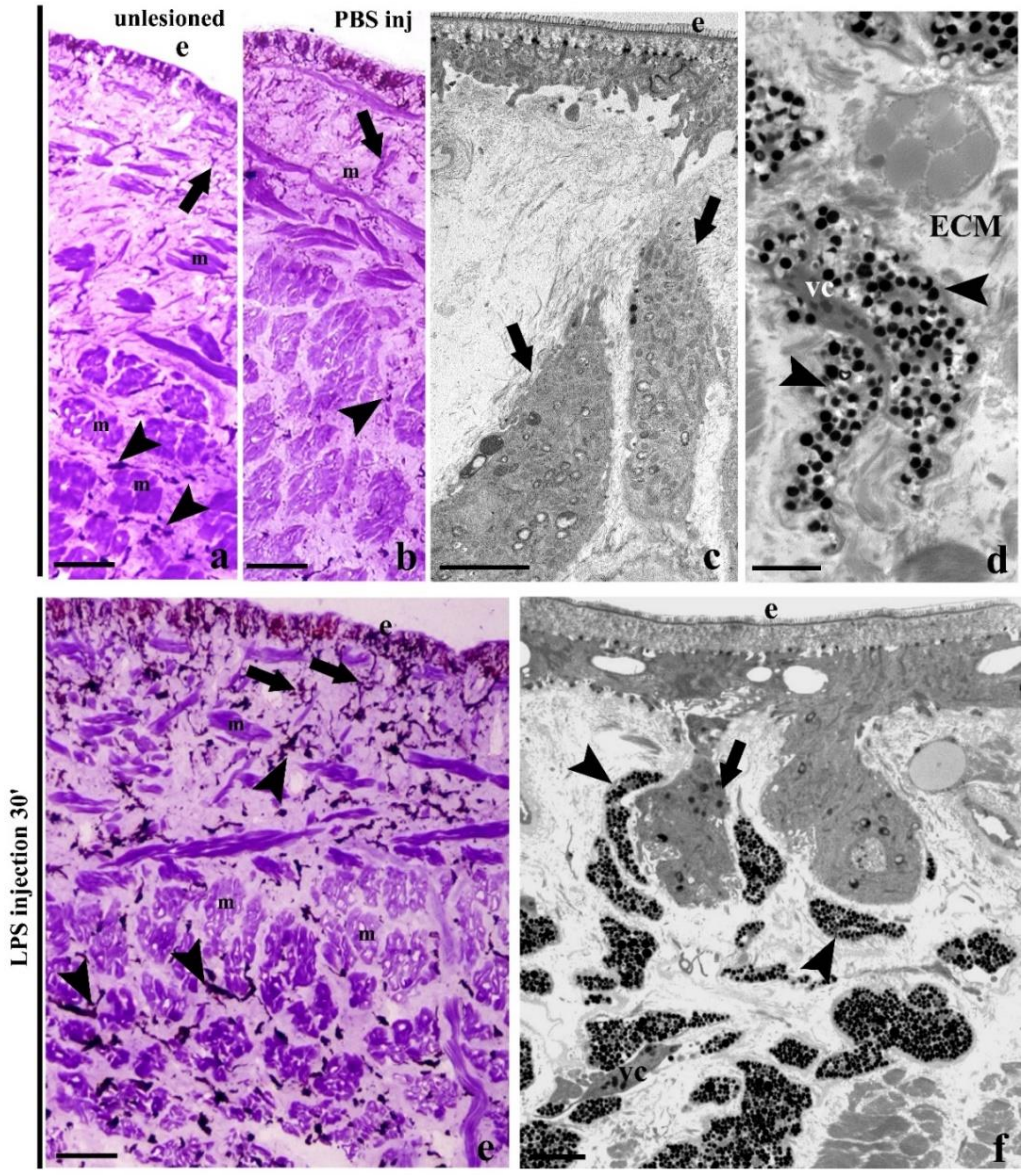

Fig. 2. Morphological analysis of leech body wall at optical and TEM microscopes 30 min after PBS and LPS injection. Few resident macrophages (arrow) and vasofibrous tissue cells (arrowheads) are visible underneath the epithelium (e) or among the muscle fibres (m) in unlesioned (**a**) and in PBS-injected animals (**b**). Detailed TEM of macrophages (arrows in **c**) and of the vasofibrous tissue (**d**) surrounded by extracellular matrix (ECM) formed by vasocentral cell (vc), with cytoplasm containing a few granules, surrounded by vasofibrous cells (arrowheads), with a cytoplasm containing numerous small highly electron-dense granules. 30 min after LPS injection (**e**, **f**), numerous vasofibrous tissue cells are recognizable by their dark color (arrowheads in **e**) among muscle fibers and underneath the epithelium (e). **f** Detailed view of type I granulocytes (arrowheads) detached from vasocentral cells (vc) and next to resident macrophages (arrow) localized in the subepithelial region (e). Bars in **a**, **b**, **e**: 100 µm; bar in **c**, **d**: 2 µm; bar in **f**: 10 nm.

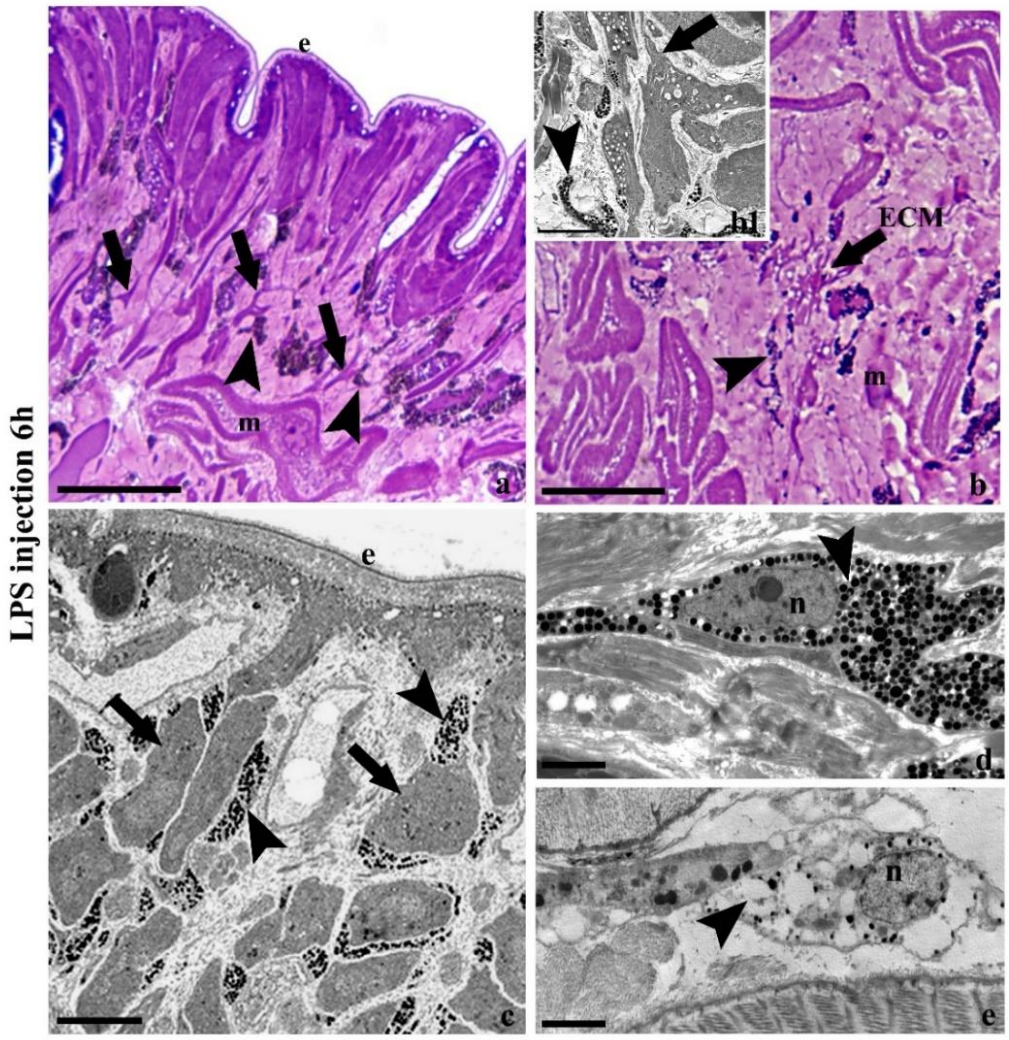

Fig. 3. Morphological analysis of leech body wall at optical and TEM microscopes 6 h after LPS injection. **a**, **b** Optical images show numerous macrophages (arrows) and type I granulocytes (arrowheads) underneath the epithelium (e) and localized in the extracellular matrix surrounding the muscle fibers (m). Ultrastructure TEM images show that macrophages (arrows in **c**) are characterized by pseudopodia (arrow in **b1**) and are in close contact with type I granulocytes (arrowheads in **b1**, **c**, and **d**), some of which are undergoing the degranulation process (**e**). n, nuclei. Bars in **a**, **b**: 20 μm; bar in **b1**: 2 μm; bar in **c**: 4 μm; bar in **d**, **e**: 2 μm.

Western Blot Analysis of AIF-1 and RNASET2 in LPS-Injected Medicinal Leech

In order to better characterize the temporal expression profiles of AIF-1 and RNASET2, Western blot assays were performed in LPS- and control PBS-injected leeches at different time points after treatment (30 min, 1, 3, 6, and 24 h) (Fig. 4a–f). Immunoblot analysis on protein extracts of tissue sections from control, PBS-injected areas confirmed the presence in leech tissues of an AIF-1-immunoreactive band at about 18 kDa, corresponding to the expected molecular weight for this protein (Schorn et al. 2015a), and of two main RNASET2-immunoreactive bands of approximately 36 kDa (the known secreted form) and 29 kDa (a known intracellular form especially visible only in a subset of the tested samples), respectively

(Campomenosi et al. 2006; Baranzini et al. 2017).

Strikingly, in the protein extracts from LPS-injected tissue areas, not only did the amount of both proteins increase significantly, their expression profile turned out to be quite different as well (Fig. 4c, d). In particular, AIF-1 expression was highly increased from the earliest phases of LPS-mediated inflammation (30 min), and then it slightly decreased up to 24 h after treatment. Interestingly, the trend for RNASET2 expression following LPS injection was quite different, since this protein showed two distinct peaks of expression, at 30 min and 6 h from stimulation.

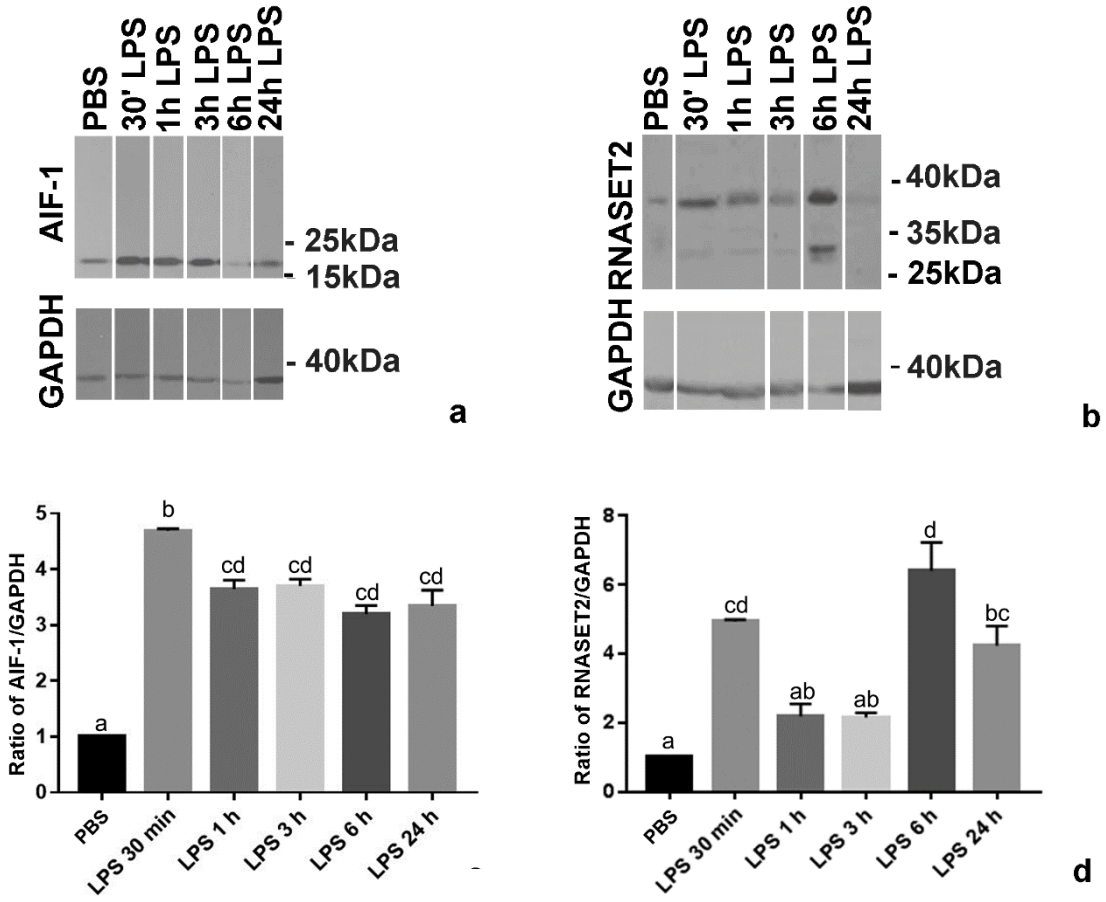

Fig. 4. AIF-1 and RNASET2 Western blot analysis. Proteins extracted from 3 PBS- and LPS-injected leeches and probed with anti-*Hm*AIF-1 (**a**) and anti-RNASET2 (**b**) antibodies. The housekeeping protein D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control, and band intensity appeared to be similar in each loaded sample. The anti-*Hm*AIF-1 antibody detected a specific immunoreactive band of about 18 kDa, while two bands of approximately 36 kDa (the extracellular form) and 29 kDa (the intracellular form) were detected by the anti-RNASET2 antibody. **c**, **d** The levels of expression were quantified by densitometry using the Image J software package, and the obtained graphs show the level of expression of the two factors. **d** The graphic is based on the RNASET2 extracellular form. The individual signals from each lane have been cropped from larger digital images, which are available as supplementary information (see www.karger.com/doi/ 10.1159/000493804 for all supplementary material). Statistical differences were calculated by one-way ANOVA followed by Tukey's post hoc test, and $p < 0.05$ was considered statistically significant (between PBS and LPS treatments). Means with different letters indicate significant difference between PBS and LPS treatments at different times. Experiments were performed in triplicate, and data represent mean values ±SEM. Statistical analyses were performed using Statistica 7.0 software (Stat-Soft Inc., Tulsa, OK, USA), and differences were calculated by one-way ANOVA followed by Fisher's post hoc test, and $p < 0.05$ was considered statistically significant.

AIF-1 and RNASET2 Tissue Localization

The expression profile of AIF-1 and RNASET2 was also analyzed by double immunofluorescence staining, using both anti-*Hm*AIF-1 and anti-RNASET2 polyclonal antibodies on cryosections obtained from unlesioned and PBS-injected leeches (Fig. 5a–c1) compared to LPS-challenged leeches (Fig. 5d–i1).

As previously demonstrated (Schorn et al. 2015b; Baranzini et al. 2017), double immunofluorescence assays confirmed that the two proteins are constitutively expressed in unlesioned (Fig. 5a) and in PBS-injected animals (Fig. 5b). However, the signal intensity detected for both proteins significantly changed following injection with LPS. In general, the number of cells positive for both AIF-1 and RNASET2 significantly increased following LPS injection, although AIF-1^{-}/RNASET2^{+} cells were also detected at specific time points. In particular, several AIF-1^{+}/RNASET2^{+} cells were detected at 30 min and 1, 3, 6, and 24 h after treatment (Fig. 5d–h), whereas AIF-1^{-}/RNASET2^{+} cells were mainly observed at 30 min and 6 h after treatment (Fig. 5d, g), in keeping with the observed pattern of RNASET2 expression shown by Western blot analysis. No signal was detected in negative control experiments in which primary antibodies were substituted with blocking solution (Fig. 5c, i) or preimmune serum (Fig. 5c1, i1).

These data suggested that, besides macrophages, other types of immune cells were expressing RNASET2, and based on morphological analysis, we hypothesized that these AIF-1^{-}/RNASET2^{+} cells might represent type I granulocytes.

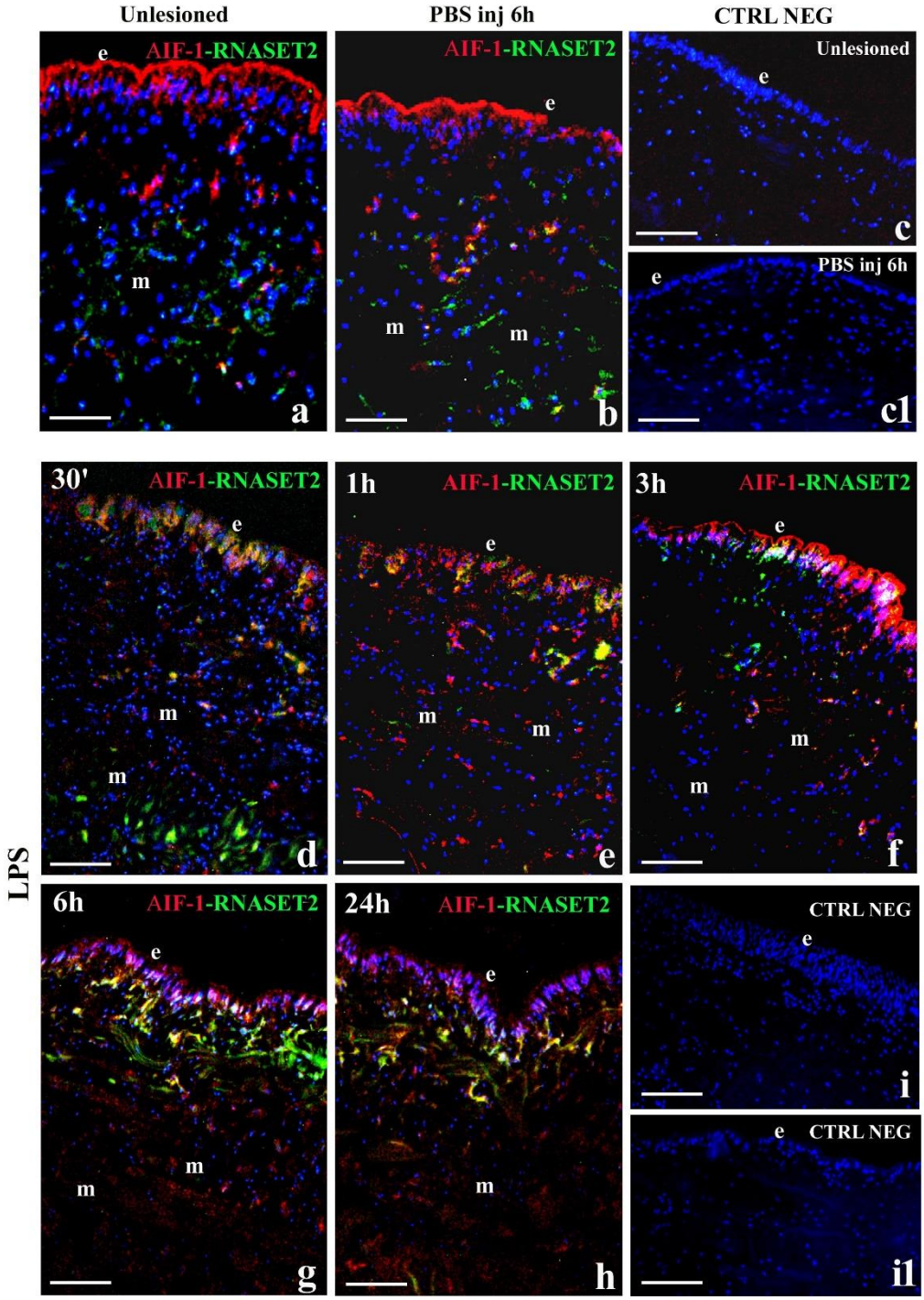

Fig. 5.Immunofluorescence analyses. Immunofluorescence assays (**a–h**) of leech body wall sections. In unlesioned (**a**) and PBS-injected animals (**b**), a few cells located close to the epithelium (e) and among muscle fibers (m) are visible and express *Hm*AIF-1 (in red) and RNASET2 (in green), whereas after LPS injection several migrating immune responsive cells are visible in the epithelial region and among muscles (m) (**d–h**). No signal is detected in negative control experiments in which the primary antibodies were omitted (**c, i**) or substituted by preimmune serum (**c1, i1**). Cell nuclei stained blue by treatment with DAPI. Bars: 100 µm.

Immunophenotype Characterization of LPS-Induced Migrating Immune Cell Populations

To further characterize the $AIF^+/RNASET2^+$ and $AIF^-/RNASET2^+$ cell populations recruited to LPS-challenged areas, we performed double-staining experiments using an anti-CD11b antibody to detect leech granulocytes (de Eguileor et al. 2000b), and an anti-RNASET2 antibody. The experiments showed that, 30 min (Fig. 6a–c) and 6 h (Fig. 6d, e) following LPS stimulation, both $CD11b^+/RNASET2^+$ and $CD11b^-/RNASET2^+$ cells gathered at the injection area and were mainly localized underneath the epithelium (Fig. 6a, d) and among the muscle fibers (Fig. 6d, e). Interestingly, cell counting performed on five representative images of $CD11b^+$ cells at each time lapse showed that $CD11b^+/RNASET2^+$ granulocytes and $CD11b^-/RNASET2^+$ macrophage cell numbers were differently distributed during the different phases of the inflammatory response. Indeed, the number of granulocytes largely increased just 30 min following stimulation, remained high at 6 h and then decreased and returned to a baseline level at 24 h from stimulation. By contrast, the number of infiltrating macrophages was lower than that of granulocytes in the early inflammatory phase, but it grew gradually in later phases to reach a peak at 6 h from stimulation.

The presence of RNASET2 in $CD11b^+/RNASET2^+$ leech granulocytes was also confirmed by immunogold staining in LPS-challenged leeches at 30 min (Fig. 7a–c). Strikingly, electron microscope analysis clearly showed the presence of RNASET2-positive gold particles specifically localized in the granules of these cells (Fig. 7b). No gold particles were detected in control experiments in which the primary antibody was omitted (Fig. 7c) or substituted with preimmune serum (Fig. 7d). Besides confirming the expression of RNASET2 in granulocytes, these data also demonstrated that this enzyme was stored in their granules.

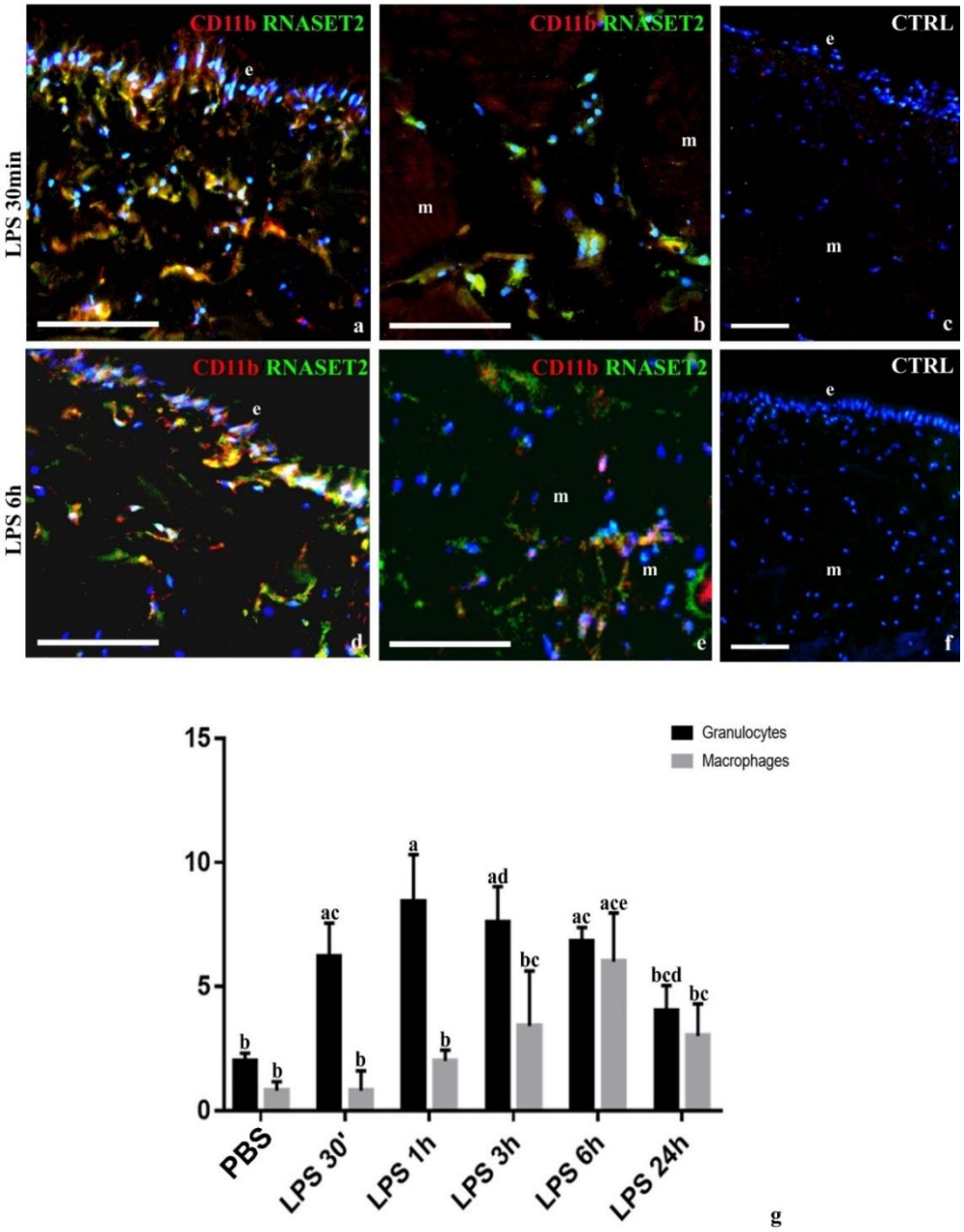

Fig. 6. Double immunolocalization of RNASET2 and CD11b or RNASET2 and *Hm*AIF-1 in animals 30 min and 6 h following LPS injection. Numerous RNASET2$^+$/CD11b$^+$ type I granulocytes (yellow in **a**, **b**) and RNASET2$^+$/*Hm*AIF-1$^+$ macrophages (yellow in **d**, **e**) migrating towards the injected area are visible under the epithelium (e) and among the muscle fibers (m). Double immunostaining was performed with anti-RNASET2 (green) anti-CD11b or anti *Hm*AIF-1 (red). **c**, **f**. No signal was detected in negative control experiments in which the primary antibodies were omitted. Cell nuclei stained blue by treatment with DAPI. Bars: 50 µm. **g** The percentages of granulocytes (RNASET2$^+$/CD11b$^+$) and of macrophages (RNASET2$^+$/CD11b$^-$) were assessed by analyzing 5 different slides (10 random fields of 45,000 µm^2 for each slide) using the Image J software package. Statistical differences were calculated by factorial ANOVA followed by Tukey's post hoc test, and $p < 0.05$ was considered statistically significant (between PBS- and LPS- challenged leeches). Means with different letters indicate significant difference between the number of granulocytes and macrophages in untreated animals.

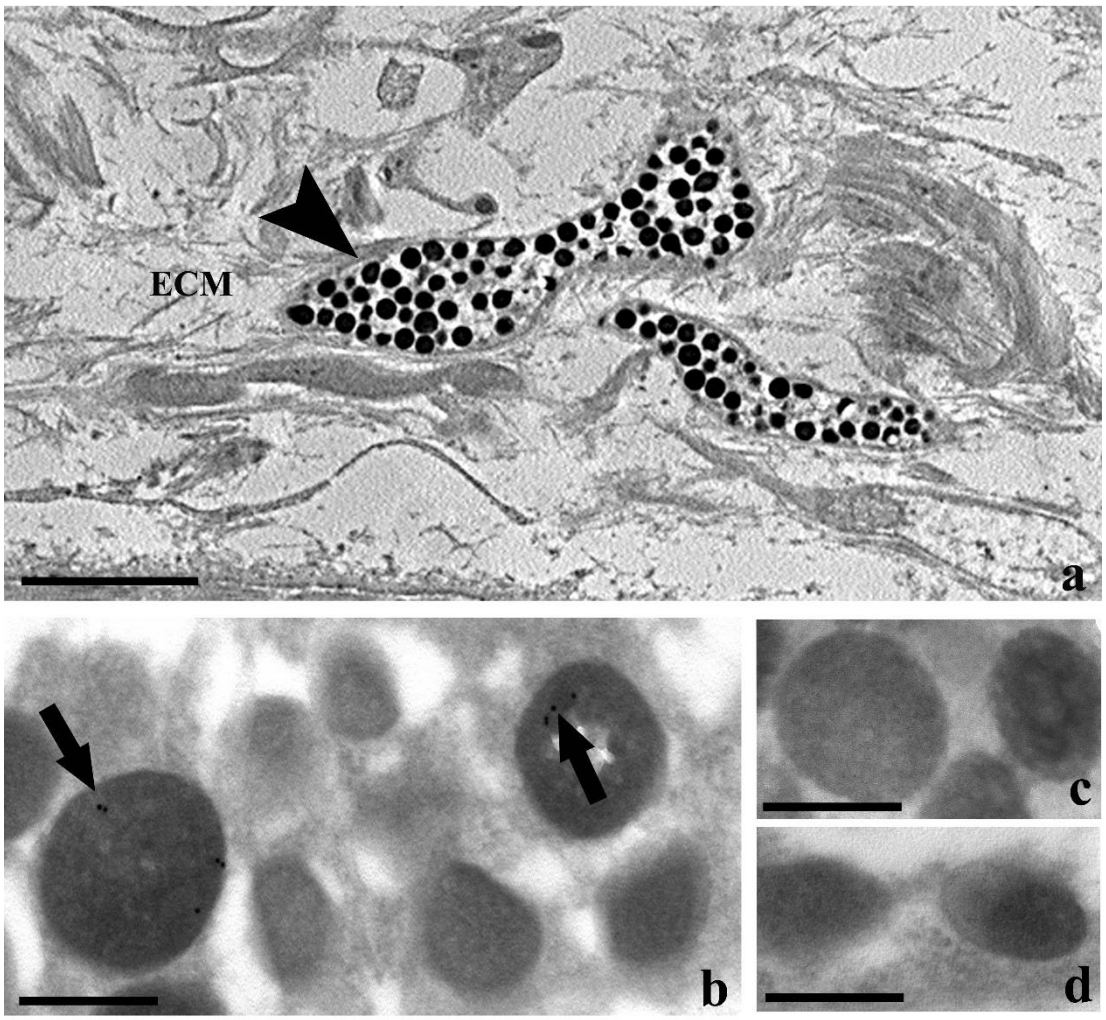

Fig. 7. Immunogold staining of RNASET2. Type I granulocytes (arrowhead in **a**) present in the extracellular matrix (ECM). **b** Detailed TEM showing the localization of gold particles in the granules (arrows). **c**, **d** Negative controls. Bar in **a**: 2.5 µm; bars in **b**, **c**, **d**: 300 nm.

Evaluation of the Antibacterial Effect of RNASET2

The RNASET2 localization pattern in the electron-dense granules of granulocytes prompted us to investigate whether this enzyme could directly or indirectly affect bacterial viability. To better evaluate this hypothesis, an *in vitro* eukaryotic-prokaryotic co-culture was set up.

The human monocytic leukemia-derived cell line (THP-1) was committed to differentiate into macrophage-like cells following treatment with phorbol esters, thus mimicking native monocyte-derived macrophages. We chose this experimental system because differentiated THP-1 cells represent a well-established model of *in vitro* macrophage differentiation when compared to other human myeloid cell lines (Auwerx 1991). In our co-culture assay, THP-1 cells, which normally express and secrete high endogenous RNASET2 levels [Scaldaferri et al., submitted], were compared to their RNASET2-silenced counterpart (Fig. 8a–d) in the presence of the GFP-expressing PAO1 *P. aeruginosa* strain described in the Materials and Methods

section.

Upon a 2-h co-culture at 37°C, bacteria cell integrity was checked by evaluating the GFP expression by fluorescence microscopy. Strikingly, the rod-like morphology of *P. aeruginosa* PAO1 cells co-cultured with parental RNASET2-expressing THP-1 cells could be hardly perceived and the GFP fluorescence signal appeared largely scattered, suggesting a bacterial stressful condition (Fig. 8a, c). By contrast, *P. aeruginosa* PAO1 cells co-cultured with RNASET2-silenced THP-1 cells maintained their cellular typical rod morphology (Fig. 8b, d). Bacterial viability has also been checked in both co-cultures with parental and RNASET2-silenced THP-1 cells, but no significant difference was observed after 2 h from the inoculum ($\sim 10^7$ CFU/ml). Based on these results, GFP-expressing *P. aeruginosa* PAO1 cells were directly injected into the leech body wall (Fig. 8e–j) to evaluate the *in vivo* effects of leech RNASET2 on bacterial viability. The microorganisms were injected with and without a neutralizing anti-RNASET2 antibody, which does not induce an inflammatory response but functionally inhibits the endogenous leech RNASET2 protein function (Baranzini et al. 2017). Leeches were sacrificed 30 min after infection, the time point at which a high expression of RNASET2 was correlated with a high number of granulocytes, and tissue sections were examined by optical and fluorescence microscopy. Strikingly, in vivo antibody-mediated blocking of the RNASET2 protein was associated with the observation of apparently undamaged bacteria, forming clusters in the connective tissue of the infected animals (Fig. 8e). These bacteria showed the typical rod shape easily recognizable by GFP (Fig. 8f) and DAPI (Fig. 8g) signals, respectively. By contrast, in leech infected in the absence of neutralizing anti-RNASET2 antibody, no bacterial clusters were observed in the tissues (Fig. 8h) and a diffused GFP signal was detectable. Only a few intact bacteria were recognizable (Fig. 8i, j). These results support a possible RNASET2-mediated bacterial cell wall damage or a failure in expression machinery given that the GFP signal was not associated with bacteria cells.

Collectively, these data support our hypothesis that the RNASET2 protein might play an antimicrobial role.

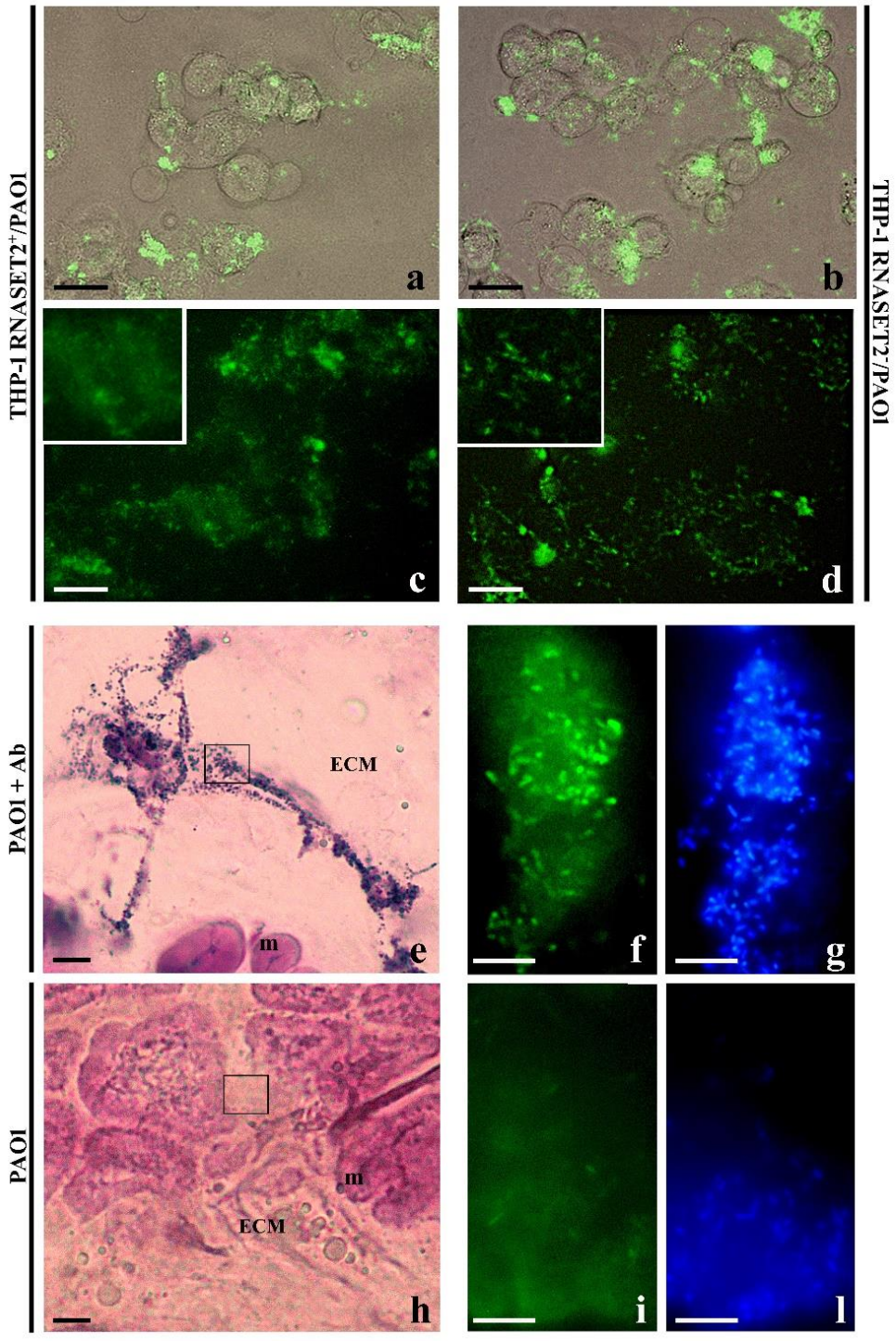

Fig. 8. In vitro and in vivo analysis. *P. aeruginosa* PAO1 cells expressing GFP and co-cultured with RNASET2 silenced THP-1 cells (**b**, **d**, and **inset**) maintained their typical rod morphology, and GFP signal was localized inside the bacteria. On the other hand, in the presence of THP-1 expressing RNASET2 cells (**a**, **c**, and **inset**), GFP signal is widespread, indicating the PAO1 are in a stressful condition. Cryosection of leech body wall injected with GFP/PAO1 cells and an antibody anti-RNASET2 (**e**-**g**) or injected with GFP/PAO1 alone (**h**–**j**). **e** Violet and fuchsin staining shows the presence of bacteria agglomerates. GFP (**f**) and DAPI (**g**) signals highlight the characteristic rod shape of these bacteria. In absence of the functional blocking antibody anti-RNASET2, no bacteria are visible (**h**), and GFP (**i**) and DAPI (**j**) show a diffuse staining. The squares in **e**, **h** indicate the magnified areas of **f**, **g**, **i**, **j**.**a**,**b**. The merge of fluorescent channel with transmission image shows the position of bacteria with respect to eukaryotic cells. Bars in **a**–**d**: 15 µm; bars in **e**–**j**: 4 µm.

DISCUSSION

Several experimental reports have demonstrated a remarkable resemblance and evolutionary conservation of both the cell types and the underlying pathways by which Hirudinea and vertebrates trigger the inflammatory and immune response processes (Grimaldi et al. 2006; Grimaldi 2016). Hirudinea are continuously exposed to attack and invasion from potential pathogens such as microorganisms or parasites (Silver et al. 2007). In order to deal with this constant threat, they are endowed with several effective strategies to recognize and destroy "not-self" molecules or microorganisms. Indeed, bacterial infections, wounds or allografts activate a complex defense response in leeches, including proliferation and migration towards the stimulated area of immunocompetent cells involved in phagocytosis, encapsulation of not-self agents, angiogenesis, fibroplasia, and reshaping of scar tissues (de Eguileor et al. 1999a, 2000b, a; Tettamanti et al. 2005). As mentioned above, these processes involve cellular mechanisms and key effector molecules that proved to be very similar to those deployed by vertebrates. Due to these remarkable similarities to vertebrates in inflammatory response and tissue repairing, medicinal leeches belonging to the *Hirudo* genus are thus increasingly exploited as an emerging, cost-effective, and valuable experimental model to investigate the mechanisms that underlie these biological processes (Grimaldi et al. 2006; Grimaldi 2016).

Within this frame, recent studies from our laboratories, focused on functional analyses of the immunomodulatory AIF-1 and RNASET2 proteins, allowed us to investigate the biological role played by these factors in the activation of the innate immune system in this animal model.

The allograft factor 1 (AIF-1) protein, a novel cytokine-like molecule involved in immunocyte recruitment into injured/grafted or infected tissues both in vertebrates (Kuschel et al. 2000; Deininger et al. 2000; Watano et al. 2001) and invertebrates (Kruse et al. 1999; Zhang et al. 2011; Ovando et al. 2012; Li et al. 2013), has recently been characterized in leech by our research group. Indeed, a gene showing high similarity with vertebrates' AIF-1, named *Hm*Iba1/alias *Hm*AIF-1, has been recently characterized in the central nervous system of the medicinal leech (Drago et al. 2014). Of note, the expression of AIF-1 in leech has been reported to increase significantly after transplantation, wound healing, or bacterial infections, suggesting its involvement in the inflammatory response and in immune system regulation. Strikingly, immunohistochemical assays with an anti-*Hm*AIF-1 polyclonal antibody revealed

the presence of this protein in leech macrophage-like cells. Moreover, recombinant AIF-1 was shown to induce a massive migration of $CD45^+/CD68^+$ macrophages towards infected of injured tissues (Schorn et al. 2015a).

In our previous work, a chemotactic role of leech cells belonging to the monocyte-macrophage lineage cells has been observed for RNASET2 as well. Indeed, injection of human recombinant RNASET2 in leeches induced a massive migration of macrophage-like cells towards the stimulated area, and endogenous RNASET2 was found to be expressed in leech's macrophages as well.

Although these previous data strongly implicate both AIF-1 and RNASET2 as evolutionary conserved mediators of the innate immune response, the specific mechanisms by which they regulate innate immune cells functions and that define their specific roles in inflammation and innate immune response activation remain largely unexplored. Our preliminary data strongly suggested that both molecules seem to play a similar role in the early stages of the inflammatory response but could also take part in later events that activate the immune response stimulating macrophage migration. However, neither the precise timing of action of both factors during the inflammatory response nor their putative functional interactions have been investigated so far.

Here, we provide further experimental evidence supporting a critical role of both factors in orchestrating a highly coordinated response against pathogen infection in medicinal leeches. To shed more light on these issues, we first analyzed the *in vivo* expression levels of both AIF-1 and RNASET2 in tissues from LPS-injected leeches by immunoblot analysis at different times after treatment. This allowed us not only to confirm that AIF-1 and RNASET2 are both activated in the early inflammatory events, but also to unveil a peculiar temporal expression profile for these two cytokines. Since medicinal leeches present a very fast inflammatory response (de Eguileor et al. 2000a, 2001a), we decided to investigate the temporal expression profiles of both proteins at 30 min and 1, 3, 6, and 24 h following LPS injection. Quantitative analyses of the data clearly showed that, whereas AIF-1 and RNASET2 were constitutively expressed at low basal level in healthy animals, LPS injection produced a marked change in their expression profile over time. In particular, AIF-1 expression significantly increased in the earliest phases of inflammation, followed by a slow but steady decline up to 24 h. By contrast, RNASET2 expression significantly peaked at 30 min and 6 h after treatment.

In medicinal leeches, LPS injection is known to induce a massive migration and activation of immunocompetent cells, which are mainly localized in the connective tissue and near the body wall epithelium (Schorn et al. 2015b; Baranzini et al. 2017). Double immunofluorescence experiments of LPS-injected leeches showed a massive recruitment of both $CD11b^{+}$/$RNASET2^{+}$ granulocytes and $AIF\text{-}1^{+}$/$RNASET2^{+}$ macrophages underneath the epithelium and near the muscular fibers, with a signal specificity for each antibody in keeping with the data obtained by the previous Western blot analysis. By contrast, control samples analyzed following PBS injection showed a tissue architecture similar to unlesioned animals, indicating that neither the mechanical stress induced by injection nor the vehicle solution alone could significantly affect AIF-1 or RNASET2 expression.

Taken together, the observed AIF-1 and RNASET2 expression pattern clearly suggests the involvement of both proteins in the early inflammatory processes of *H. verbana*.

Moreover, the expression of RNASET2 by granulocytes, which to our knowledge has not been reported before, prompted us to evaluate the possible involvement of leech granulocytes in RNASET2 production and secretion. Our optical, ultrastructural, and immunohistochemical analyses of LPS-injected leeches confirmed that $CD11b^{+}$/$RNASET2^{+}$ granulocytes, representing the first immunocompetent cells that are activated to trigger a defense response against microbial infections, are early recruited at the site of LPS injection. Moreover, RNASET2 turned out to be specifically confined in the granules of these cells, suggesting a possible antibacterial role of this protein. It is important to stress that several ribonucleases are known to be endowed with a strong antibacterial activity such as the RNASE A superfamily members RNase 3, 6, 7, and 8, whose antibacterial activity has been associated with disruption of the bacterial membrane (Nitto et al. 2006; Pizzo et al. 2008). In fact, these enzymes display a strong affinity for LPS of Gram-negative bacteria, take contact with the bacterial cellular membrane and subsequently destabilize the phospholipid double layer, finally triggering the agglutination and depolarization of the bacterial membrane (Torrent et al. 2010; Pulido et al. 2013). Interestingly, several members of this RNase superfamily are often expressed in different types of immune cells (Gupta et al. 2013). For instance, EDN/RNase2 and ECP/RNase3 have been detected in the secondary granules of eosinophils (Shamri et al. 2011), whereas Rnase2 and RNase3 represent two actively secreted proteins that were found in the granules of these cells during an inflammatory response (Koczera et al. 2016).

Following the detection of RNASET2 in the granules of RNASET2^{+}/CD11b^{+} cells in LPS-challenged leeches, we set up in vitro and in vivo assays to evaluate the ability of *Hirudo* RNASET2 to affect *P. aeruginosa* PAO1 integrity. Strikingly, both assays strongly suggested that RNASET2 affects bacterial integrity. Although several antimicrobial peptides produced by medicinal leech have been described (Schikorski et al. 2008; Tasiemski and Salzet 2017), to our knowledge, this is the first report of the occurrence of an antibacterial activity of a ribonuclease protein in this animal model. The expression of RNASET2 seemed not to interfere with bacterial viability as outlined from viability assay in co-cultures. However, *P. aeruginosa* co-cultured with parental THP-1 cells released in the extracellular environment the GFP, suggesting a modification of membrane permeability. Although GFP release was apparently not able to influence cellular viability under the tested conditions, it could nevertheless represent the first part of a multistep *in vivo* antimicrobial response involving other immune system cells. The observed release of the GFP in both *in vitro* co-cultures and *in vivo* could therefore be attributed to the direct effect of RNase T2 on the bacterial envelope. For instance, the cationic residues of RNASET2 could bind to the anionic bacterial membrane to induce cell death (Pizzo et al. 2008). However, an indirect antimicrobial effect of RNase T2 cannot be ruled out, and further investigations are needed to shed light on both hypothesized antimicrobial mechanisms.

Collectively, these results suggest that the innate immune response could be activated and modulated in medicinal leeches by the establishment of an AIF-1/RNASET2-mediated cross talk involving the recruitment and activation of granulocytes and macrophages, resulting in an effective defense against bacterial infections.

Thus, we can envisage an early inflammatory response in leeches, where both residents, LPS-activated AIF-1^{+} macrophages, and CD11b^{+} LPS-recruited granulocytes actively produce and secrete RNASET2, whose effect is to send an alarm signal to nearby healthy tissue in order to recruit further innate immune cells and at the same time to carry out a direct antibacterial activity.

Moreover, the AIF-1 protein actively secreted by LPS-activated, resident macrophages contributes to immune cell recruitment as well (Schorn et al. 2015b, a). At later stages (6 h after treatment), a second boost of RNASET2 expression ensues to maintain the inflammatory state by means of recruitment of further macrophages (as shown by the resumed AIF-1 expression detected by Western blot analysis at 24 h) in order to clean the infected area from

bacterial debris.

CONCLUSION AND FUTURE PERSPECTIVES

Taken together, our data strongly suggest that, in addition to the well-established role of AIF-1 in triggering the innate immune response, the early phase of the inflammatory response in leeches is characterized by the contemporary release of RNASET2 and AIF-1 from immunocompetent cells, which might carry out different but complementary roles, namely bacterial killing and further innate immune cell recruitment. Moreover, at later stages of infection, RNASET2 might act mainly as a chemokine in order to attract new macrophages (which in turn produce RNASET2 by themselves) to further strengthen the inflammatory state (as schematically shown in Fig. 9).

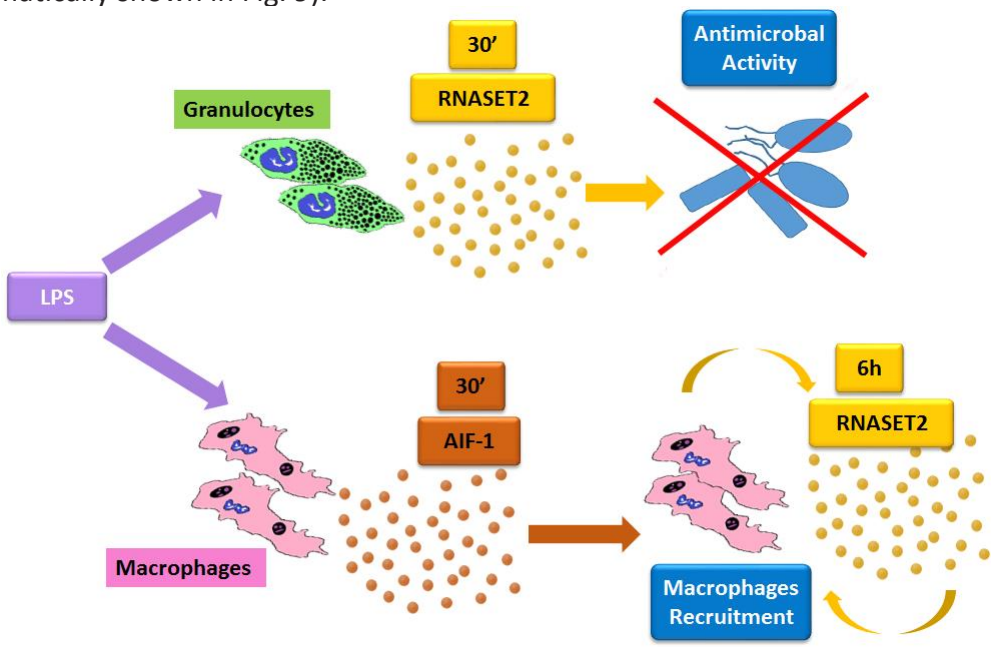

Fig. 9. Representation to explain the different but complementary roles of RNASET2 and AIF-1 in early inflammatory response. 30 min after LPS stimulation, activated granulocytes secrete RNASET2, whose first effect is to carry out a direct antibacterial activity. In parallel, LPS-activated macrophages release AIF-1 in order to recruit other macrophages. These cells, releasing RNASET2, maintain the inflammatory state by recruiting other macrophages involved in cleaning the infected area from bacterial debris

Such dual role (antibacterial activity and innate immunity stimulation) played by RNASET2 represents a further demonstration of the pleiotropic role carried out by this class of ancient, evolutionary conserved ribonucleases. To confirm the evolutionary conserved function of RNASET2 in regulating the innate immune response, the role of the *H. verbana* RNASET2 gene in the response to bacterial infection will be investigated as well following the cloning of the corresponding gene's coding sequence. Moreover, we are currently implementing experimental protocols for endogenous *RNASET2* gene knockdown in leeches,

in order to develop functional assays aimed to further dissect the role of RNASET2 in inflammatory response.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.G. and F.A. conceived and designed the experiments and wrote the main manuscript text; N.B. and M.V. performed the morphological, immunofluorescence, and immunogold analyses; L.M. performed Western blot assays; R.G. performed statistical analysis; D.S. cultured and differentiated THP-1 cells; F.B. transformed PAO1 for GFP expression; V.T.O. analyzed data of Figure 7; G.T. and M.d.E. provided expertise for TEM and imaging; J.V. provided anti-*Hm*AIF-1 antibody and expertise for histocytochemistry and imaging; R.T. contributed to reagents. All authors critically reviewed the manuscript.

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CHAPTER 3

Antimicrobial role of RNASET2 protein during innate immune response in the medicinal leech *Hirudo verbana*

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ABSTRACT

The innate immune response represents a first-line defense against pathogen infection that has been widely conserved throughout evolution. Using the invertebrate *Hirudo verbana* (Annelida, Hirudinea) as an experimental model, we show here that the RNASET2 ribonuclease is directly involved in the immune response against Gram-positive bacteria. Injection of lipoteicoic acid (LTA), a key component of Gram-positive bacteria cell wall, into the leech body wall induced a massive migration of granulocytes and macrophages expressing TLR2 (the key receptor involved in the response to Gram-positive bacteria) towards the challenged inoculated. We hypothesized that the endogenous leech RNASET2 protein (*Hv*RNASET2) might be involved in the antimicrobial response, as already described for other vertebrate ribonucleases, such as RNase3 and RNase7. In support of our hypothesis, *Hv*RNASET2 was mainly localized in the granules of granulocytes and its release in the extracellular matrix triggered the recruitment of macrophages towards the area stimulated with LTA. The activity of *Hv*RNASET2 was also evaluated on *Staphylococcus aureus* living cells by means of light, transmission and scanning electron microscopy analysis. *Hv*RNASET2 formed *S. aureus* clumps following a direct interaction with the bacterial cell wall, as demonstrated by immunogold assay. Taken together, our data support the notion that, during the early phase of leech immune response, granulocytes-released *Hv*RNASET2 triggers bacterial clumps formation and, at the same time, actively recruits phagocytic macrophages in order to elicit a rapid and effective eradication of the infecting microorganisms from inoculated area.

Keywords: RNASET2, antimicrobial activity, LTA, innate immunity, medicinal leech.

INTRODUCTION

The RNases T2 family is represented by extracellular ribonucleolytic enzymes that act at an optimal pH of about 4.0-5.0 and have been found variously distributed throughout *taxa*, from viruses to higher eukaryotes (1). Strikingly, despite their highly conserved biochemical and structural features, T2 RNase from different organisms show a marked pleiotropic nature, being involved in an impressive range of biological functions, often related to stress response and host defense (1,2).

Indeed, T2 RNases have acquired throughout evolution a range of biological functions, such as control of cell senescence (3), induction of oxidative stress-mediated apoptosis (4), cytotoxicity (5) regulation of cell motility/migration by cytoskeletal reassembly (6), modulation of angiogenesis (7), regulation of self-incompatibility in plants (8,9) and tumor suppression (10). In addition, T2 RNases in plants counteract harmful agents to protect germinal seeds from different type of pathogens, such as virus or bacteria (11).

The pleiotropic roles of T2 RNase are further suggested by the observation that several biological processes regulated by members of this enzyme family do not depend by their catalytic activity (1). Moreover, besides extracellular compartments T2 ribonucleases have been reported in cytoplasmic vacuoles, lysosomes, P-bodies and mitochondria (6,12–14), further arguing in support to their involvement in wide range of key biological processes.

Recently, our group begun investigating the role of T2 RNases in the medicinal leech *Hirudo verbana*, in order to better define the host defense role of this class of proteins in an invertebrate model showing a very simple anatomy, coupled to a marked similarity with vertebrates concerning the cellular and molecular effectors involved in inflammatory processes (15). Noteworthy, we were able to confirm the pro-inflammatory role of T2 RNases in this invertebrate model, carried out by means of recruitment and activation of cells from the monocyte/macrophage lineage, as previously defined in mammalian experimental models (10). This finding clearly suggests that stimulation of innate immunity-mediated host defense represents a key evolutionarily conserved role for T2 ribonucleases (16,17).

Furthermore, we found preliminary experimental evidence in support of a putative role for T2 RNases in host defense against bacterial infections as well, since lipopolysaccharide (LPS) injection into the leech body wall triggered a marked increase in the expression levels of

endogenous RNASET2 protein in both host macrophages and granulocytes and recombinant RNASET2 was apparently able to affect bacterial cell integrity *in vivo* (16,17).

Although the molecular mechanisms by which T2 enzymes from plants and medicinal leech act in the antimicrobial process are still unknown, this ability is reminiscent to that previously described for some members of the RNase A superfamily which, unlike T2 RNases, has been described only in vertebrates (18). For instance, the class A human RNase3 protein, also called eosinophil cationic protein (ECP) (19), acts as a strong eosinophils-mediated antimicrobial protein or peptide (AMPs) independently from its ribonucleolytic activity (20). ECP is released during eosinophil activation from the inner secondary cytoplasmatic granules to the extracellular environment and, after specific interaction with bacterial cells, it permeabilizes their external membranes in order to disrupt them (21–23). ECP is active against different types of bacteria (24) and shows a high affinity to lipopolysaccharides (LPS), a component of the outer membrane of Gram-negative bacteria. By binding to bacterial cell membranes and subsequent destabilizing them, ECP shows a carpet-like anti- bacterial mechanism which recalls many host defense antimicrobial proteins or peptides (20). In addition, its N-terminal region induces the formation of bacterial clumps, thus promoting a systematic elimination by immune cells (25).

Other class A ribonucleases, such as RNase 2 and RNase 7, act as “alarmins”, molecules passively released by necrotic cells or actively secreted by immune or epithelial cells in order to signal to the innate and adaptive immune system the occurrence of a wide range of dangerous events, such as those occurring during pathogen infection or tumor progression. As such, alarmins promote inflammatory responses, usually mediated by Toll-like receptors family members (TLRs) (26,27). Among TLRs, TLR2 and TLR4 represent the most significant group of PRRs (Pattern Recognition Receptors), which are evolutionary conserved both in vertebrate and in invertebrate species (28–30) and are expressed by immune cell membranes. These receptors mediate the recognition of conserved biomolecules known as Pathogen-Associated Molecular Patterns (PAMPs), such as lipoteichoic acid (LTA) and lipopolysaccharide (LPS), which are normally displayed in the external membrane of Gram-positive and Gram-negative bacteria, respectively. Noteworthy, human RNASET2 has also been suggested to act as an alarmin, being actively release under a wide range of stressful conditions (4,6,31).

Starting from these assumptions, using *H. verbana* as an experimental model to gain more insights into the role of RNASET2 as a host defense protein, we have confirmed and

characterized the antimicrobial action of RNASET2 against Gram-positive bacteria in both *in vivo* and *in vitro* experimental settings.

MATERIALS AND METHODS

Hirudo verbana recombinant r*Hv*RNASET2: cloning and expression

The mRNA sequence of *H. verbana* RNASET2 was obtained from an in silico leech transcriptome database (http://genomes.sdsc.edu/leechmaster/database/) (32): the >EN-124k-90-group2043 coding the full sequence was selected. In parallel, mRNA extraction from the leech body wall was performed and the sample was treated with DNase (Turbo DNA-freeTM Kit- Invitrogen) to remove all DNA traces. After reverse transcription with oligo dT (High-Capacity cDNA Reverse Transcription Kit - Applied Biosystems™), different couples of primers were used resulting in a partial amplifications of the coding sequence. Therefore, an overlap extension PCR was performed to obtain the full coding sequence of *Hv*RNASET2. The primers used in the first two PCR amplifications were:

First sample: Fw: 5'-CGTAGAATTCAAGTAATTAAATCTGATTCGGAGTG-3' (fw1-e); Rev: 5' ATATTGCAGTGGTTCATTACGTGGA 3'.

Second sample: Fw: 5' TCCACGTAATGAACCACTGCAATAT 3'; Rev: 5' CGTAGAATTCTGTGTAAAAGGGAATATTAGATCAAG 3' (rev7-e).

Both the products were used as template in a third PCR performed using the external primers (fw1-e+ rev7-e - the underlined bases represent restriction site for EcoRI). After digestion with EcoRI, the product was cloned in pBluescript.

Subsequently, the leech *Hv*RNASET2 coding sequence (BankIt2095553 Hirudo MH325331) was amplified without the predicted signal for secretion, using the following primers:

5' CGTAGAATTCAGGCCTCTGAAGGAAGAATT 3'; 5' CGTATCTAGACCTGAGTTTGAATGAATTTGGTT 3' as forward and reverse primer, respectively (the underlined sequences represent restriction sites for EcoRI and XbaI). After digestion with EcoRI and XbaI, the product was cloned into the pPICZαA expression vector for heterologous expression in the yeast *Pichia pastoris*, as previously described (33). To introduce a 6XHIS tag at the N-terminus of the protein, a useful sequence for protein purification, the pPICZαA-*Hv*RNASET2 construct was digested at EcoRI site. The tag was introduced by amplification using the following primer pair (as an insert):

5’ AATTCCATCACCACCATCATCACG 3’; 5’ AATTCGTGATGATGGTGGTGATGG 3’.

Recombinant DNA was purified from several clones and, after control sequencing (BMR, Padova, Italy), the pPICZαA-*Hv*RNASET2 expression vector was used to transform the X33 *P. pastoris* strain. Briefly, the RNASET2-coding construct was linearized within the 3'AOX region with PmeI restriction enzyme and transformed into yeast with the lithium chloride method (34). The methanol utilization test was carried out as a control to verify the correct yeast phenotype.

Subsequently, a selected clone was inoculated into 50 mL of BMGY medium (BMGY/BMMY: 1% yeast extract, 2% bactopeptone, 1.34% yeast nitrogen base, 4x10-5% biotin and either 1% glycerol or 0.5% methanol). Cultures were grown at 30 °C overnight until they reached an Optical Density at 600 nm (OD_{600}) between 2 and 6. After centrifugation, cell pellets were resuspended in 1.3 L BMMY at a starting OD_{600} of 1 for induction of protein expression. Every day (for 7 days) fresh methanol (with a final concentration of 0.5%) was added to cultures.

Purification of the recombinant r*Hv*RNASET2

The supernatant was concentrated by ultrafiltration using an Amicon stirred cell (Merk Millipore) equipped with a 10-kDa membrane to a final volume of 40 mL, and extensively dialyzed against 20 mM sodium phosphate, 150 mM NaCl, pH 7.5. The sample was added of NaCl up to 1 M final concentration and the protein was purified using a HiTrap chelating affinity column (5 mL) (GE Healthcare) previously loaded with 100 mM NiCl2 and equilibrated with 20 mM sodium phosphate, 1 M NaCl, pH 7.5. The column was washed with this buffer until the absorbance value at 280 nm was that of the buffer. r*Hv*RNASET2 was eluted with the same buffer added of 100 mM imidazole; the fractions were equilibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5 by a gel- permeation chromatography (PD10 column, GE Healthcare). The amount of protein was determined by using the absorbance intensity at 280 nm and a molar extinction coefficient of 66 mM^{-1} cm^{-1} (6). The recombinant r*Hv*RNASET2 was isolated as a single band at ≈ 36 kDa with >90% purity as judged by SDS-PAGE analysis: ≈ 3.5 mg of purified enzyme per L of fermentation broth were obtained.

Endotoxin removal

Endotoxins were removed from the purified protein according to the procedure reported in (35). Briefly, the protein sample was added of 1% Triton X-114, incubated at 4°C for 30 min

and then at 37°C for 10 min, and finally centrifuged at 16,000 g for 15 min, at RT. The supernatant was recovered and all the steps were repeated two times, plus a final step without Triton X-114. The removal of endotoxins was assessed with a LAL test (PYROGENT™ Gel Clot LAL Assay – LONZA).

Animals and treatments

Adult leeches (*Hirudo verbana*, Annelida, Hirudinea, from Ricarimpex, Eysines, France), measuring 10 cm were kept in lightly salted water (NaCl 1.5 g/mL) in aerated tanks at 19°-20°C. Before injection and/or dissection, leeches were anesthetized with a 10% ethanol solution and all treatments were performed at the 80th superficial metamere from the oral sucker. Animals were randomly split into separate experimental groups (three individuals for each time point) and submitted to various protocols and treatments, as described below:

Group 1: injection with 100 µL 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) followed by evaluation at 30 min, 1 hours, 3 hours, 6 hours and 24 hours time-points, to confirm that PBS alone does not induce an immune response.

Group 2: injection with 100 µL of PBS containing 100 ng/mL of LTA from *Bacillus subtilis* (Sigma Aldrich, St. Louis, MO, USA) followed by evaluation at 30 min, 1 hours, 3 hours, 6 hours and 24 hours time-points, to stimulate an inflammatory response and to evaluate the expression of TLR2 and TNF-α in cells involved in the immune response. The optimal LTA concentration required to induce significant cell migration in leeches was determined based on our previous work (36).

Group 3: injection with 100 µL of PBS containing 1 µg/ml of CyP, a LPS-like molecule extracted from the cyanobacterium *Oscillatoria Planktothrix* FP1 (cyanobacterial product [CyP]) that acts as a potent and selective antagonist of bacterial LPS (30,37) followed by evaluation at 30 min, 1 hours, 3 hours, 6 hours and 24 hours time-points. This treatment was performed to exclude any possible interaction between LTA and the LPS receptor TLR4 (30).

Group 4: injection with 100 µL of PBS containing 1 µg/ml of CyP plus 100 ng/mL of LTA obtained from *Bacillus subtilis* (Sigma Aldrich) followed by evaluation at 30 min, 1 hours, 3 hours, 6 hours and 24 hours time-points. This treatment was performed to confirm the TLR2

specificity in LTA recognition and to exclude any possible interaction between LTA and TLR4 (30).

Group 5: injection with 100 µL of PBS containing methicillin susceptible *Staphylococcus aureus* ATCC 6538P (10^7 CFU/mL) followed by evaluation at 3 hours.

Group 6: injection with 100 µL of a PBS solution containing *S. aureus* ATCC 6538P (10^7 CFU/mL) and 10 µM recombinant *Hirudo verbana* RNASET2 protein (*Hv*RNASET2, accession number BankIt2095553 Hirudo MH325331) followed by evaluation at 3 hours.

Group 7: injection with 100 µL of a PBS solution containing *S. aureus* ATCC 6538P (10^7 CFU/mL) and 10 µM r*Hv*RNASET2 pre-treated with 1 µL of an anti-RNASET2 antibody followed by evaluation at 3 hours, as a specificity control to functional block *Hv*RNASET2 activity (30).

Light and electron microscopy

Leech tissues, dissected from the area of injection in each experimental group, were fixed for 2 h in 0.1 M cacodylate buffer at pH 7.4 containing 2% glutaraldehyde. After several washes in the same buffer, tissue samples were postfixed for 1 hour with 1% osmium tetroxide in cacodylate buffer, pH 7.4 and subsequently embedded in an Epon-Araldite 812 mixture (Sigma-Aldrich, Milan, Italy), after serial ethanol dehydration (70%, 90%, 100%). Tissue sections were obtained with a Reichert Ultracut S ultratome (Leica, Wien, Austria). Semi-thin sections (0,7µm) were stained by conventional methods, using crystal violet and basic fuchsin (according to (38) and observed under a light microscope Nikon Eclipse Ni (Nikon, Tokyo, Japan). Data were recorded with a DS-5M-L1 digital camera system (Nikon). Ultrathin sections (80 nm) were collected on copper grids (300 mesh, Sigma- Aldrich, Milan, Italy), counterstained by uranyl acetate and lead citrate, and observed with a Jeol 1010 EX transmission electron microscope TEM (Jeol, Tokyo, Japan). Data were recorded with a MORADA digital camera system (Olympus, Tokyo, Japan).

S. aureus ATCC 6538P cells were grown overnight in Müller Hinton broth 2 (MHB2, 0.3% beef infusion solids, 1.75% casein hydrolysate, and 0.15% starch) with continuous shaking at 200 rpm at 37°C and then transferred to fresh medium to reach the exponential growth phase. Subsequently, bacteria were suspended in PBS with the recombinant enzyme r*Hv*RNASET2 (10 µM) for 3 hours at 20°C, and then centrifuged for 10 minutes at 12.000 rpm. After surnatant removal, bacterial pellets were fixed with Karnovsky fixative (2% paraformaldehyde and 2.5%

glutaraldehyde in 0.1 M cacodylate Buffer, pH 7.2) for 1 hour at 4°C and then processed for TEM microscopy as above described.

3D imaging was obtained by scanning electron microscopy (SEM). After 3 hours from r*Hv*RNASET2 (10 μM) treatment, bacteria were fixed in Karnovsky fixative for 30 minutes, washed in 0.1 M cacodylate buffer (pH 7.2) and post-fixed in a solution of 1% osmium tetroxide and potassium ferrocyanide for 1 h. After several washes in PBS (pH 7.2) and dehydration with an increasing scale of ethanol, 20 μL of bacterial pellet resuspended in ethanol 100% were dried onto glass slides and finally subjected to critical point drying with hexamethyldisilazane. Images were acquired using the SEM-FEG XL-30 microscope (Philips, Eindhoven, The Netherlands).

Immunogold staining at TEM

Samples were fixed for 2 h at 4°C with 4% paraformaldehyde and 0.5% glutaraldehyde in PBS, dehydrated in ethanol series and embedded in an Epon-Araldite 812 mixture (Sigma-Aldrich). Ultrathin sections, obtained as above, were collected on gold grids (300 mesh, Sigma-Aldrich). After etching with 3% NaOH in absolute ethanol (39) they were incubated for 30 min in blocking solution containing PBS, 1% bovine serum album (BSA), and 0.1% Tween and then with the polyclonal primary antibody rabbit anti-human RNASET2 (40) diluted at 1:20 in blocking solution. After several washings with PBS, the primary antibody was visualized by immunostaining with the secondary goat anti-rabbit IgG (H+L)-gold conjugate antibody (GE Healthcare, Amersham, UK; particle size, 10 nm) diluted at 1:100 in blocking solution for 1 h. In control experiments, the primary antibody was omitted and sections were treated with BSA containing PBS and incubated only with the secondary antibodies. Sections were counterstained with uranyl acetate in water, observed at TEM and data were recorded with a digital camera system as previous described.

Immunofluorescence assays

Tissue samples, dissected from differently treated leech body wall, were embedded in Polyfreeze tissue freezing medium (OCT, Polysciences, Eppelheim, Germany), immediately frozen in liquid nitrogen. Cryosections (7 μm) from *S. aureus* injected leeches were obtained with a cryotome (Leica CM1850), collected on gelatinous slides and counterstained with crystal violet and basic fuchsin for morphological analysis or with 0,1 mg/mL 4,6-diamidino-2-

Phenylinedole (DAPI, excitation and emission filter 360/420 nm).diluted 1:5000 in PBS to highlight bacterial DNA.

For immunofluorescence assays, slices were incubated for 30 min in blocking solution and then for 1 h at 37°C with the following polyclonal primary antibodies diluted in the same blocking solution: rabbit anti-RNASET2 (40), expressed by macrophages and granulocytes of leech (17) diluted 1:200; goat anti-CD11b (Santa Cruz Biotechnology, CA, USA, sc-28664) that specifically stains leech granulocytes (41) diluted 1:100; rabbit anti-*Hm*AIF-1 (kindly donated by Prof. Jacopo Vizioli, University of Lille 1, France), reacting with leech macrophages (36,42), diluted 1:1000; rabbit anti-TNF-α (Abcam, Cambridge, UK, ab6671) diluted 1:200 reacting with leech homologous protein (30); rabbit anti-TLR2 (Abcam, Cambridge, UK, ab213676, recognizing an epitope corresponding to amino acids 730–780 mapping to an internal region of TLR2 of human origin) diluted 1:200. After washing in PBS, samples were incubated for 45 minutes at room temperature respectively with an anti-goat or anti-rabbit Cy5-conjugated (Jackson Immuno Research Laboratories, West Grove, USA) secondary antibodies (excitation filter 650 nm, emission filter 672 nm) diluted 1:250 in blocking solution. Double labelling experiments to detect cells co-expressing CD11b/TLR2 or CD11b/RNASET2; were performed combining the following polyclonal primary antibodies: goat anti-CD11b and rabbit anti-TLR2 or goat anti-CD11b and rabbit anti-RNASET2. After washing in PBS, sections were incubated with a mix of the appropriate secondary antibodies: donkey anti-goat fluorescein isocyanate (FITC) conjugated (excitation filter 493 nm, emission filter 518 nm) and goat anti-rabbit Cy5-conjugated (Jackson Immuno Research Laboratories), diluted 1:200. To detect MyD88/TLR2, *Hm*AIF-1/TLR2, *Hm*AIF-1/RNASET2 co-expressing cells, since the primary antibodies were raised in the same species, the method previously described was used (16). The primary antibodies rabbit anti-MyD88 or rabbit anti *Hm*AIF-1 were applied first, then sections were incubated with the secondary antibody goat anti-rabbit (FITC)-conjugated. Before the second staining cycle, sections were treated with rabbit IgG (Jackson Immuno Research Laboratories) diluted 1:25 for 2 h (43) and incubated with rabbit anti-TLR2 or anti-RNASET2 Subsequently, the sections were treated with the secondary goat anti-rabbit (Cy5)-conjugated diluted 1:200. Tissue autofluorescence was reduced by treating sections with 1 mM $CuSO_4$ in 50 nM ammonium acetate buffer (pH 5.0) for 15 min (44). In all sections, nuclei were counterstained for 5 minutes with DAPI. The primary antibodies were omitted in the negative control experiments and sections were incubated only with the secondary antibodies. All samples

were mounted with Citifluor (Citifluor Ltd, UK) and examined with a Nikon Eclipse Ni (Nikon, Tokyo, Japan) light and fluorescence microscope. Data were recorded with a Nikon digital sight DS-SM (Nikon), and combined with Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Acid phosphatase reaction (ACP)

Leech tissues, taken from injected areas, were embedded in OCT and frozen in liquid nitrogen. Cryosections (7 µm) were rehydrated with PBS for 5 min and stained as previously described (16).

Western blot analysis

Leech tissues, dissected from the LTA challenged areas, were promptly frozen in cryovials and homogenized with a mortar. Homogenates (10 µL per mg of tissue) were suspended in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0) in the presence of protease and phosphatase inhibitors and kept O/N on a rotation mixer at 4°C. The particulate was removed by centrifugation at 13,000 rpm for 20 minutes at 4°C in a refrigerated Eppendorf Minispin microcentrifuge (Hamburg, Germany). After denaturation at 95°C for 5 minutes, protein concentrations were assayed with Coomassie Brilliant Blue G-250 protein assay (Pierce, Rockford, IL, U.S.A.) and BSA was used as standard. 10 µL of denatured proteins (2 mg/mL final concentration) were loaded on gel 12% acrylamide minigels for SDS-PAGE analyses. The SDS- PAGE separated proteins were transferred onto a nitrocellulose filter by means of a gel transfer system by applying 350 mA for 2 h. After pre-incubation for 2 hours in continuous stirring with a blocking solution containing 5% milk in Tris buffered saline (TBS: 50 mM TrisHCl 7.5, 150 mM NaCl), membranes were treated O/N at 4°C with the following primary rabbit polyclonal antibodies: anti-RNASET2 (40), anti-TLR2 antibody (Sigma-Aldrich) and anti-TNF-α (Abcam) diluted 1:250 in blocking solution. After several washing with TBST (0.1% Tween20 in TBS) nitrocellulose membranes were incubated with a secondary anti-rabbit IgG antibody horseradish peroxidase conjugated (Jackson Immuno Research Laboratories) diluted 1:7500 in blocking solution for 1 hour at room temperature. To reveal the immunocomplexes, the membranes were incubated with luminol LiteAblot® PLUS Enhanced Chemiluminescent Substrate (EuroClone S.p.A., Pero, Italy) and exposed to a with Kodak X-Omat AR film. Subsequently, nitrocellulose membranes were placed in stripping solution (62.5 mM TrisHCl pH 6.7, 2% (w/v) SDS, and 100 mM β-

mercaptoethanol) for 30 min at 50°C, washed in TBS, incubated with blocking solution for 30 min and then with a rabbit anti-human polyclonal antibody IgG recognizing the housekeeping protein D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) diluted 1:1000 (Proteintench, Chicago, USA). Immunolabeled bands were detected using an anti-rabbit secondary antibody peroxidase-conjugated (Jackson Immuno Research Laboratories) diluted 1:7500 in blocking solution for 1 hour at room temperature. The processed blots, before and after stripping, were scanned and, for quantification analysis, were subjected to densitometry analysis using ImageJ software package (http://rsbweb.nih.gov/ij/download.html). The recorded intensities of the GAPDH bands were used as an internal control to correct for differences in the samples loading on the gels and the bands were normalized with GAPDH using the ImageJ software package. The expression levels of *Hv*RNASET2, TLR2, TNF-α were reported relatively to control PBS injected animals.

Bacterial viability assay

Viable counts (expressed as colony-forming units per mL, CFU/mL) were estimated by employing the plate count technique: a volume (0.1 or 0.01 mL) of undiluted or serially diluted samples was plated on nutrient agar plates and incubated for 24 h at 37°C to evaluate the viable cells.

Statistical analysis

Western blot and immunofluorescent experiments were performed in triplicate and data represent the mean values ± SD. The percentages of $CD11b^+$ and *Hm*AIF-1^+ cells were assessed by analyzing 5 different slides (random fields of 45,000 μm^2 for each slide) for each experimental time point using the Image J software package. Cells in the chosen fields were counted by hand as granulocytes if they were $CD11b^+$ Cy5 labelled or as macrophages if they were *Hm*AIF-1^+ Cy5 labelled. Statistical analyses were performed using (GraphPad Prism 7, GraphPad Software, La Jolla, CA, USA), differences were calculated by one-way ANOVA followed by Fisher's post hoc test and $p < 0.05$ was considered statistically significant.

RESULTS

Starting from our previous results, showing that RNASET2 was detected in leech granulocytes and was able to actively recruit macrophages in an infected area (17), we investigated the RNASET2 antimicrobial action towards Gram-positive bacteria. To this aim, *in vivo* experiments were first performed to characterize the leech immune cells involved in the inflammatory response induced by LTA injection, a key component of the bacterial cell wall used to simulate a Gram-positive bacterial infection. Subsequently, the direct antimicrobial effect of the recombinant r*Hv*RNASET2 was evaluated by *in vitro* assays.

MORPHOLOGICAL CHARACTERIZATION OF IMMUNE CELL TYPES INVOLVED IN THE INFLAMMATORY RESPONSE INDUCED IN LEECHES BY LTA INJECTION

By means of light microscope analysis, the cross-sectioned body wall of PBS-injected leeches (Figure 1A) showed a typical cutaneous muscle sac, formed by well-defined epithelial and avascular muscular layers, where muscle fibers were arranged in distinct groups surrounded by a scant extracellular matrix (ECM). Among muscle fields, only a few resident immunocompetent cells were detectable.

By contrast, starting from 30 minutes following LTA injection, newly formed vessels and many infiltrating immune cells were clearly observable underneath the epithelium and in the ECM surrounding the muscle fibers (Figure 1B-H). In particular, several granulocytes were clearly recognizable both by light (Figure 1D) and TEM microscope analysis (Figure 1G) (17,30). At 6 hours post-injection, a high number of macrophages infiltrating the injected area (characterized by a ruffled surface due to the presence of pseudopodia, a typical feature of migrating cells) were readily detected as well (Figure 1E,F,H). LTA injection thus triggered a typical antibacterial response in leeches.

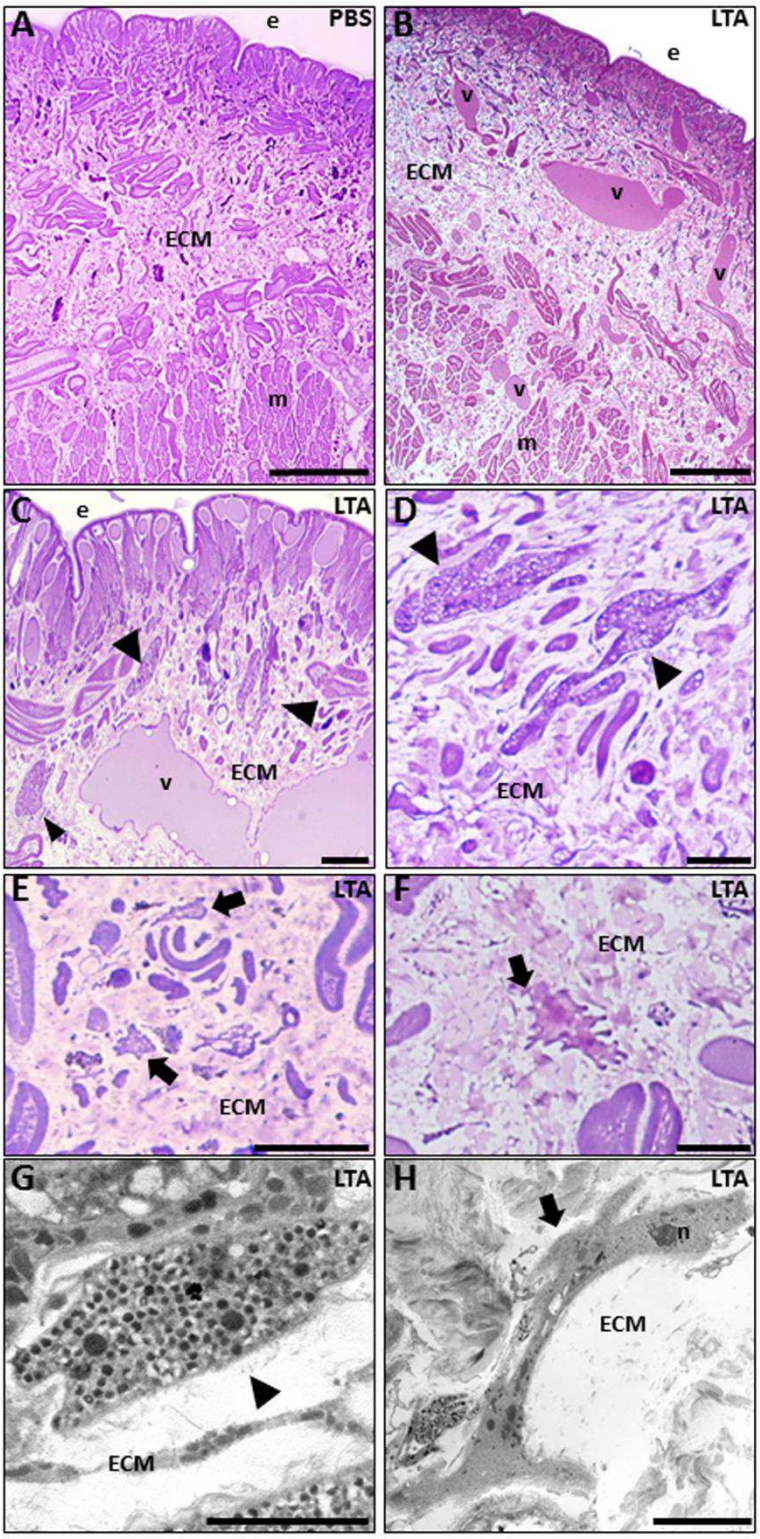

FIGURE 1. Images of morphological analyses of cross sectioned leech body wall at light **(A-F)** and TEM microscopes **(G-H)**. In PBS injected leeches **(A)**, tissue appears essentially avascular with few resident cells underneath the epithelium (e) and in the extracellular matrix (ECM) surrounding the muscle fibers (m). After LTA treatment **(B-D)** new vessels (v), numerous granulocytes **(**arrowheads in **C,D)** and macrophages **(**arrows in **E,F)** are clearly visible among muscles and underneath the epithelium. TEM details show granulocytes **(**arrowhead in **G)**, with dark granules inside the cytoplasm, and macrophages **(**arrow in **H)** characterized by the presence of pseudopodia. Bars in **(A- C)**: 100 µm; bars in **(D,F)**: 10 µm; bar in **(E):** 50 µm; bar in **(G)**: 2 µm; bar in **(H)**: 5 µm. n: nuclei.

CD11b AND TLR2 EXPRESSION

Since these morphological observations correlated well with our previous finding following Gram-negative bacterial infection in leeches, which showed the massive recruitment of $CD11b^+$ granulocytes expressing the TLR4 specific LPS receptor towards the challenged area (30), we focused on the immunophenotype of leech granulocytes activated in response to LTA injection. As expected, in control/PBS injected leeches (Figure 2A) a low CD11b signal was detectable, indicating that the mechanical stress induced by the injection or the vehicle solution alone did not trigger a significant inflammatory effect.

By contrast, starting from 30 minutes up to 3 hours following LTA injection, an increased number of $CD11b^+$ granulocytes were clearly visible underneath the epithelium and crossing the ECM surrounding the muscle fibers (Figure 2B-D). The number of granulocytes decreased after 6 hours (Figure 2E) and was drastically reduced 24 hours post-treatment (Figure 2F). Double immunofluorescence assays coupling anti-CD11b and anti-TLR2 antibodies showed that these two markers were co-expressed in the granulocytes (Figure 2G), suggesting that the TLR2 receptor might be involved in Gram-positive bacteria recognition during the early phase of immune response in leeches. To confirm that the recognition of LTA did not involve the TLR4 pathways, which is specific for Gram-negative bacteria recognition, further analyses were performed by injecting the cyanobacterium selective TLR4 antagonist CyP (29,30). In detail, leeches were injected with CyP (as a control) or with CyP added to LTA. Our data showed that the immune response caused by LTA treatment was not affected by Cyp (Figure 2H) and CD11b expression in LTA/Cyp stimulated leeches was comparable to that found in tissues of leeches injected with LTA only (Figure 2I). Therefore, we could assume that CyP is closely connected to LPS-TLR4 interaction in leech and does not interfere with LTA-TLR2 specific signal transduction pathway. In control experiments, where the primary antibody was omitted, no signals were detected (Figure 2J). Furthermore, as demonstrated by cell counting performed on 5 representative images of each time lapse, the number of $CD11b^+$ granulocytes changed during the different phases of inflammation and its increase was statistically significant in the earliest LTA and CyP/LTA induced inflammatory phase (Figure 2K).

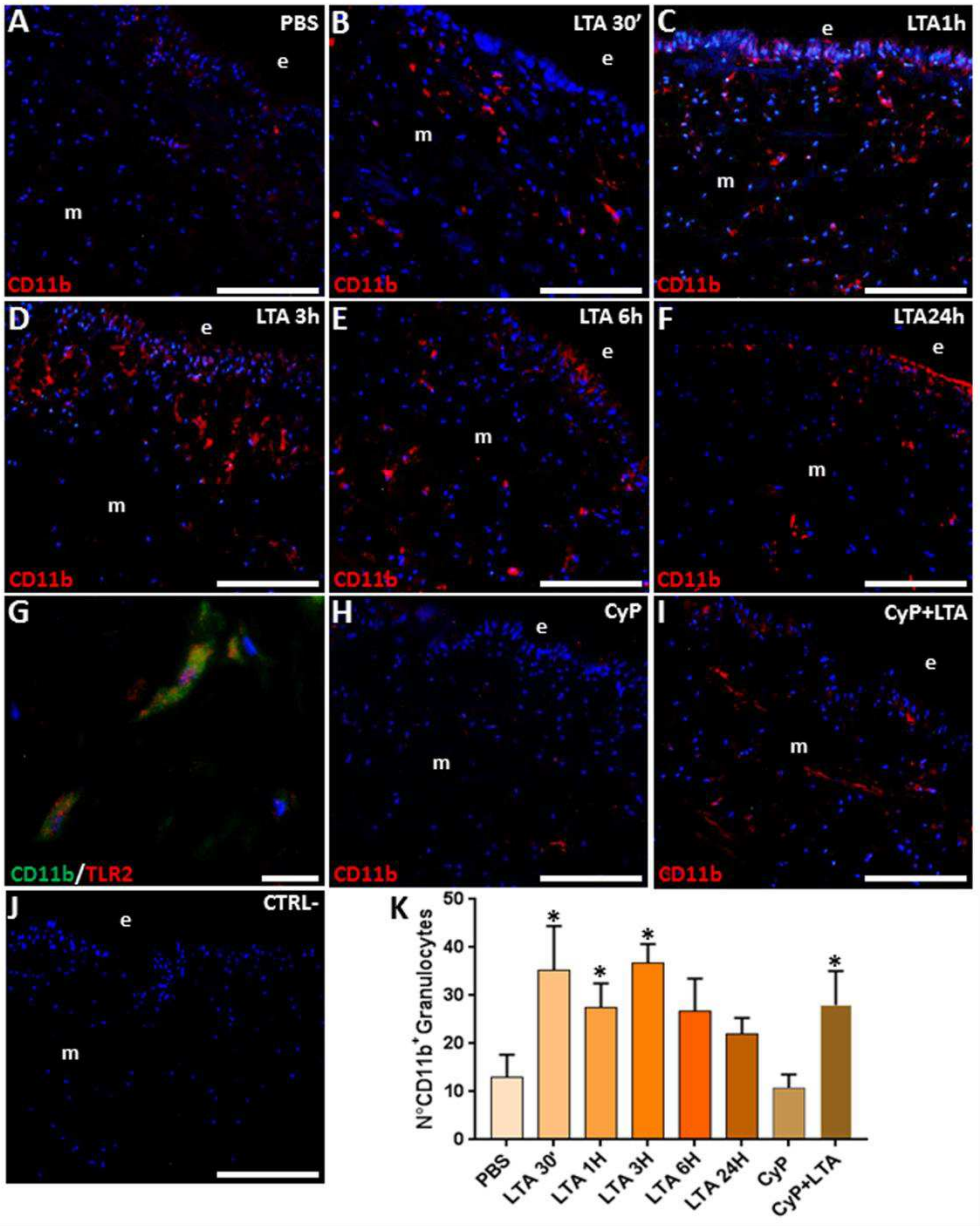

FIGURE 2. Immunofluorescence analyses on cryosections from leech body wall injected with PBS **(A)**, LTA **(B-G)**, CyP **(H)** and CyP+LTA **(I)**. In PBS **(A)** and CyP **(H)** treated leeches, few CD11b^{+} granulocytes are detectable under the epithelium (e) and in the ECM surrounding muscles (m).The signal considerably increases after 30 minutes **(B)**, reaches a peak after 1 and 3 hours **(C, D)** and decreases after 6 **(E)** and 24 hours from treatment **(F).** In CyP/LTA samples **(I)**, signal appears to be similar to LTA challenged leeches. In negative control experiments **(J)**, where the primary antibody is omitted, no positive cells are detected. Double immunofluorescence assays **(G)**, using anti CD11b (green) coupled to TLR2 antibody (red), reveal that granulocytes express TLR2. The cell count is obtained on CD11b fluorescence signal for each treatment **(K)**. Cell nuclei were stained in blue by treatment with DAPI. Bars in **(A-F, H-J)**: 100 µm; bar in **G:** 10 µm.

The expression of TLR2 and its downstream signalling pathway components was then evaluated by means of immunofluorescence and western blot analyses. In PBS-injected control leeches (Figure 3A), a low fluorescent signal for TLR2 was visible while, 30 minutes after LTA injection, an increased number of TLR2^{+} cells was detected, reaching a peak after 3 hours (Figure 3B-D) to decrease at 24 hours post-treatment (Figure 3E,F). Moreover, double

immunofluorescent assays showed a co-localization of MyD88 and TLR2 in the same cells (Figure 3G) confirming a MyD88-dependent activation pathway via TLR2 (45) in leech. Further studies using the CyP agonist confirmed that LTA recognition involved only TLR2 and its signalling pathway, while TLR4 was excluded. Indeed, a TLR2 signal comparable to that of control and of 3hours LTA-injected leeches was detected in both CyP- and Cyp/LTA-treated leeches, respectively (Figure 3H,I). No signal was detected in the negative control experiments in which tissue sections were incubated only with the secondary antibody (Figure 3J).

The increased expression level of TLR2 in LTA injected leeches was also confirmed by immunoblot assays, showing the presence of a 109 kDa band, corresponding to the expected molecular weight of vertebrate TLR2 (Supplementary Figure 1). As shown in the figure, TLR2 expression was highly increased in LTA-treated samples when compared to PBS-treated controls (Figure 3K).

Since one of the key targets of TLR2 signalling pathway is TNF-α (46), we also evaluated its expression level in control and LTA-stimulated leeches. Immunofluorescent assays detected a basal TNF-α expression in PBS-treated leeches (Figure 4A), whereas, as expected, its level markedly increased 30 minutes following LTA injection (Figure 4B), reached a peak at 1 and 3 hours (Figure 4C,D) and then decreased at 6h post injection (Figure 4E,F). As expected, TNF-α expression in CyP and CyP/LTA injected leeches was comparable to that observed for TLR2 (Figure 4G,H) confirming once again that the immune response caused by LTA treatment is not affected by blocking the TLR4 pathway. No signal was detected in the negative control experiments (Figure 4I). Western blot analysis of PBS- and LTA-injected leech tissues revealed the presence of an immunoreactive 36 kDa band (Supplementary Figure 1), corresponding to the expected molecular mass of TNF-α in leech (30). A quantitative analysis confirmed the expression profile of this pro-inflammatory cytokine as already observed in immunofluorescence experiments (Figure 4J).

Taken together, these data showed that LTA injection in leeches triggered the expected innate immune response molecular signalling cascade, thus properly mimicking the effects of a real bacterial infection.

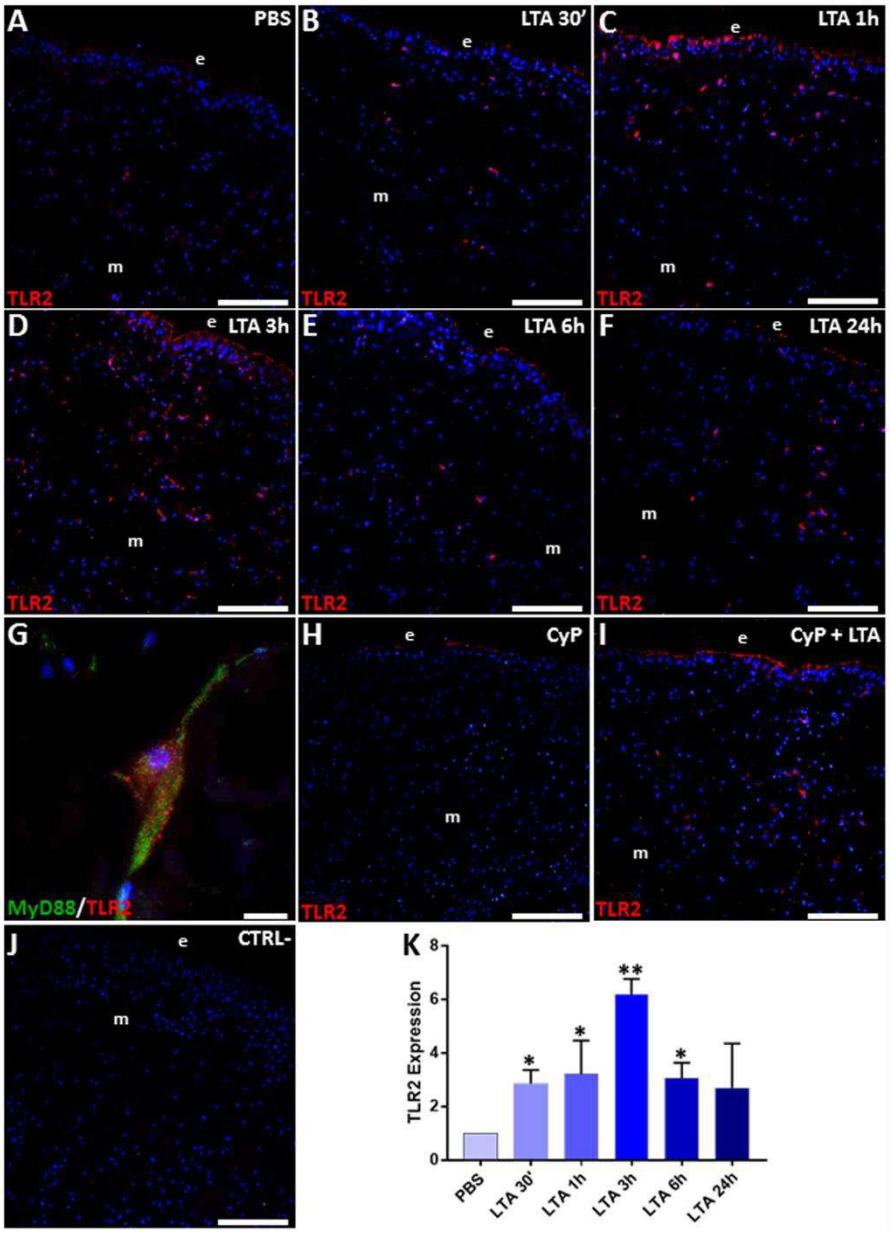

FIGURE 3. Immunofluorescence assays on cryosections from PBS **(A)**, LTA **(B-G)**, CyP **(H)** and CyP+LTA **(I)** injected leeches. In PBS **(A)** injected leech, few TLR2^{+} cells were visible, whereas an increasing number of migrating immune-responsive cells located under the epithelium (e) and among muscles (m) are detected starting from 30 min until 3 hours following LTA injection **(B-D).** The signal turns out to decrease at 6 and 24 hours post treatment **(E,F)**. In CyP (**H**) and Cyp/LTA samples **(I)**, the number of TLR2^{+} cells appears to be similar to that of PBS and of LTA challenged leeches respectively. No positive cells are detected in negative control experiments **(J).** Detail of double immunofluorescence stained with anti-TLR2 (in red) and MyD88 (in green) **(G)**. Cell nuclei are stained in blue with DAPI. The graph, relative to the western blot analysis (**see supplementary Figure1**) showing the TLR2 expression profile **(K)**. Bars in **(A-F, H-J)**: 100µm; bar in **(G)**: 10µm.

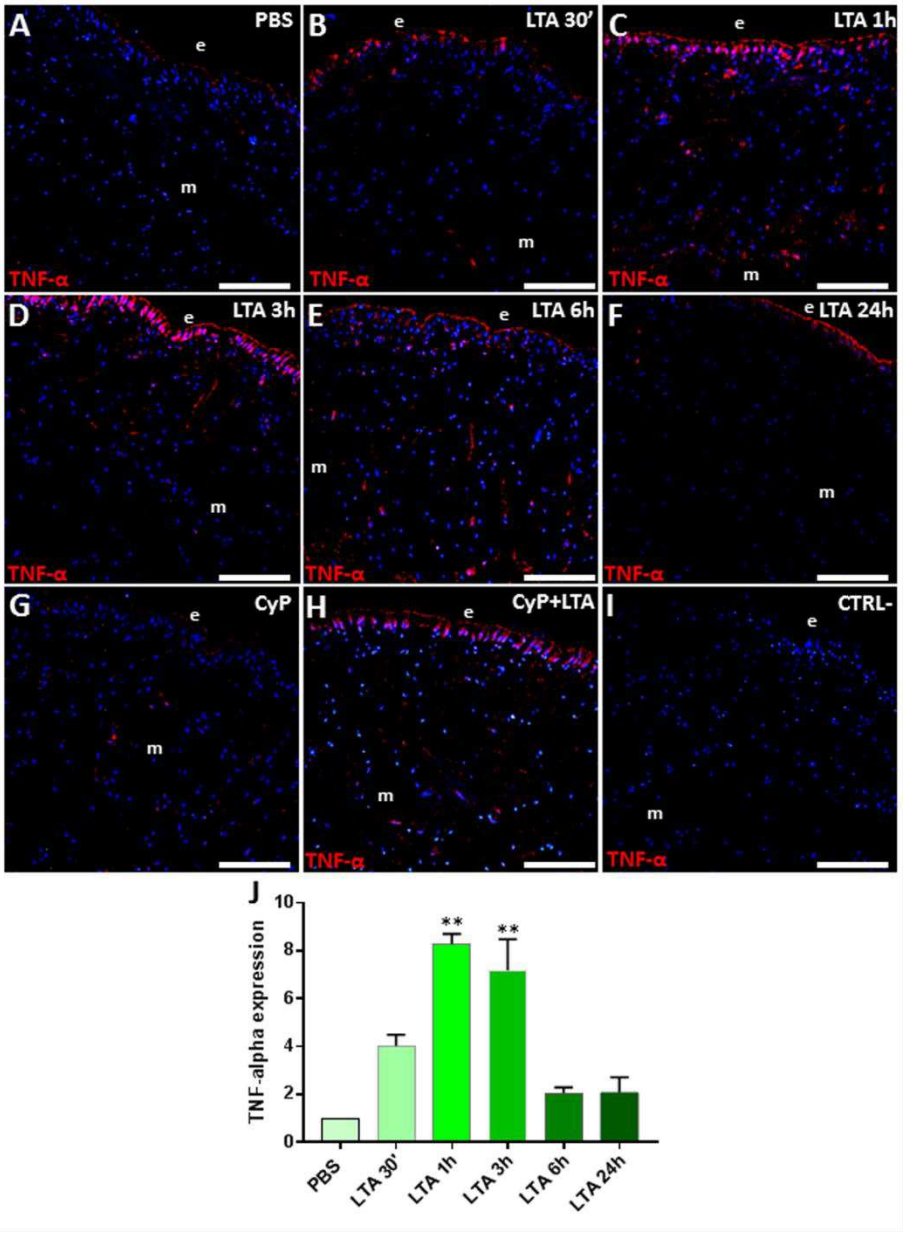

FIGURE 4. Immunofluorescence analyses on cryosection from PBS **(A)**, LTA **(B-F)**, CyP **(G)** and CyP+LTA **(H)** injected leeches. In PBS **(A)** injected leech, few TNF-α cells were visible, whereas an increasing number of immune-responsive cells located underneath the epithelium (e) and in the ECM surrounding muscles (m) are detected starting from 30 min to 3 hours following LTA treatment **(B- D),** then decreases at 6 and 24 hours **(E-F)**. In CyP (**G**) and Cyp/LTA samples **(H)**, the number of TNF-α^{+} cells is similar to that of PBS and of LTA challenged leeches respectively. In negative control experiments **(I)**, no positive cells are detected. The graph, relative to the Western blot analysis (**see supplementary Figure1**), shows the TNF-α expression profile **(J)**. Bars in **(A-I)**: 100 µm.

RNASET2 EXPRESSION IN IMMUNE COMPETENT CELLS

We next carried out both double immunofluorescence assays with anti-RNASET2 and anti-CD11b antibodies and immunogold experiments at TEM, in order to show the presence of *Hv*RNASET2 in the granules of leech granulocytes (Figure 5A,B''), as previously reported in LPS-stimulated leeches (17). In addition, *Hv*RNASET2 immunolocalization on cryosections from

control and LTA-injected leech body wall showed that in control, PBS-injected animals, *Hv*RNASET2 was expressed at a basal level (Figure 5C), whereas 30 min after LTA treatment its expression gradually increased, reaching a peak after 6 and 24 hours from stimulation (Figure 5D-H). No signal was visible when sections were incubated with the secondary antibody only (Figure 5I).

The *Hv*RNASET2 temporal expression profile in LTA-injected leeches was also evaluated by western blot analyses. A 37 kDa immunoreactive band, corresponding to the molecular weight of the extracellular RNASET2 isoform was detected in all samples (Supplementary Figure 1). As expected, unlike control samples, the expression level of *Hv*RNASET2 gradually increased in LTA- treated samples, reaching a peak at 6 h post treatment (Figure 5J).

Taken together, our results not only confirmed that *Hv*RNASET2 was produced by leech granulocytes, but also suggested that LTA-induced increase of this protein might be functionally involved in its potential antimicrobial activity against Gram-positive bacteria.

Immune and enzyme histochemical characterization of LTA recruited macrophages

The massive migration of leech granulocytes into the injected area during the earliest phase of the innate immune response was followed by a later macrophage recruitment towards the same area. Indeed, immunofluorescence analysis using the specific *Hm*AIF-1 macrophage marker unveiled the presence of many *Hm*AIF-1^{+} macrophages in the LTA-challenged area, while in control PBS-injected leeches only few resident cells were detectable next to the epithelium and in the ECM surrounding the muscle fibers (Figure 6A-F).

Strikingly, the signal co-localization observed by double immunofluorescences assays with anti-*Hm*AIF-1 and anti-TLR2 or anti-RNASET2 antibodies, respectively, suggested a direct involvement of these macrophages in LTA-response through the TLR2 pathway and confirmed that these immunocompetent cells expressed *Hv*RNASET2 as well during LTA-induced innate immune response, as previously observed after LPS challenging (16,17) (Figure 6G-I). Indeed, *Hm*AIF-1^{+} cell count performed on 5 representative images of each time lapse confirmed a significant migration of macrophages, especially at 6 and 24 hours post-treatment (Figure 6J) Moreover, an enzymatic histochemical ACP assay (Figure 6K-P) revealed that these macrophages were actively involved in a phagocytic activity (16,47–49), since PBS-injected leeches displayed a negligible number of ACP^{+} cells (Figure 6K), whereas their number significantly increased in relation to the time elapsed after LTA-treatment, reaching a peak at

6 and 24 hours from LTA injection (Figure 6M-N). The fact that CyP treatment did not induce any migration of phagocytic cells (Figure 6O) and at the same time did not reduce the LTA effects (Figure 6P) prompted us to postulate that the phagocytic activity of macrophages against Gram-positive bacteria was mediated by TLR2.

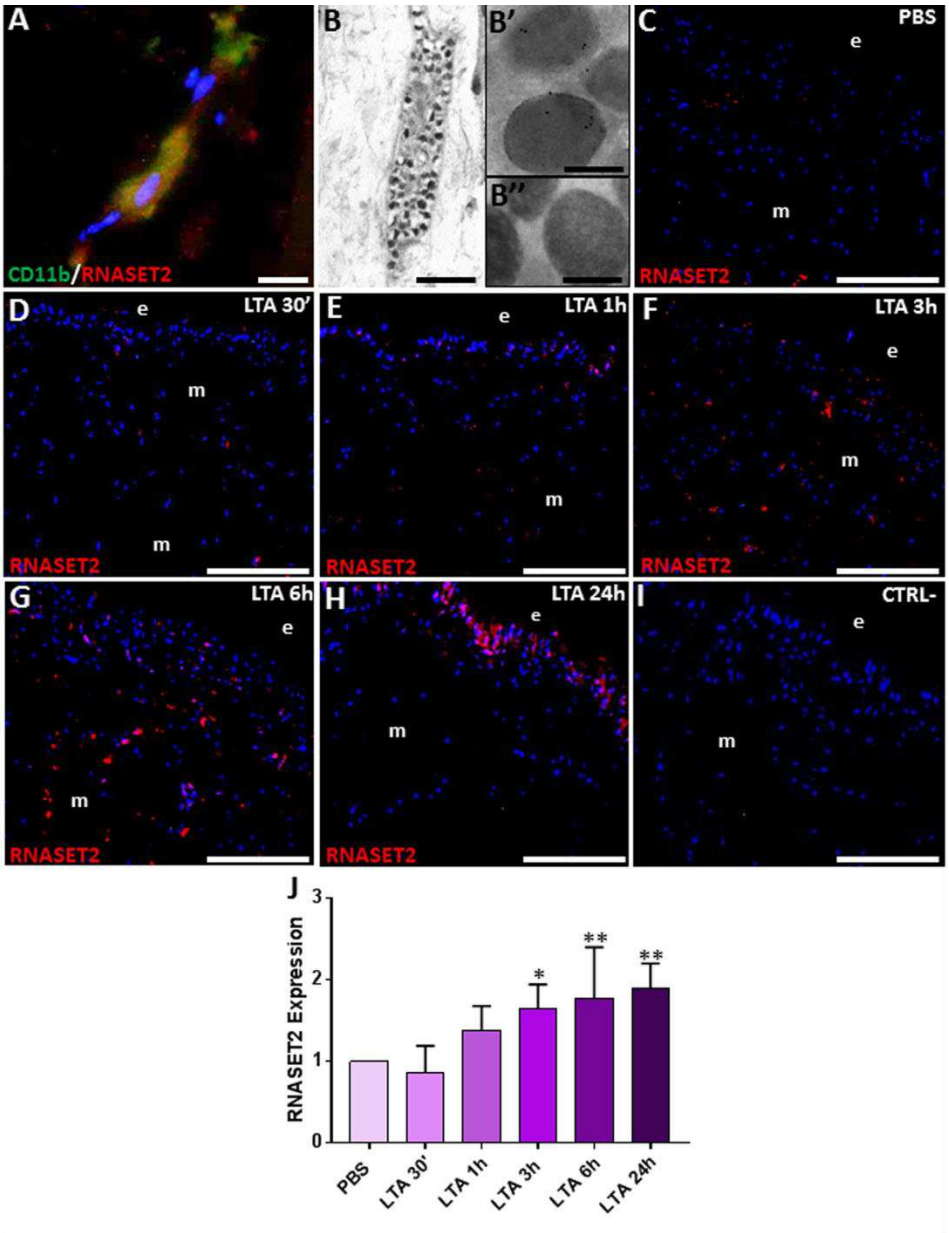

FIGURE 5. Double immunolocalization of RNASET2 (in red) and CD11b (in green) **(A)**. Immunogold staining shows that *Hv*RNASET2 is localized in the electron-dense granules of granulocytes **(B')**. No gold particles are detected in the negative control experiments, in which primary antibody is omitted **(B'')**. Immunofluorescence analyses on cryosection from PBS **(C)** and LTA **(D-H)** injected reveals an increasing RNASET2 signal starting form 30 minutes post LTA treatment. Negative control **(I).** The graph, relative to the Western blot analysis **(seesupplementary Figure1)**, shows the *Hv*RNASET2 expression profile **(J)**. Bar in **(A)**: 10 µm; bar in **(B)**: 2 µm; bars in **(B', B'')**: 200 nm; bars in **(C-I)**: 100 µm.

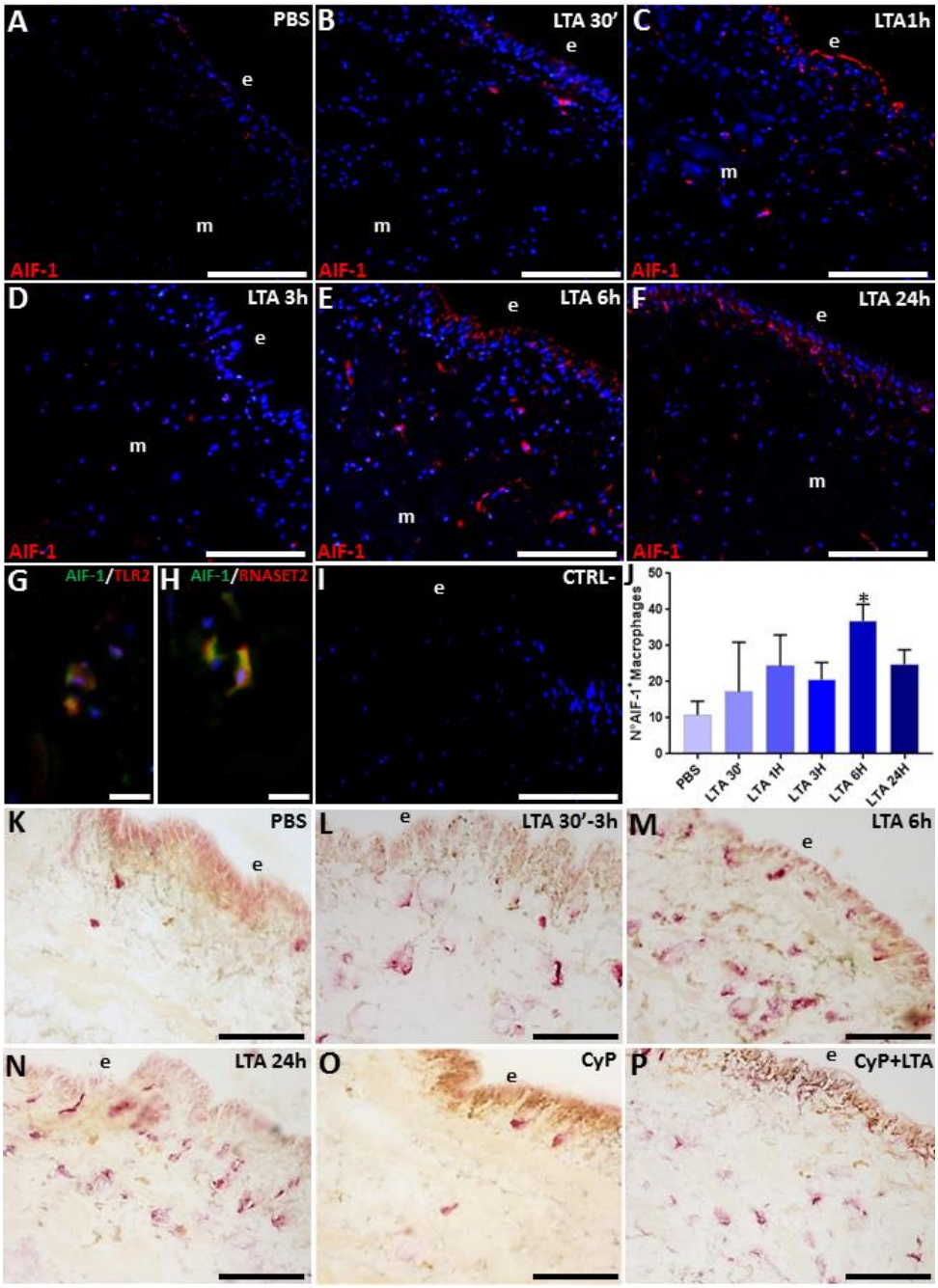

FIGURE 6. Immunofluorescence images of cryosection from PBS **(A)** and LTA **(B-F)** injected leeches. In PBS **(A)** injected leech, few *Hm*AIF-1^{+} macrophages are visible, while starting from 30 minutes up to 24 h from LTA injection an increasing number of positive cells were detectable underneath the epithelium (e) and among the muscle fibers **(B-F).** Double immunofluorescent assays **(G, H)**, using anti-*Hm*AIF-1 (in green) coupled to anti-TLR2 (in red) or anti-RNASET2 (in red) antibodies, reveal that macrophages (in yellow) are *Hm*AIF-1^{+}/ TLR2^{+} and *Hm*AIF-1^{+}/RNASET2^{+}. Negative control experiment **(I).** The cell count is obtained on *Hm*AIF-1 fluorescence signal for each treatment **(J)**. The acid phosphate (ACP) reaction **(K-P)** shows an increasing cytosolic lysosomal activity in phagocytic macrophages after LTA challenging **(L-N).** In CyP **(O)** and Cyp/LTA samples **(P)**, the number of ACP^{+}cells is similar to that of PBS and of LTA challenged leeches respectively. Bars in **(A-F, I, K-P)**: 100 µm; bars in **(G, H)**: 10 µm.

IN VITRO AND *IN VIVO* ASSAYS TO EVALUATE THE r*Hv*RNASET2 EFFECT

To further shed light on the putative *Hv*RNASET2 antimicrobial mechanisms, we performed morphological analyses by means of both light, TEM and SEM microscopy on bacterial cell cultures upon incubation with r*Hv*RNASET2 at a final concentration of 10 μM (Figure 7A-I).

Unlike control, PBS-treated samples (Figure 7A,B,G), adding *Hv*RNASET2 clearly induced the formation of bacterial clumps (Figure 7D,E,H,I). Strikingly, immunogold TEM analysis, using an anti-RNASET2 antibody, showed several gold particles localized on the bacterial cell surface, indicating a direct interaction between *Hv*RNASET2 and one or more bacterial cell wall components (Figure 7C,F). Moreover, several blebs could be observed by SEM analysis following *Hv*RNASET2-treatment, suggesting a local destabilization taking place at the bacterial cell surface (Figure 7I).

Of note, r*Hv*RNASET2 protein also triggered bacterial aggregation in *in vivo* experiments (Figure 7L-Q). Indeed, both light and fluorescence images showed that, in leeches injected with either *S. aureus* alone or a mixture of the same bacterial cells and r*Hv*RNASET2 protein pre-incubated with a neutralizing anti-RNASET2 antibody, bacterial cells appeared randomly distributed throughout the leech ECM. By contrast, the extracellular matrix of leech co-injected with *S. aureus* and r*Hv*RNASET2 was consistently characterized by clusters of aggregated bacteria surrounded by macrophages (Figure 7M,P). This finding is clearly consistent with a role for r*Hv*RNASET2 in stimulating a macrophage-mediated antimicrobial response, likely carried out by bacterial aggregation followed by ingestion by r*Hv*RNASET2-recruited macrophages.

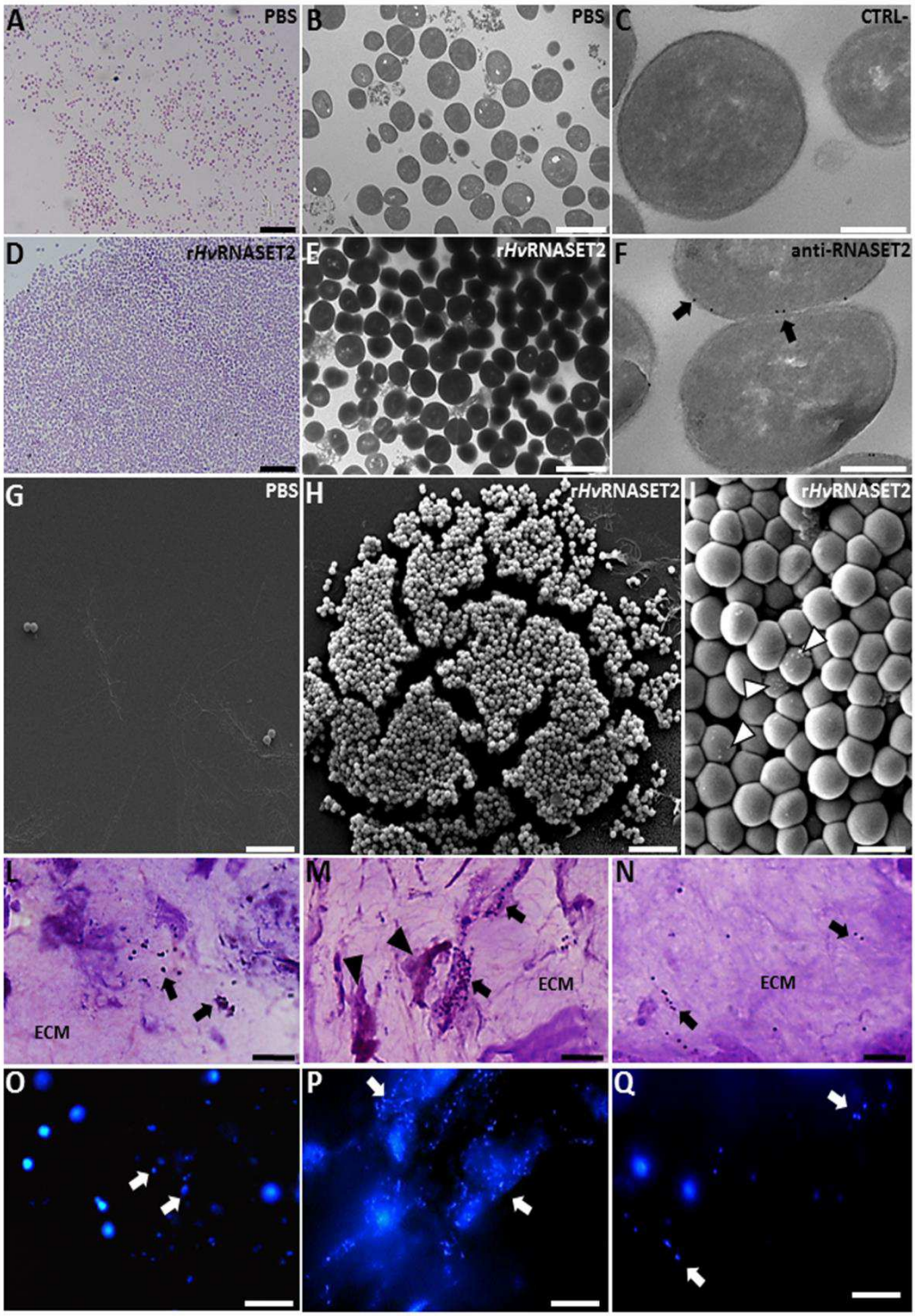

FIGURE 7. Evaluation of r*Hv*RNASET2 antibacterial activity by i*n vitro* **(A-I)** and *in vivo* **(L-Q)** assays. Images at light **(A, D)**, TEM **(B, E)** and SEM (**G-I**) microscopy of *S. aureus* incubated for 3 h *in vitro* with PBS or with r*Hv*RNASET2 show that only r*Hv*RNASET2 treatment induces a bacterial agglutination and the formation of several blebs on the bacterial cell surface **(**arrowheads in **I)**. Immunogold assay **(F, C)** demonstrates a direct interaction between r*Hv*RNASET2 and the bacterial cell wall. *In vivo* experiments, performed by injecting in the leech body wall *S. aureus* alone **(L, O)** or *S. aureus* together with r*Hv*RNASET2 **(M, P)** or with r*Hv*RNASET2 pre-incubated with a specific blocking antibody **(N, Q)**. The agglutination effect of r*Hv*RNASET2 on *S. aureus* is highlighted with violet and fuchsin colorant **(L-N)** and with fluorescent DAPI staining **(O-Q)**. Bars in **(A, D, G, H, L- Q)**:10 µm; bars in **(B, E)**: 2 µm; bars in **(C, F)**: 250 nm; bar in **(I)**: 1 µm.

DISCUSSION

Although in the last decades several studies have demonstrated that members of the human RNase A superfamily play a crucial role in the defense against bacterial infection (50), very little is known about the potential antibacterial activity of T2 RNase family members and their possible involvement in microbial recognition.

We have previously demonstrated that the leech ribonuclease RNASET2 not only acts as chemoattractant for granulocytes and macrophages, being thus involved in modulating inflammatory processes, but it also plays an effective response against Gram-negative bacteria infection (16,17). Indeed, our previous data clearly demonstrated that LPS bacterial injection in the leech body wall induced an increased expression of endogenous *Hv*RNASET2 in both granulocytes and macrophages. Interestingly, *Hv*RNASET2 is released by granulocytes in the early phase of the inflammatory response and likely plays an antibacterial role against Gram-negative bacteria, by recruiting macrophages in the challenged area (16). Both macrophages and granulocytes are able to recognize Gram-negative bacteria by expressing on their membranes TLR4, the specific receptor for LPS (30). Recombinant RNASET2 did not apparently influence cellular viability *in vitro*, but it nevertheless affected *Pseudomonas aeruginosa* cell wall, triggering a change in the typical rod morphology of these cells (17).

Here, we have analyzed the antibacterial activity of *Hv*RNASET2 against Gram-positive bacteria, by investigating the role of this ribonuclease in regulating and orchestrating the innate immune response induced by stimulation with LTA in the medicinal leech *H. verbana*. This invertebrate has been increasingly used as an experimental model to study innate immune response processes, due to its cost-effective use, easy manipulation and lack of significant ethical considerations related to regulatory restrictions, coupled to the occurrence of immune response processes that are very similar to those reported in vertebrates (15,51–53).

By means of morphological, ultrastructural and immunofluorescence analyses, we have first shown that LTA injection in the leech body wall induces a marked recruitment of $CD11b^+$ granulocytes, which are the first immune cells to be activated following leech bacterial infection (17,30). These immune cells were shown to express TLR2, the key receptor involved in the response to Gram-positive bacteria. Moreover, as in vertebrates, TLR2 triggering induced intracellular signalling events involving MyD88, a key molecular intermediate for the

activation of TLR2 signalling pathways (54) that ultimately leads to the production of the proinflammatory cytokines, including TNF-α (29,30,55). Functional studies carried out by injecting the cyanobacterium selective TLR4-antagonist CyP strongly suggested that, as in vertebrates, the recognition of LTA (a component of Gram-positive cell wall) in leeches did not involve the TLR4 pathway but was specific for the TLR2 pathway (54).

Interestingly, after 3 hours from stimulation with LTA, $CD11B^{+}/TLR2^{+}$ granulocytes also expressed a high amount of *Hv*RNASET2, as demonstrated by double immunofluorescence and western blot assays. This ribonuclease, as previously demonstrated following Gram-negative bacterial challenge, promoted macrophage migration and activation, since cells expressing the specific leech macrophage *Hm*AIF-1 (47,56) were clearly recruited in the challenged area and showed a high phagocytic activity, as demonstrated by their positivity for the hystoenzymatic ACP reaction. We propose that such cells have a dual role following bacterial infection in leeches, being involved both in cleaning up the infected area from bacteria and producing, 24 hour following LTA stimulation, a second wave of *Hv*RNASET2 for further macrophage recruitment. Of note, the phagocytic activity of macrophages was apparently facilitated by the aggregating properties of *Hv*RNASET2 on bacterial cells. Indeed, *in vitro* experiments clearly show that, following *Hv*RNASET2 treatment, *S. aureus* formed cell clusters. This aggregation was likely mediated by a specific interaction between *Hv*RNASET2 and the bacterial cell wall, as demonstrated by immunogold experiments at TEM.

Of note, the aggregating ability of *Hv*RNASET2 was also demonstrated by *in vivo* experiments, since clusters of *S. aureus* cells surrounded by macrophages were detected in the injected leech body wall in the presence of *Hv*RNASET2.

Taken together, our results highlight that the antimicrobial properties of leech recombinant *Hv*RNASET2 protein are comparable to other class A RNases, such as RNase 3 and 7, and correlate to bacterial clumps forming activities (57). Indeed, similarly to RNase 7, *Hv*RNASET2 triggered local blebs in the bacterial wall (58) and promoted a marked inflammatory response, mediated by TLR2 activation and followed by the release of inflammatory stimuli, such as TNF-α (26). Moreover, as reported for RNase 3, the ability of *Hv*RNASET2 to bind bacterial cell wall seems of special relevance for its role in bacterial clumps formation (58).

To our knowledge, this study represents the first report describing a marked antibacterial activity for a T2 RNase member in an invertebrate experimental model and

plausible mechanism of action. Of note, the similarity of the biological role between *Hv*RNASET2 and some of class A RNases open the door to the production of new peptide-derived antimicrobials drugs, which could help to counteract the ever-growing problem of the dramatic worldwide emergence of antibiotic-resistant strains.

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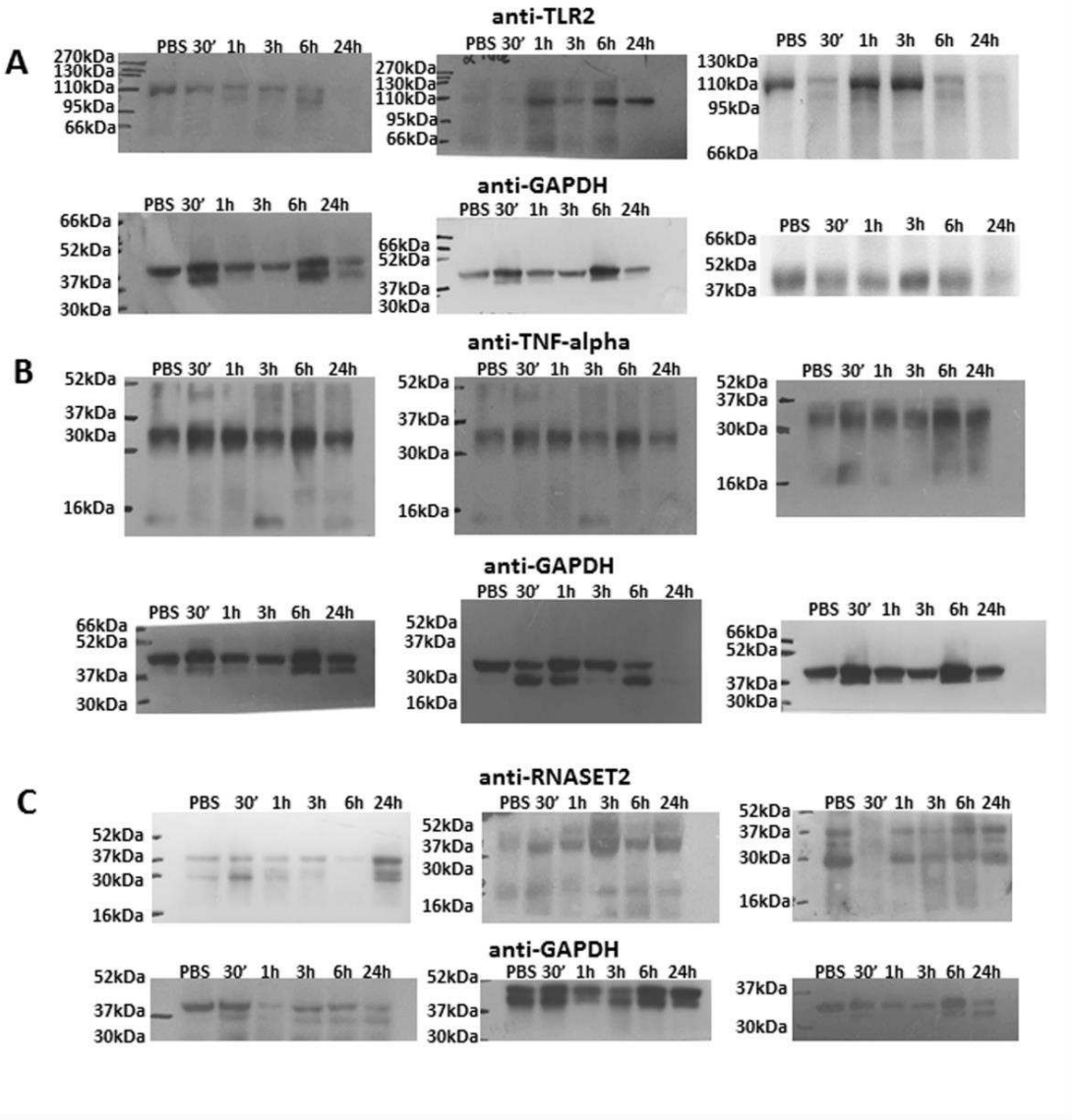
Supplementary material



SUPPLEMENTARY Figure 1 Western blot analysis performed on PBS or LTA injected leeches. Proteins extracted from leech body wall are probed with anti-TLR2 **(A)**, anti- TNF-α **(B)** and anti- RNASET2 **(C)** antibodies respectively. Each experiment is quantified on the expression level of D- glyceraldyde-3-phosphate dehydrogenase (GAPDH), used as a control. Immunoreactive bands of about 109, 34 and 37 kDa are respectively detected for anti-TLR2, anti-TNF-alpha and anti-RNASET2.

CHAPTER 4

Recombinant *Hv*RNASET2 protein induces a marked connective tissue remodelling in the invertebrate model *Hirudo verbana*

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SUBMITTED TO CELL AND TISSUE RESEARCH

Abstract

The RNASET2 ribonuclease, belonging to the highly conserved RH/T2/s RNase gene family, has been recently shown to modulate inflammatory processes in both vertebrates and invertebrates. Indeed, the RNASET2 protein acts as a chemoattractor for macrophages in both *in vitro* and *in vivo* experimental settings and its expression significantly increases following bacterial infections. Moreover, we have recently observed that injection of human recombinant RNASET2 protein in the body wall of the medicinal leech (a consolidated invertebrate model for both immune response and tissue regeneration investigations) not only induced immune cell recruitment but apparently triggered a massive connective tissue remodelling as well. Based on these data, we evaluated here a possible role of leech recombinant RNASET2 protein (r*Hv*RNASET2) in connective tissue remodelling, by characterizing the cell types involved in this process through histochemical, morphological and immunofluorescent assays. Moreover, a time-course expression analysis of newly synthesized pro-collagen1α1 (COL1α1) and basic FGF receptor (bFGFR, a known fibroblast marker) following r*Hv*RNASET2 injection in the leech body wall further supported the occurrence of r*Hv*RNASET2-mediated matrix remodelling. Human MRC-5 fibroblast cells were also investigated in order to evaluate their pattern of collagen neosynthesis driven by r*Hv*RNASET2 injection. Taken together, the data reported in this work provide compelling evidence in support of a pleiotropic role for RNASET2 in orchestrating an evolutionarily conserved cross-talk between inflammatory response and regenerative process, based on macrophage recruitment and fibroblast activation, coupled to a massive extracellular reorganization.

Keywords: RNASET2, fibroblasts, COL1α1, connective tissue remodelling, medicinal leech

INTRODUCTION

Tissue regeneration in metazoa requires the accurate coordination of multiple processes, which include recruitment, proliferation and activation of progenitor cells, immune modulation, angiogenesis, extracellular matrix (ECM) remodelling and innervation of the newly forming tissue. Recently, several studies have highlighted the importance of the immune system in restoring damaged organs and tissues. In this context, injury-induced wound healing typically entails an acute inflammatory response, which acts not only to counteract possible infections, but also to restore tissue homeostasis and functional integrity (Mescher et al. 2015; Eming et al. 2009). Indeed, the release of immunomodulatory molecules such as cytokines and growth factors by injury-activated immune cells is now known not only to support the recruitment of different inflammatory cells to the damaged site (Frantz et al. 2005), but also to actively participate in tissue remodelling, by establishing a crosstalk between immune cells and several stromal cellular component, such as fibroblasts (Glaros et al. 2009). Given the fundamental importance of innate immunity in both defence against pathogens and tissue regeneration, further investigations are necessary to shed further light on the cellular and molecular mechanism(s) by which immune-mediated debris clearance and tissue regenerative process are coordinated in a way that leads the immune system to act as both a “friend and foe” to the damaged tissue.

In this context, the pleiotropic and evolutionary conserved *RNASET2* gene, which has been involved in both innate immune response and tissue remodelling (Baranzini et al. 2017, 2018), represents an interesting candidate whose role in the abovementioned biological processes is worth investigating. RNASET2 belongs to the Rh/T2/S family of extracellular ribonucleases~~,~~ which, unlike other RNase families, is widely distributed throughout most *taxa,* from viruses to humans. Of note, besides the canonical RNA processing or degradation roles (whose function has only partially been described), T2 RNases are known to be involved in an impressively wide range of biological processes, some of which do not require the enzyme’s ribonuclease activity (Luhtala and Parker 2010). For instance, human RNASET2 has been involved in the control of tumorigenesis *in vivo* in several human cancer models independently of its catalytic activity (Acquati et al. 2011), affecting cancer growth by establishing of a complex cross-talk between cancer cells and the tumor microenvironment, which entails both the recruitment and activation of innate immune cells endowed with anti-tumor activity and

an extensive ECM remodelling (Acquati et al. 2011). Moreover, recent experimental data from *in vitro* ovarian cancer model systems reported that RNASET2-mediated cancer growth suppression involves the ECM/integrin signalling pathway (Roggiani et al. 2019).

Of note, further *in vivo* studies have shown the ability of human recombinant RNASET2 protein to induce fibroplasia, connective tissue remodeling and ECM rearrangements following injection in the medicinal leech *Hirudo verbana*, coupled to a chemotactic role for cells of the monocyte-macrophage lineage (Baranzini et al. 2017).

To better define the role of RNASET2 in ECM remodelling in a more physiological experimental setting, in this work we analysed the effects of *H. verbana* recombinant RNASET2 (r*Hv*RNASET2) on leech tissue remodelling and ECM reorganisation.

The medicinal leech represents a powerful experimental model system to gain novel insight on these topics (Grimaldi et al. 2018). Indeed, the response to several experimental manipulations (such as wounds or bacterial infections) can be easily detected in leeches, due to their small size and anatomical structures (de Eguileor et al. 2004, 2003; de Eguileor et al. 1999; Grimaldi et al. 2006, 2004), allowing unambiguous assessment of the different cell types involved in wound healing and tissue repair processes, which typically extend over the whole thickness of body wall (Tettamanti et al. 2004). Following experimental wound or bacterial infection, the reorganization of the leech's connective tissue is known to be regulated by several diffusible factors such as EGF (epithelial growth factor), bFGF (basic Fibroblast Growth Factor) and Cathepsin B, produced by fibroblasts and immune cells (Grimaldi et al. 2004; Tettamanti et al. 2005). By activating fibroblast proliferation, these molecules promote *ex novo* collagen synthesis. Moreover, both innate immune response and regenerative process in leeches have been shown to be strikingly similar to those reported in vertebrates, since they involve similar molecular and cellular effectors and a common equipment of key molecules playing pivotal roles for regulating immune competent cells and fibroblast activation.

We report here that injection of recombinant r*Hv*RNASET2 in the leech body wall triggers a rapid reorganization of the connective tissue and a subsequent activation of bFGFR$^+$ fibroblasts engaged in new fibril production, such as those normally recruited after a wound to assemble a new collagen scaffold (Tettamanti et al. 2003).

Moreover, we investigated the effect of leech RNASET2 on the human MRC-5 fibroblast cell line to evaluate a possible evolutionary conservation between the leech and human proteins in terms of their role in ECM remodelling. By establishing a coordinated *in vivo* cross-

talk between inflammatory response and connective tissue remodelling, two key biological processes whose functional interconnection is still poorly defined, RNASET2 can be ascribed as a putative key novel player in this field which deserves further investigations.

Materials and methods

Hirudo verbana recombinant r*Hv*RNASET2: cloning and expression

An *in silico* leech transcriptome database (http://genomes.sdsc.edu/leechmaster/database/) was used to retrieve the mRNA sequence of *H. verbana* RNASET2 (*Hv*RNASET2) (Macagno et al. 2010b)**.** We then selected a sequence representing a putative full-length coding sequence (>EN-124k-90-group2043). To clone the corresponding cDNA, total mRNA extraction from the leech body wall was performed and the sample was treated with DNase (Turbo DNA-*free*^{TM} Kit-Invitrogen) to remove all DNA traces and reverse transcribed with an oligo dT primer (High-Capacity cDNA Reverse Transcription Kit - Applied Biosystems™). By using different primer combinations, we obtained partial amplicons representing the coding sequence.

We therefore carried out an overlap extension PCR to obtain the full-length coding sequence of *Hv*RNASET2.

The PCR products were used as a template in a third PCR performed using the external primers bearing artificially-introduced EcoRI sites. The resulting PCR product was digested with EcoRI and cloned in pBluescript. Finally, the leech *Hv*RNASET2 coding sequence (BankIt2095553 Hirudo MH325331) was PCR-amplified to remove the predicted signal for protein secretion from the coding sequence. The PCR product was cloned into the pPICZαA expression vector for heterologous expression in the yeast *Pichia pastoris,* as previously described (Campomenosi et al. 2011).

All primer sequences are available upon request.

*Hv*RNASET2 recombinant protein and purification have been described elsewhere (Baranzini et al. 2019, manuscript submitted).

Endotoxin removal

Endotoxins removal from *rHv*RNASET2 preparations was carried out according to the procedure reported in (Liu et al. 1997).

Animals and treatment

Adult leeches (*H. verbana*, Annelida, Hirudinea, from Ricarimpex, Eysines, France) measuring 10 cm were kept in lightly salted water (NaCl 1,5 g/l) at 20°C in aerated tanks and fed weekly with calf liver. Animals were randomly split into separated experimental groups (five individuals for each time point). Each treatment was performed at the level of the 20th metamere on leeches anaesthetized with a 10 % ethanol solution. Treated and untreated (control) animals were anesthetized and then dissected to remove body wall tissues at specific time points. Samples were processed for optical and electron microscopy, immunofluorescence and western blot protocols as previously reported (Baranzini et al. 2017, 2018).

Group 1: unstimulated control or PBS-injected leeches (100 µL), to verify that the mechanical stress induced by the injection or the vehicle solution alone did not exert a significant effect on COL1α1 and bFGFR expression in the body wall of challenged animals.

Group 2: leeches injected with 100 µl sterilized PBS containing 100 ng of recombinant protein r*Hv*RNASET2.

Optical and electron microscopy

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

Masson trichrome staining

Tissues were fixed in 4 % paraformaldehyde for one hour and then washed for three times in PBS. After washings, samples were dehydrated in an ethanol series and paraffin embedded.

Sections obtained with paraffin microtome (5µm thick) have been processed for Trichrome Masson staining (Trichromica kit, Bio Optica, Milano, Italy), as suggested by the data sheet. This colouring technique allows us to observe in blue the collagen and the reticular fibers and in red cells cytoplasm. Images have been obtained using the Nikon Digital Sight DS-SM optical Microscope (Nikon, Tokyo, Japan).

Sirius Red staining

The paraffin sections obtained as described above have been processed for 10 min in 0,1% Sirius Red staining (0.1 g of Sirius Red powder in 100 mL of picric acid), washed with distilled water and finally were dehydrated with an increasing scale of alcohols. Sirius Red is a red dye that allows to stain collagen fibres.

Immunofluorescence assays

Tissue samples just collected from the leech body were embedded in Polyfreeze tissue freezing medium (OCT, Tebu-Bio, Le Perray-en-Yvelines, France)/(Polysciences, Eppelheim, Germany) and immediately frozen in liquid nitrogen. They were then kept at -80°C. Cryosections (7 µm), obtained with a cryotome (Leica CM1850), were collected on gelatinous slides and kept at -20°C.

For immunofluorescence assays, sections were rehydrated with PBS 1X (NaCl 8 g/l; KCl 0,2 g/l; Na_2HPO_4 1,44 g/l; pH 7,4) for 10 minutes and then pre-incubated for 30 minutes in blocking solution (2% Bovine Serum Albumin and 0.1% Tween20 in PBS). The same blocking solution was used to dilute primary and secondary antibodies. Samples were incubated with the primary antibodies for 1 hour. After 3 washes of 5 minutes each with PBS 1X, specimens were incubated for 45 minutes with the secondary antibodies diluted in blocking solution. Following other 3 washes, nuclei were counterstained with 4.6-diamidino-2-Phenylinedole (DAPI, 0.1 mg/ml in PBS) for 3 minutes and, after the last 3 washes, slides were mounted with Cityfluor (Cityfluor Ltd, UK). In negative control experiments, primary antibodies were omitted, and sections were incubated only with the secondary antibodies.

For bFGFR and COL1α1 immunofluorescence assays the primary antibodies respectively used were anti-bFGF receptor Flg (rabbit, Santa Cruz Biotechnology, sc-121), diluted 1:150 and anti-COL1α1 (rabbit, polyclonal, EMD Millipore, ABT257), diluted 1:200. Subsequently, samples were incubated with the respectively secondary antibody goat anti-rabbit TRITC-conjugated (goat, Jackson ImmunoResearch Laboratories, Baltimora Pike, West Grove–PA), diluted 1:300.

The percentages of positive bFGFR$^+$ cells were assessed after r*Hv*RNASET2 injection by analysing five different slides (random fields of 45000 μm^2 for each slide) for each experimental timing using the Image J software package. Cells in the chosen fields were counted as fibroblasts if were bFGFR$^+$ (TRITC labelled). Statistical analyses were performed using GraphPad Prism 7, (GraphPad Software, La Jolla, CA, USA). Statistical differences were calculated by one-way ANOVA followed by Fisher's post-hoc test and $p < 0.05$ was considered statistically significant. Means with different letters significant difference between PBS and r*Hv*RNASET2 injected leeches at different times elapse.

Double-labelling experiments were performed as previously described (Baranzini et al. 2017) to detect RNASET2/COL1α1 and COL1α1/ bFGFR co-expressing cells. Samples were first incubated with the respective primary antibodies: anti-RNASET2 (Acquati et al. 2011), diluted 1:200, anti-bFGFR (rabbit, Santa Cruz Biotechnology), diluted 1:100. After several washes in PBS, sections were incubated with the secondary antibody goat anti-rabbit (Cy5)-conjugated (goat, Abcam England), diluted 1:200. 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 Laboratories, West Grove, USA) at 1:25 for 2 h. After several washings, samples were incubated with the primary antibody COL1α1 (rabbit, polyclonal, EMD millipore), diluted 1:200. Subsequently, sections were treated with the secondary (FITC)-conjugated goat anti-rabbit antibody (Abcam England), diluted 1:300. Slides were examined with a Nikon Eclipse Ni (Nikon, Japan) optical and fluorescence microscope equipped with three different excitation/emission filters: 360/420nm, for DAPI nuclear staining; 488/532nm, for TRICT/FITC signals and 650/672nm, for Cy5 signals. Data were recorded with Nikon Digital Sight DS-SM (Nikon, Tokyo, Japan) digital camera and images were combined with Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Protein extraction, SDS-PAGE, Western blot

Tissues extracted from the PBS-injected body wall or from injected areas were immediately frozen in liquid nitrogen and then homogenized with a mortar. The homogenates were suspended (10 µl per mg of tissue), in RIPA buffer (150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris-HCl, pH 8.0) in the presence of a protease inhibitor cocktail (Sigma, Milan, Italy). The particulate material was removed by centrifugation at 13,000 rpm for 10 min at 4°C in a refrigerated Eppendorf Minispin microcentrifuge (Hamburg, Germany). Supernatants containing total protein extracts were denatured at 100°C for 5 min and loaded on 12 % acrylamide minigels for SDS-PAGE analyses. Molecular weights were determined by concurrently running broad range standards from Bio-Rad (Richmond, MA, USA). 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 an anti-COL1α1 antibody (rabbit, polyclonal, EMD millipore) diluted 1:500. After, the membrane was washed three times with TBS-Tween 0.1 %, and antigens were revealed with a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories, West Grove, USA), diluted 1:5000. After a further washing step, immunocomplexes were revealed with luminol LiteAblot PLUS Enhanced Chemiluminescent Substrate (EuroClone, Pero, Italy). Bands were normalized by using the ImageJ software package (http://rsbweb.nih.gov/ij/download.html), with the housekeeping protein D-glyceraldehyde-3-phosphatedehydrogenase (GAPDH), which was detected with a rabbit polyclonal anti-human GAPDH IgG (Proteintech, Chicago, USA), diluted 1:7500. The expression level of COL1α1 in treated leeches were compared to that of control animals. Statistical analyses were performed using GraphPad Prism 7, (GraphPad Software, La Jolla, CA, USA). Statistical differences were calculated by one-way ANOVA followed by Fisher's post-hoc test and $p < 0.05$ was considered statistically significant. Means with different letters significant difference between PBS and r*Hv*RNASET2 injected leeches at different times elapse.

Human MRC-5 fibroblast cell culture

The human fibroblast MRC-5 cell line was cultured at 37°C in DMEM medium supplemented with 10% FBS and 1% penicillin/streptavidin solution. Cells were washed with phosphate-buffer solution (PBS) and dissociated with 0.25% Trypsin solution. Cells were then diluted in

fresh medium and seeded into 96-well plates (8.0 X 10^4) for performing luciferase assays, into 48-well plates (3.0 X 10^5) for mRNA extraction and into 8-plates LabTek (1.0 X 10^5) for immunofluorescence assays.

Dual-luciferase assay system

Luciferase assay was performed to evaluate the effects of the recombinant *Hv*RNASET2 on MRC-5 fibroblast collagen I expression. After 24 h, the medium of cultured fibroblasts was removed and cells were co-transfected by Lipofectamine 3000 Reagent (Invitrogen, Waltham, MA) with pCol1-Luc, a collagen I promoter-driven firefly luciferase recombinant construct (0.25 μg/μl) and PRL-TK (promega) an HSV-TK promoter-Renilla luciferase construct as an internal control (0.25 μg/μl) in a 90μl volume of fresh DMEM medium. Tranfected fibroblasts were then treated with different concentrations of recombinant *Hv*RNASET2 (0, 50 and 200 ng/μl) for 24 h. The medium was then removed and the Dual-Glo Luciferase assay (Promega Madison, WI) was carried out by adding 75μl of Dual-Glo® Luciferase Reagent. The firefly luciferase luminescence was measured using a multilabel plate reader after 10 min (Victor – PerkinElmer Waltham, MA). The Dual-Glo® Stop & Glo® Reagent, which quenches the luminescence from the firefly reaction and provides the substrate for Renilla luciferase, used as internal control for normalizing values, was directly added after 20 min and a second measure was performed. Cells treated with TGF-β were used as positive control. The ratio between the two measurements from each sample was obtained and statistical differences were calculated using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Statistical differences were calculated by t-test and $p < 0.05$ was considered statistically significant.

In vitro immunofluorescent assays

MRC-5 cells grown in LabTek and treated with or without r*Hv*RNASET2, were fixed with 4% paraformaldehyde at room temperature, washed 3 times for 5 min with PBS 1X and treated for 10 mins with 0.5% Triton X100 (Sigma-Aldrich St-Louis, MI) to permeabilize cell membranes. Following a 30 min incubation with BSA blocking solution (4% in PBS), cells were treated overnight with anti-collagen I (rabbit polyclonal, Abcam, Cambridge, MA), diluted 1:500, primary antibodies. After 3 washes of 5 min with PBS1X, fibroblasts were incubated for 45 min with secondary antibodies: anti-rabbit Cy3-conjugated (Jackson Immunoresaerch Inc), diluted 1:200, and with 400 μM Phalloidin-Atto488 (Sigma-Aldrich) to detect actin. Nuclei

were counterstained with 2µM Hoechst 33342 (Sigma-Aldrich) for 10 sec and, after one quick wash, slides were mounted in Fluoromount-G mounting medium (Interchim, Montluçon, France).The intensity of anti-collagen I signal was assessed by analyzing ten different slides (random fields of 45000 μm^2 for each slide) for each experimental group using the Image J software package. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Statistical differences were calculated by t-test and $p < 0.05$ was considered statistically significant.

RNA extraction from MRC-5 fibroblasts and qRT-PCR

MRC-5 cells treated with different concentration of HvRNASET2 were lysed with the lysis buffer solution from Nucleospin RNA Plus XS kit (Macherey-Nagel, Dueren Germany). Total RNA was extracted according to the manufacturer's instructions. RNAs were retro-transcribed into cDNA using Superscript II Reverse Transcriptase and qPCR was carried out in triplicate on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Reaction mixtures had a final volume of 10 µl, consisting of 2 µl of cDNA, 5 µl of LightCycler® 480 SYBR Green I Master and 0.5 µM primers. After initial denaturation, amplification was performed at 95°C (10 s), 60°C (5 s) and 72°C (10 s) for 45 cycles.

RESULTS

Recently, our group reported that RNASET2 injection in leeches triggered an inflammatory process coupled to extracellular connective tissue remodelling. Indeed, injection of *human* recombinant RNASET2 in the leech body wall was shown to induce both fibroplasia and the recruitment of infiltrating cells expressing both granulocyte and macrophage-specific markers (Baranzini et al. 2017).

To further support this experimental evidence in a more physiological context and to rule out the occurrence of putative species-specific differences affecting the above mentioned process, we evaluated the effect of leech *H. verbana* recombinant RNASET2 protein (r*Hv*RNASET2 - BankIt2095553 Hirudo MH325331) injection on leech connective tissue remodelling, under the assumption that such biological response might provide a scaffold for the subsequent migration of endothelial and immunocompetent cells at the injection site.

To investigate these aspects, the *H. verbana* RNASET2 coding sequence was amplified by RT-PCR and cloned into a *P. pastoris* expression vector for heterologous production of recombinant r*Hv*RNASET2. Endotoxin-free r*Hv*RNASET2 was then injected into the leech body wall as described in Material and Methods. We first carried out both morphological and immunocytochemical assays to evaluate the presence and distribution pattern of leech fibroblasts and their possible activation in the earliest phases of leech inflammation by means of an anti-bFGFR antibody, which is a known marker for leech fibroblasts (Tettamanti et al. 2003). Western blot assays were also carried out to evaluate newly synthesized collagen I expression levels. Furthermore, double immunocytochemical assays were performed to investigate the putative interplay between RNASET2, fibroblasts activation and fibrillar collagen production.

r*Hv*RNASET2 injection leads to leeches ECM reorganization

A morphological analysis by means of both optical and transmission electron microscopy (TEM) was initially carried out. A thick muscular layer made of tightly packed helical muscle fibers, embedded in a scarce and loose connective tissues (de Eguileor et al. 1999; Sawyer 1986; Tettamanti et al. 2004), was observed in the body wall of control, PBS-injected leeches (Fig. 1a,b).

Ultrastructural TEM analysis confirmed that the amount of fibrillar collagen in the extracellular matrix (ECM) surrounding muscle fibers of control leeches was almost negligible, since only a few collagen fibres were detectable (Fig. 1b). Accordingly, few fibroblasts were found embedded in this loose matrix (Fig. 1a) and showed a typical roundish shape, with the cytoplasm almost completely filled by spherical lipid droplets (Fig. 1c).

Noteworthy, 30 min following r*Hv*NASET2 injection, ECM deposition among the muscle fibers already appeared to be increased. Moreover, its gross organization was markedly changed, showing an even looser appearance (Fig 1d,e). Of note, a marked increase in the number of fibroblasts embedded in the newly-laid ECM was easy detectable in the injected area (Fig. 1d,e). These cells appeared to be massively activated from the earliest phases of r*Hv*NASET2 injection, as suggested by both the marked deposition of a massive amount of collagen and their clear change in morphology, detected under both optical microscopy (Fig. 1d) and TEM (Fig. 1e) analyses. Indeed, r*Hv*NASET2-activated fibroblasts appeared elongated and star-shaped (Fig. 1d-i), being characterized by the presence of multiple projections of cytoplasmic laminae stretching towards the extracellular space (Fig. 1f) in which new collagen fibril deposition was evident (Fig. 1g,h). Such cytoplasmic projections are known to be involved in the spatial organization of collagen bundles and in the control of fibril orientation (Tettamanti et al. 2004, 2005). Of note, abundant fibroblast-derived collagen deposition has been reported to be used also by immune cells to reach the area affected by injury and/or infection (Baranzini et al. 2017). Accordingly, a marked increase in the number of infiltrating macrophages and granulocytes (de Eguileor et al. 2000b, a; Girardello et al. 2019) was detected in r*Hv*NASET2 injected leeches (Fig 1d,e,i).

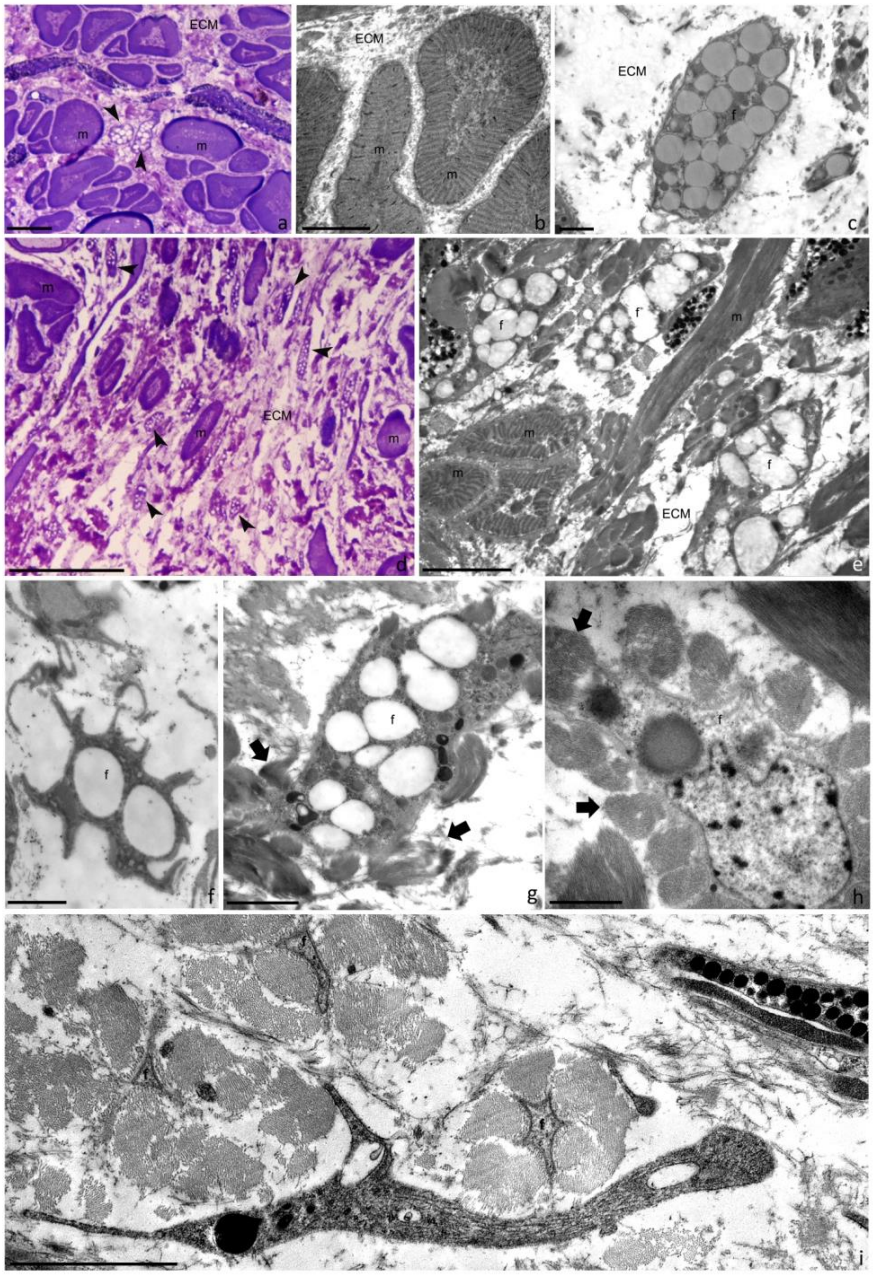

Fig. 1 Images of cross-sectioned leech body wall at optical (**a, d**) and TEM (**b-c,e-i**) microscopes. Organized and compact extracellular matrix (*ECM*) with few fibroblasts (*arrowheads*) is visible among muscle fibers in control animals (**a,c**). Detail at TEM of a roundish fibroblast with a cytoplasm containing a large quantity of lipid droplets (**c**). After the rHvRNASET2 injection (**d,e**) the ECM appears less compact and a large number of fibroblasts (*arrowheads*) are detected between the muscles. Details at TEM of fibroblasts with a stellate shape (**f**) in active fibrillogenesis (*arrow*) are observable (**g,h**). In the membrane folding of fibroblasts, collagen fibers with a precise geometrical organization were visible (**i**). *Bar* (**a**) 10µm, *Bar* (**b**) 2,5µm, *bars* (**c,g,i**) 2µm, *bar* (**d**) 50µm, *bar* (**e**) 5µm, *bars* (**f,h**) 1µm

Colorimetric Staining of rHvRNASET2-induced ECM

We next used collagen-specific Masson's trichrome and Sirius red (Fig. 2) staining to further investigate the effects of direct intramuscular injection of r*Hv*RNASET2 on ECM remodelling.

In control, PBS-injected leeches, the ECM organized among muscle fibers (Fig. 2a-c) appeared homogenously stained by both staining assays. By contrast, a remarkable rearrangement of

the collagen-based connective tissue was observed following r*Hv*RNASET2 injection. In particular, the connective tissue assumed a rather lax appearance already 30 min following treatment (Fig. 2d-f) and such loose organization was maintained until 6h post-injection (Fig. 2g-i). After 24h, a newly synthesized compact collagenous scaffold surrounding the groups of muscle fibers was evident (Fig. 2j-l).

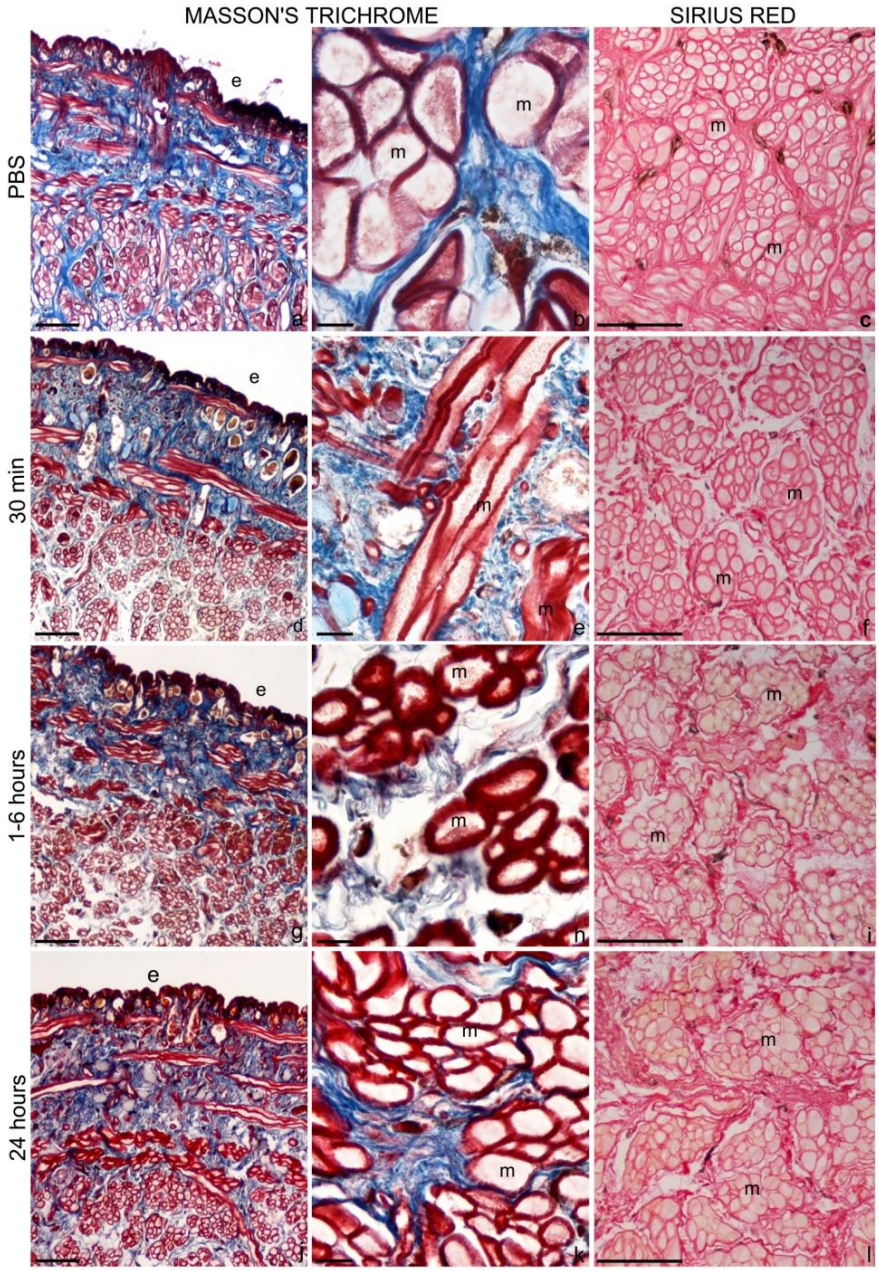

Fig. 2 Images of cross-sectioned leech body wall at optical microscope stain with Masson's Trichrome and Sirius Red staining, in which collagen fibers are stained in blue and red respectively. In control PBS injected animals, tightly packed collagen fibers are visible under the cuticle and among muscle fibers (**a-c**). After 30 minutes from the recombinant r*Hv*RNASET2 injection (**d-f**) collagen fibers seem to lose their precise organization. Between 1 and 6 hours newly synthetized collagen is present (**g-i**) and after 24 hours the fibers appear to be re-arrange in a regular way and connective tissue is similar to that observe in control leech (**j-l**). *Bars* (**a,c,d,f,g,l,j,l**) 100µm, *bars* (**b,e,h,k**) 10µm. *e*: epithelium, *m*: muscle fibers

Effect of rHvRNASET2 on COLα1α1 production and bFGFR expression

To better define the cell types involved in collagen neo-synthesis following r*Hv*RNASET2 injection and thus confirm the occurrence of a RNASET2-driven fibroplasia and connective tissue remodelling, immunofluorescence assays were performed with anti-COL1α1 and bFGF receptor/Flg polyclonal antibodies, to detect collagen I (Fig. 3) and fibroblasts (Fig. 4), respectively. Immunofluorescence analysis of cryosections from the body wall of PBS-injected control leeches showed a basal level of bFGFR expression from few resident cells (Fig. 3a), whereas a striking increased in the number of bFGFR$^+$ cells was detected among muscles fibers following r*Hv*RNASET2 treatment (Fig. 3b-f). In particular, cell counting performed on 5 representative images from each time point confirmed a statistically significant change in bFGFR$^+$ fibroblast number during different phases of the inflammatory response was (Fig. 3g). Indeed, the number of fibroblasts expressing bFGFR immediately increased following r*Hv*RNASET2 treatment, reached a peak at 1 h and remained high for 6 h before decreasing to a baseline level after 24 h. Moreover, unlike the pattern observed in control leeches, the different morphology acquired by these cells, represented by the starry appearance typical of activated fibroblasts involved in collagen production, was confirmed (Fig. 3h).

When we turned to evaluate COL1α1 expression pattern, control PBS-injected leeches displayed a barely detectable signal (Fig. 4a), whereas expression of this marker progressively increased at different time points following r*Hv*RNASET2 injection (Fig. 4b-f).

The temporal expression profile of COL1α1 after r*Hv*RNASET2-injection was also evaluated by Western blot analysis. The observed expression pattern confirmed the presence of a 140 kDa bands (corresponding to the molecular weight of this type of collagen) (Fig. S1a-f), that was shown by quantitative analyses to be expressed at low basal level in PBS-injected control leeches and at much higher levels following r*Hv*RNASET2 injection (Fig. 4g).

Indeed, COL1α1 expression began to increase in the early stages of treatment (at 30 min post injection) until the 24 h post-treatment. To better characterize the cells expressing COL1α1, we performed double-staining immunofluorescence assays by coupling the anti-COL1α1 antibody to antibodies raised against either the common fibroblast marker bFGFR or RNASET2. Strikingly, these assays not only confirmed that r*Hv*RNASET2-activated fibroblasts produced COL1α1 (Fig. 4h), but also showed that COL1α1$^+$ fibroblasts co-expressed RNASET2 as well (Fig. 4i). In the negative control experiments no signals were detected (Fig. S1g-i).

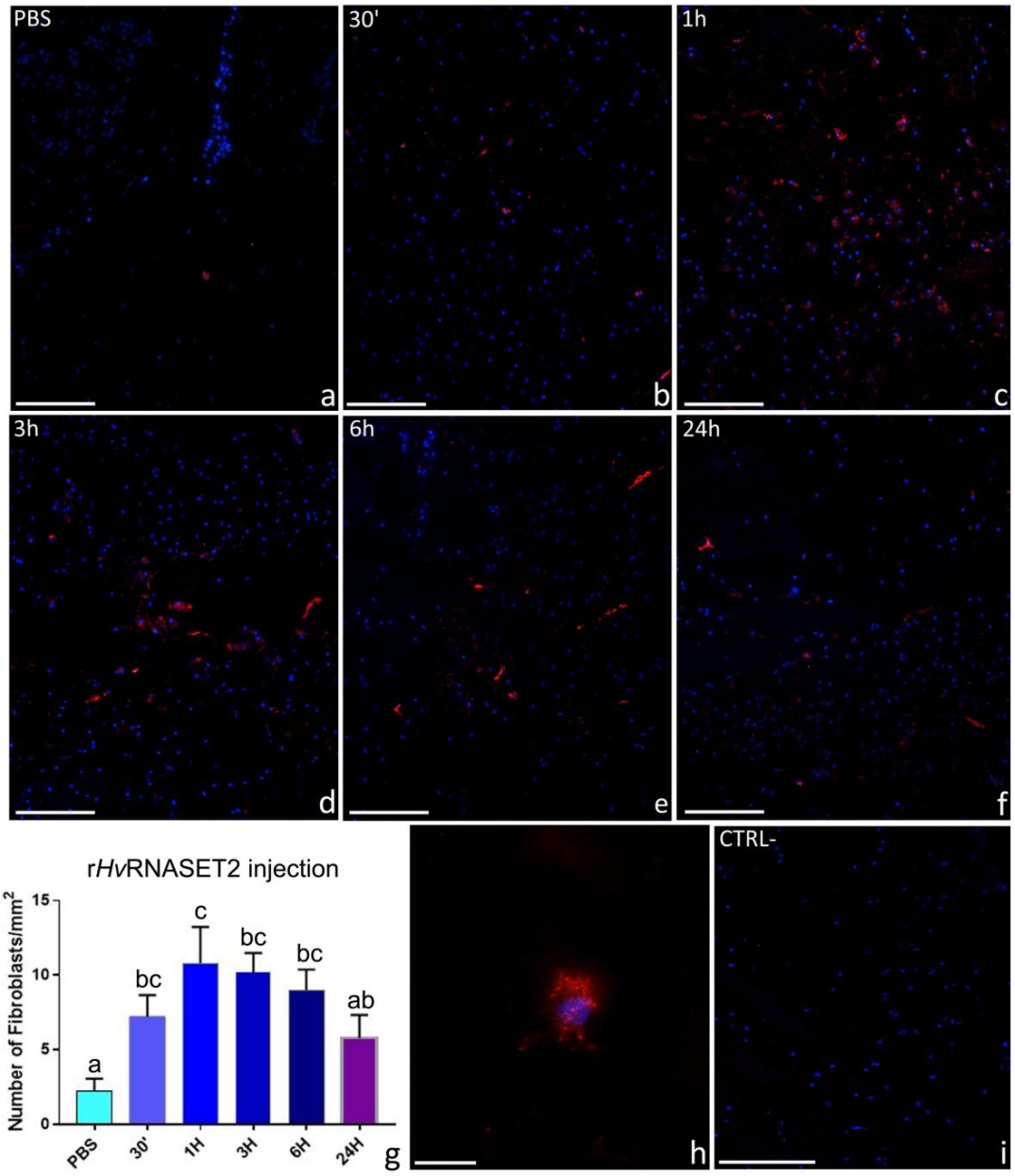

Fig. 3 Immunofluorescent analysis of cryosectioned PBS- (**a**) and r*Hv*RNASET2 injected leech body wall (**b-i**). After 30 minutes (**b**) and 24 hours (**f**) from recombinant protein injection, the antibody anti-bFGFR stains in red a few cells among muscle fibers, whereas the presence of several positive cells is detected at 1, 3 and 6 hours (**c-e**). Cell nuclei are stained in blue with DAPI. The graph shows the percentage of positive fibroblasts (bFGFR^{+}) in each time point after treatment (**g**). Statistical analyses were performed using Statistica 7.0 software (StatSoft, Inc, Tulsa, OK, USA) and differences were calculated by one-way ANOVA followed by Fisher's post-hoc test. Means with different letters represent a significant difference between PBS and rHvRNASET2 injected animals at different time elapse. Detail of fibroblast showing the typical stellate shape following activation (**h**). No signal is present in negative control experiments in which primary antibody is omitted (**i**). *Bars* (**a-i**): 100µm

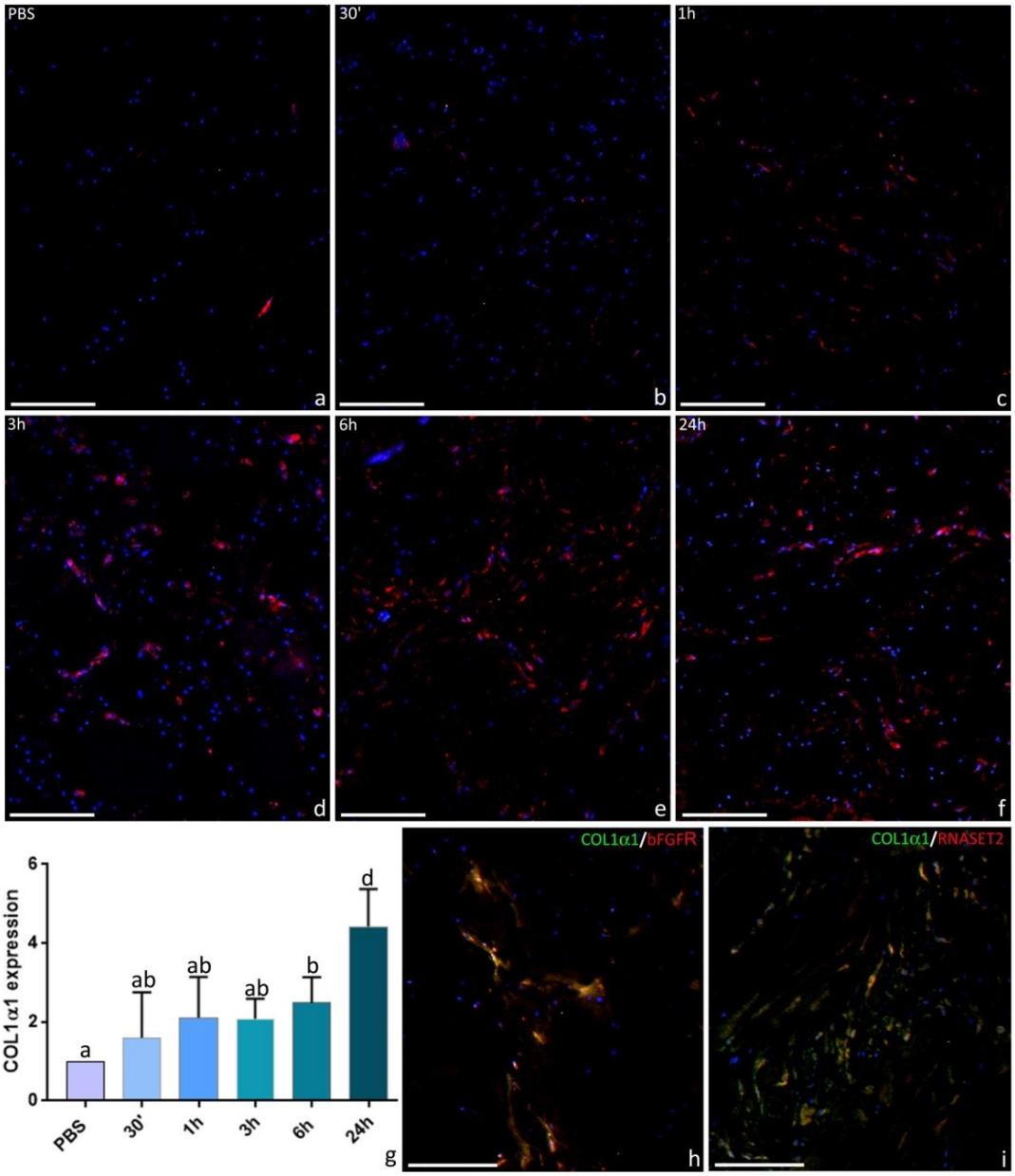

Fig. 4 Immunofluorescent analysis of cryosection from PBS (**a**) and r*Hv*RNASET2 (**b-h**) injected leeches. The antibody anti-COL1α1 stains in red the new produced collagen fibrils. In control PBS animals (**a**) a low signal is detectable, while after 30 minutes, but especially starting from 1 hour (**b**), it begins to increase. The positivity remains high and continue to increase until the 24 hours (**c-f**). Cell nuclei are stained in blue with DAPI. The graph shows the level of expression of the newly synthetized COL1α1 in each time point quantified by densitometry with ImageJ software (**g**). Statistical analyses were performed using Statistica 7.0 software (StatSoft, Inc, Tulsa, OK, USA) and differences were calculated by one-way ANOVA followed by Fisher's post-hoc test. Means with different letters represent a significant difference between PBS and rHvRNASET2 injected animals at different time elapse. Double immunolocalization of COL1α1 (in green) and bFGFR (in red) (**h**) and COL1α1 (in green) and RNASET2 (in red) (**i**) showing that bFGFR^{+} activated fibroblasts (in yellow) expressed both pro-collagen I and endogenous RNASET2. No signals are detected in the negative control experiments, in which the primary antibodies are omitted. *Bars* (**a-i**) 100µm

Analysis of in vitro MRC5 fibroblast cell line following HvRNASET2 treatment

We next turned to the human MRC5 fibroblast cell line to evaluate the collagen neosynthesis rate following treatment with leech recombinant *Hv*RNASET2 at different concentrations (Fig. 5).

By means of realtime qPCR assays, human COL1α1 mRNA expression was shown to increase in *rHv*RNASET2-treated MRC5 cells (Fig. 5a), suggesting that the RNASET2-mediated effect on collagen neosynthesis is conserved across species and, at the same time, indicating that the induction of *Col1* expression by *Hv*RNASET2 in fibroblastic cells apparently acts at the transcriptional level.

The effect of r*Hv*RNASET2 treatment on COL1α1 expression pattern was further evaluated by a luciferase reporter assay, as described in Material and methods. The observed levels of luciferase expression at different concentrations of r*Hv*RNASET2, related to collagen I promoter activation are shown in Fig. 5b-c. At each timepoint evaluated, a statistically significant increase in luminescence was observed when a r*Hv*RNASET2 recombinant protein concentration of 200 ng/ml was used. Therefore, these data provided further support to the notion of a r*Hv*RNASET2-mediated increase in collagen expression.

Finally, the COL1α1 expression pattern was investigated in control and r*Hv*RNASET2-treated MRC5 fibroblasts by immunofluorescent assay (Fig. 5d-f). Human fibroblasts showed a positive staining for COL1α1 antibody both in the presence or absence of r*Hv*RNASET2 (Fig. 5c-e). However, signal intensity measurements revealed a statistically significant increase following recombinant leech protein treatment (Fig. 5g), suggesting the occurrence of a r*Hv*RNASET2-mediated increase in collagen I neosynthesis by these cells.

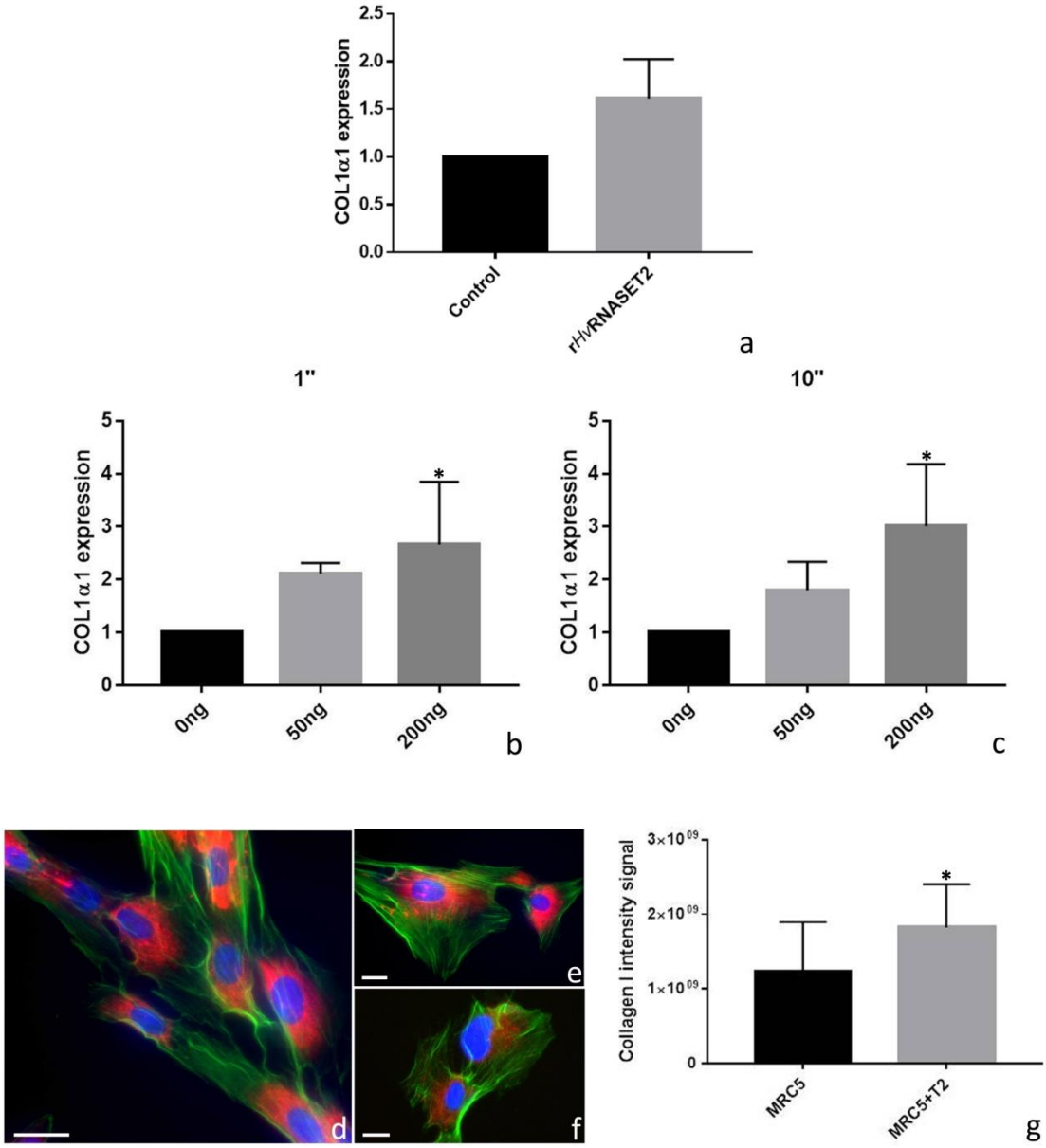

Fig. 5 In vitro experiments conducted on MRC5 human fibroblast cell line. (**a**) The graph shows COL1α1 mRNA expression in MRC5 fibroblasts treated with PBS or r*Hv*RNASeT2. (**b, c**) Graphs showing the intensity of the firefly luciferase luminescence following treatment with different concentrations of r*Hv*RNASET2. Immunofluorescent assays performed on treated (**d, e**) and untreated fibroblasts (**f**) using specific anti-collagen I antibody. The graph shows the significant different intensity of signal between r*Hv*RNASeT2 treated and untreated samples (**g**). *Bar* (**d**) 50µm, *bars* (**e,f**) 20µm

DISCUSSION

In both vertebrates and invertebrates, complex interactions based on several intracellular and extracellular events occur to restore and renew damaged tissues and regulate tissue homeostasis in response to wounds or infections, thus representing the key events in regenerative processes. To restore the original tissue reorganization, different types of cells such as immune cells, endothelial cells and fibroblasts are recruited and activated to modify their expression profile and produce several effector molecules, which in turn lead to cell differentiation and proliferation (Gurtner et al. 2008). In this context, the extracellular matrix (ECM) plays a key role, by acting as a molecular scaffold to affect and guide these cellular processes and to maintain the correct organization of organs and tissues (Daley et al. 2008).

A better understanding of the molecular and cellular basis of tissue repair is one of the main targets of regenerative medicine. Interestingly, several discoveries in the field of regenerative processes that have been reported in low-complexity eukaryotic species, such as amphibians and invertebrates, have brought significant insights into human research (Gurtner et al. 2008).

Among these alternative animal models, the medicinal leech represents a well-consolidated tool for wound healing investigation. Indeed, in leech as in vertebrates, ECM degradation and reassembly are necessary for both immune cell recruitment and new vessel orientation during the inflammatory response (de Eguileor et al. 2004; Tettamanti et al. 2004). In this context, the Cathepsin B protease produced by both fibroblasts (Tettamanti et al. 2005) and macrophages (Grimaldi et al. 2004) is probably involved in degrading different components of ECM such as laminin, fibronectin and collagen. Either immune cells, endothelial cells or fibroblasts are known to exploit such degradation of the collagenous matrix in order to migrate and change their spatial position in the body wall during the reparative process. However, other molecular effector besides Cathepsin proteins are likely involved in the coordination of such a complex biological process as tissue repair.

We have previously reported that injection of the human rhRNASET2 recombinant protein in leeches triggers both a strong innate immune system activation and a significant collagen production, leading to a marked remodelling of the connective tissue (Baranzini et al. 2017).

To further investigate the role of this highly pleiotropic ribonuclease in these distinct but functionally related biological processes, we investigated here the effect of the endogenous *H. verbana* RNASET2 protein in leech ECM remodelling. To this aim, both *in vivo* and *in vitro* assays have been carried out to evaluate the role of recombinant *Hv*RNASET2 protein injection in collagen synthesis, following the cloning of the *H. verbana* orthologous cDNA into a proper expression vector for recombinant protein production.

Morphological and colorimetric analyses clearly demonstrated that, already at 30 min following *in vivo* r*Hv*RNASET2 treatment, the ECM surrounding the muscular fields underwent a marked remodelling, becoming looser and disorganized. This pattern was maintained up to 6 h from injection, whereas after 24 h post-treatment collagen neosynthesis was initiated to replace the previously degraded collagen, as already reported following the injection of the human recombinant protein rhRNASET2 (Baranzini et al. 2017). Moreover, a highly increased number of fibroblasts embedded in the extracellular network was easy detectable following r*Hv*RNASET2 injection. Of note, these cells appeared to be actively involved in the production of a massive amount of collagen following their activation.

As previously described upon experimental surgical lesions or cytokines injection in leeches (de Eguileor et al. 2001a; Tettamanti et al. 2005; Schorn et al. 2015b), the phenotype of r*Hv*RNASET2-induced fibroblasts appeared very similar to that seen in vertebrates activated fibroblasts, with numerous laminar cytoplasmic projections stretching into the extracellular matrix. These cytoplasmic laminae allow~~s~~ the assembly of a specific microenvironment in which not only fibrillogenesis occurs, but also new collagen bundles are spatially organized and orientated (Tettamanti et al. 2004). These new fibrils, in cooperation with other molecular components such as proteoglycans and glycosaminoglycan, have been reported to serve as a solid and robust scaffolding favouring cells and new vessels migration towards the injured area (Tettamanti et al. 2005). As expected, the abundant production of collagen induced by r*Hv*RNASET2 was also coupled by a numerical increase in fibroblast cells, as shown by both morphological analysis and cell counts in immunofluorescence images, where a high increase in bFGFR expression, a typical fibroblast marker, was unveiled. Indeed, the observed bFGFR expression pattern not only confirmed the activated state of fibroblasts in r*Hv*RNASET2-treated leeches, but also allowed us to demonstrate by cell count assays that the number of these activated cells was mainly increased in the earliest phases post treatment.

Furthermore, immunofluorescence assays and western blot analysis showed a continuous synthesis of new collagen fibrils over time, resulting with an ever-increasing COL1α1 signal in r*Hv*RNASET2-treated leeches when compared to controls, in which mechanical stress induced by injection of the vehicle solution alone did not exert a significant effect. Therefore, following r*Hv*RNASET2 injection, both bFGFR and COL1α1 expression were found to be significantly increased in the connective tissue surrounding the muscle fibers fields.

The observed ECM remodelling following r*Hv*RNASET2 injection in the leech body wall is of key relevance, since it allowed us to rule out the occurrence of an aspecific response due to injection of a non-self protein in our previous data reported with human recombinant rhRNASET2 (Baranzini et al. 2017).

Moreover, the evolutionarily conserved role of RNASET2 in ECM remodelling was further suggested by our *in vitro* experiments, which confirmed the ability of r*Hv*RNASET2 to induce collagen I production at the transcriptional level in the human MRC5 fibroblast cell line as well. Since recruitment and possibly activation/polarization of human cells from the monocyte/macrophage lineage by RNASET2 was also recently recapitulated in leeches by our group (Grimaldi et al. 2004; Baranzini et al. 2017, 2018), the data presented in this work are in keeping with the notion of RNASET2 as a highly evolutionarily conserved protein involved in a key ancient host defense process, represented by tissue repair following injury or infections, based on induction of an inflammatory process coupled to a marked ECM remodelling likely mediated by tissue fibroblasts.

In this context it is worth noting that, following bacterial infection in leeches, a massive recruitment of RNASET2-expressing macrophages is observed (Baranzini et al. 2017). Since RNASET2 is known to be actively secreted by cells expressing it, it is tempting to speculate that the recruited RNASET2^{+} macrophage and granulocyte populations represent the source of extracellular RNASET2 which might in turn activate resident fibroblast in order to carry out ECM remodelling. If confirmed by future studies, the data reported in this work would therefore point at RNASET2 as a master regulator of a highly coordinated cellular crosstalk involved in a crucial biological process.

Although further experiments will be necessary, our data also suggest for the first time a direct role of a T2 ribonuclease in the regulation of collagen synthesis in human cells, opening new possibilities related to its functionalities and its applications in the therapeutic field.

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Disclosure of potential conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Supplementary material

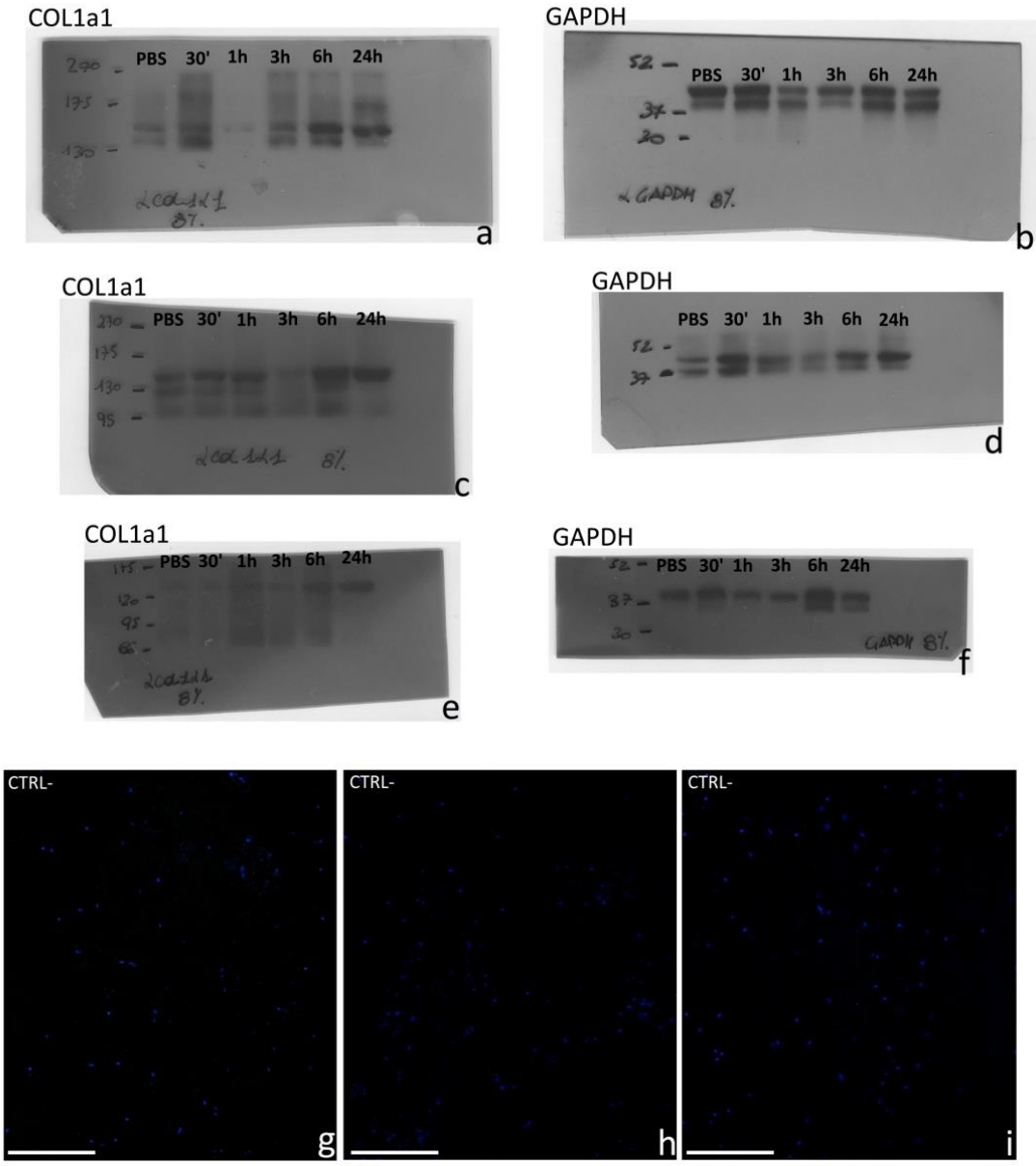

Figure S1 Western Blot analysis. The proteins extracted from the body wall of the leeches injected with PBS and r*Hv*RNASET2 have been marked with anti-COL1α1 antibody (**a,c,e**). In all samples, the antibody detected a specific immunoreactive band of about 140 kDa. The respectively housekeeping gene GAPDH (**b,d,f**) were used as internal control. Negative control experiments performed on leech cryosections related to the COL1α1 immunofluorescent assay (**g**) and to double immunofluorescent experiments showed in Figure 4 (**h,i**) in which the primary antibodies were omitted

DISCUSSION

Following injuries or infections, a highly coordinated response involving intracellular and extracellular processes is critically involved in triggering a homeostatic rescue and restoring the original tissue reorganization. These mechanisms are considered key events in regenerative processes and are based on specific and complex molecular intereactions.

To restore the original tissue structure, different types of cells such as immune, endothelial and fibroblasts cells are recruited and activated to change their expression profile in order to express a wide range of effector molecules, which in turn lead to cell differentiation and proliferation (**Gurtner et al. 2008**). Given the key role of innate immunity in both defense against pathogens and in tissue repair, innovative studies are critically needed to clarify how the molecular and cellular bases regulating the clearance of immune-mediated debris and the regenerative process are connected.

Several findings related to innate immunity and regenerative processes reported in eukaryotic species with lower complexity, such as amphibians and invertebrates, have recently led to a significant advance for human research (**Gurtner et al. 2008**). Among these alternative animal models, leeches represent a well-consolidated tool for studying innate immune response and wound healing processes. Indeed, as observed in vertebrates, the degradation and subsequent reassembly of the ECM are necessary in leeches as well, both for immunocompetent cell recruitment and for the orientation of new vessels (**de Eguileor et al. 2004; Tettamanti et al. 2004**). In particular, the Cathepsin B protease expressed by both fibroblasts (**Tettamanti et al. 2005**) and macrophages (**Grimaldi et al. 2004**) is probably involved in the degradation of laminin, fibronectin and collagen, all essential components of ECM. Of note, such degenerative events of the collagenous matrix are exploited by immune and endothelial cells or fibroblasts to migrate and vary their spatial position in the body wall during the regenerative processes.

In this contents, our preliminary data obtained in leeches clearly suggest a key role of recombinant hRNASET2 not only in the activation of the innate immune response and in immune cells recruitment towards the injured/grafted or bacterial infection areas, but also in rearrangement of the extracellular matrix (**Baranzini et al. 2017**).

These observations prompt us to clone and produce the leech RNASET2 recombinant protein (*Hv*RNASET2) in order to evaluate the effect of the endogenous enzyme in leech. As previously

observed for human recombinant RNASET2, the injection of *Hv*RNASET2 induces fibroplasia, connective tissue remodeling and the recruitment of numerous infiltrating $CD11b^+$ granulocytes and *Hm*AIF-1^+ macrophages (**Schorn et al. 2015a, b; Baranzini et al. 2017, 2018, 2019a, submitted**).

Interestingly, bacterial stimulation enhances the expression of both *Hm*AIF-1 and *Hv*RNASET2 in leeches, key molecules involved in early events that trigger inflammation. However their temporal expression profile is quite different (**Schorn et al. 2015a; Baranzini et al. 2018**), suggesting that these two cytokines can play a different role in activation and modulation of innate immune response and, in particular, that *Hv*RNASET2 might display a specific antibacterial role.

The antibacterial activity of *Hv*RNASET2 in regulating and organizing the innate immune response, induced by stimulation with LPS and LTA, was confirmed through morphological, ultrastructural and immunofluorescence assays. Indeed, after 3 hours from the injection of both these PAMPs in the leech body wall, a strong recruitment of $CD11b^+$ granulocytes occurred, and these represent the first activated immunocompetent cells following bacterial infection (**Baranzini et al. 2018; Girardello et al. 2019**). These cells also expressed a significant amount of *Hv*RNASET2, as demonstrated by double immunofluorescence and western blot analyses. In addition, this ribonuclease promoted *Hm*AIF-1^+ macrophage migration towards the challenged area (**Drage et al. 2009; Drago et al. 2014**), which showed a high phagocytic activity, as demonstrated by their positivity for the hystoenzymatic ACP reaction (**Schorn et al. 2015a**; **Baranzini et al. 2019b, submitted**). We thus propose that these cells have a dual role during bacterial infection in leeches, being involved in both cleaning up the infected area from bacteria and producing, 24 hour following LPS and LTA stimulation, a second wave of *Hv*RNASET2 in order to recruit further macrophages. In this context, both granulocytes and macrophages were shown to express TLR4 and TLR2 (**Girardello et al. 2019; Baranzini et al. 2019b**), the key receptor involved in the response to Gram-negative and Gram-positive bacteria respectively (**Dziarski and Gupta 2000; Schröder et al. 2003; Bäckhed et al. 2003; Esen et al. 2003; Lu et al. 2008; Rahman et al. 2014**). Moreover, as in vertebrates, both PRRs triggering induced intracellular signalling events involving MyD88, a key molecular intermediate for the activation of TLR4 and TLR2 signalling pathways (**Girardello et al. 2019; Baranzini et al. 2019b, submitted**) that ultimately leads to the production of TNF-α (**Kawai and Akira 2010; Molteni et al. 2016; Girardello et al. 2019**). In addition, functional studies

carried out by injecting the cyanobacterium selective TLR4-antagonist CyP clearly demonstrated that TLR4 is specific for LPS recognition, while LTA likely involved the TLR2 pathway **(Takeuchi et al. 1999**).

Although both *Hv*RNASET2 and hRNASET2 recombinant proteins did not apparently influence cellular viability *in vitro* (**Baranzini et al. 2018, Baranzini et al. 2019b, submitted**), they nevertheless affected *Pseudomonas aeruginosa* cell wall, triggering a change in the typical rod morphology of these cells (**Baranzini et al. 2018**) or induced *Staphylococcus aureus* clumps formation (**Baranzini et al. 2019b, submitted**). Of note, the phagocytic activity of macrophages was apparently facilitated by the aggregating properties of *Hv*RNASET2 on bacterial cells, as demonstrated by *in vivo* experiments, where clusters of *S. aureus* surrounded by macrophages were detected in the injected leech body wall in the presence of *Hv*RNASET2. Moreover, *in vitro* experiments confirmed that, following *Hv*RNASET2 treatment, *S. aureus* formed cell clusters. This aggregation was likely mediated by a specific interaction between *Hv*RNASET2 and the bacterial cell wall, as demonstrated by immunogold experiments at TEM. These results suggested that the antimicrobial properties of the leech recombinant *Hv*RNASET2 protein are comparable to those of other RNases, such as the A RNases 3 and 7, and are in close relation to bacterial clumps forming activities (**Pulido et al. 2016**). Indeed, as reported for RNase 7, *Hv*RNASET2 induced the formation of local blebs in the cell wall of bacteria (**Torrent et al. 2010**), promoting a marked inflammatory response, mediated by TLR2 activation and followed by the release of several pro-inflammatory stimuli, such as TNF-α (**Harder and Schroder 2002**). Moreover, similarly to RNase 3, the ability of *Hv*RNASET2 to bind bacterial cell wall seems of special relevance for its role in bacterial clumps formation (**Torrent et al. 2010**).

Of note, this ability to agglutinate bacteria reminds to that observed for some lectins or lectin-like proteins, both in vertebrates (**Sahly et al. 2008**) and in invertebrates (**Fisher and DiNuzzo 1991; Vazquez et al. 1996**). Indeed, lectins are defensive proteins involved in the recognition of potential invasive microorganisms that, not only recruit pahgocityc or other immunocompetent cells in order to eliminate pathogens, but also can directly agglutinate or opsonize bacteria, inducing the membranes disruption (**van Kooyk and Rabinovich 2008; Stowell et al. 2010**). In particular, C-type lectins (CTLs) represent a well-known conserved group, discovered in many invertebrates models (**Schulenburg et al. 2008; Wang and Wang 2013; Wang et al. 2013**) that directly agglutinate different microbial invaders, stimulating the

production of melanin, the rectuitment of hemocytes and promoting the humoral response (**Li et al. 2014**). The calcium dependent immulectin-2, discovered in the plasma of the tobacco hornworm *Manduca sexta*, presents a specific LPS-binding, agglutinating gram-negative bacteria also at low concentration (**Yu and Kanost 2000**). Given these evidences it is possible to assume that analogies exist between the leech enzyme HvRNASET2 and lectins.

Taken together, the results presented in this work are in keeping with the notion of RNASET2 as a highly evolutionarily conserved protein involved in a key ancient host defense process, represented by tissue repair following injury or infections based on induction of an inflammatory process coupled to a marked ECM remodelling, likely mediated by tissue fibroblasts.

CONCLUSION

The data reported in this work provide compelling evidence in support of a pleiotropic role for RNASET2 in orchestrating an evolutionarily conserved cross-talk between inflammatory response and regenerative process, based on granulocytes and macrophage recruitment and fibroblast activation, coupled to a massive extracellular remodelling.
Moreover, to our knowledge, this study represents the first report describing a marked antibacterial activity for a T2 RNase member in an invertebrate experimental model and plausible mechanism of action.

Of note, the similarity of the biological role assigned for some class A RNases and for T2 RNases in plants (**Irie 1999**) and lower invertebrate, such as leeches, and the common cellular and structural features maintained by enzymes belonging to these two families, such as the preference of substrate and the affiliation with the extracellular pathways (**Hillwig et al. 2009**), lead us to hypothesize a possible evolutionary relations among T2 and A enzymes.

We reckon that these working hypothesis pave the way to further targeted studies that deal with the possible evolutive origins of the T2 and A RNases enzymes and to the production of new peptide-derived antimicrobials drugs, which could help to counteract the ever-growing problem of the dramatic worldwide emergence of antibiotic-resistant strains.

Moreover, our data show that the medicinal leech, which present similar morpho-functional and molecular characteristics with Vertebrates, can be considered a useful invertebrate model to investigate all these mechanisms involved in immune response, wound healing, regeneration and control of tissue homeostasis. Indeed, studies conducted on leech should represent a helpful tool for conducing functional studies for better understanding the close connection between immune response and tissue regeneration and the specific role of the main immune modulators. Keeping in mind that in invertebrates all the biological processes and mechanisms are incredibly similar to those observed in Vertebrates, indicating a significant evolutive conservation, this should be a new emergent and reliable model for studying biological responses, cell types and related molecules. Moreover, as suggested by other invertebrate models, fine information can be obtained about the inflammation process, which resulted faster than in mammals. In addition, the use of powerful methods such as the MG biopolymer, allow to reduce the gap existing between in vitro and in vivo data.

Finally, the use of leeches in scientific research actually avoids some inconveniences associated to the increasing number of restrictions, i.e. ethical considerations, restrictions about the experimental animals use and the number of species accessible for any experimentation, and animal welfare.

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