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REGULAR ARTICLE

The medicinal leech as a valuable model for better understanding the role of a TLR4-like receptor in the inflammatory process

Rossana Girardello[1] · Nicolò Baranzini[1] · Monica Molteni[2] · Carlo Rossetti[2] · Gianluca Tettamanti[1] · Magda de Eguileor[1] · Annalisa Grimaldi[1]

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Abstract

Despite extensive investigation focused on both the molecular characteristics and the expression level of Toll-like receptors (TLRs) during the inflammatory response in vertebrates, few data are available in the literature on the role of these proteins in invertebrate's immune response. Here, we propose the medicinal leech as a valuable model to better elucidate the role of TLR4 and its related products, such as tumor necrosis factor (TNF-α), after activation of the leech peripheral immune system with the endogenous medicinal leech recombinant allograft inflammatory factor-1 (r*Hm*AIF-1) or with an exogenous stimulus, such as lipopolysaccharide (LPS). Our results indicate that activated macrophages (*Hm*AIF-1$^+$) and granulocytes (CD11b$^+$) express both TLR4 and its coreceptor CD14. Moreover, functional studies performed by injecting a cyanobacterium selective TLR4 antagonist CyP demonstrated that only the TLR4 pathway was blocked, while the immune response caused by lipoteichoic acid (LTA) treatment is not affected. These results are consistent with literature on vertebrates, indicating that TLR4 functions as a LPS receptor while the recognition of LTA may involve other pathways.

Keywords TLR4 · LPS · Cyanobacterial product · Macrophage · Granulocyte

Introduction

Innate immunity is the first line against microbial infection and provides defense mechanisms to protect organisms from pathogens (Wakabayashi et al. 2006). The essential step to fight microbial infection is their recognition by specific receptors, such as the pattern recognition receptors (PRRs) (Mahla et al. 2013). Among PRRs, Toll-like receptors (TLRs) have been found throughout the animal kingdom and have a crucial role as primary sensors of invading pathogens in both vertebrate and invertebrate species (Coscia et al. 2011; Molteni et al. 2016). These highly conserved receptors have evolved to recognize products unique to microbial metabolism. In mammalians, TLR4 has been described as the key receptor involved in the response both to exogenous stimuli, such as pathogen-associated molecular pattern (PAMPS) and to several endogenous molecules (known as DAMPS, danger or damage-associated molecular patterns) produced during tissue damage in a noninfectious inflammatory response (Molteni et al. 2016). TLR4 triggering induces intracellular signaling events involving myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways, leading to the activation of downstream transcription factors, such as NF-κB, AP-1 and IRF3, involved in the activation of several cellular responses including the production of pro-inflammatory cytokines and type I interferons (IFNs) (Kawai and Akira 2010; Molteni et al. 2016).

Despite extensive investigation focused on TLR4 function especially in humans, due to its role in dysregulated inflammation occurring in several infectious and noninfectious diseases (Molteni et al. 2016), few data are available in the literature on the role of TLR4 in invertebrate's immune response. The study of TLRs and innate immunity has mainly focused on deuterostomes such as sea urchins and tunicates (Rast et al. 2006). TLRs and Toll signaling pathway components have

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✉ Annalisa Grimaldi
annalisa.grimaldi@uninsubria.it

[1] Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy

[2] Department of Medicine and Surgery, University of Insubria, Varese, Italy

been detected also in other lower invertebrates such as sponges and unicellular organisms, such as the amoeba *Dictyostelium* (Chen et al. 2007). Among invertebrates, protostomes that have been analyzed for the presence of TLRs and their role in immune response are primarily representatives of the Ecdysozoa superphylum (e.g., *Drosophila* and *Caenorhabditis*). Conversely, protostome organisms within the Lophotrochozoan superphylum, such as annelids, have been poorly analyzed. The first identification of TLR genes in annelids was done by Davidson C.R. and colleagues in 2008 (Davidson et al. 2008). More recently, the analysis of genome and transcriptome data from the leech *Hirudo medicinalis* showed that at least five TLRs and several splice variants close to vertebrate TLRs and TLR1 were completely cloned and characterized (Macagno et al. 2006; Coscia et al. 2011). In addition, the immune function of TLR1, regulating the release of the cytokine endothelial monocyte-activating polypeptide II (*Hm*EMAPII) and considered a modulator of inflammatory reactions within the mammal peripheral innate immune response, was demonstrated in the *H. medicinalis* central nervous system (CNS) (Schikorski et al. 2009). Analysis of transcriptome database also showed the presence in leeches of sequence homologs of several products involved in TLR4 signaling pathways, such as gram-negative lipopolysaccharides (LPS)-binding protein (LBP), Toll-interacting protein (TOLLIP), interleukin-1 receptor-associated kinase 4 (IRAK-4), TNF receptor-associated factor 6 (TRAF-6), MyD88, NF-kB subunits, IRF, AP-1; closely related to vertebrate counterparts (Macagno et al. 2010; Tasiemski and Salzet 2017). Intriguingly, in *H. medicinalis*, MyD88 induction by LPS challenge was observed, suggesting that the medicinal leech might respond to LPS through a MyD88-dependent signaling pathway (Rodet et al. 2015).

In the present work, we investigate, both in vivo and in vitro, the response to LPS, used as exogenous stimuli PAMP and to the recombinant cytokine allograft inflammatory factor-1 (r*Hm*AIF-1, Drago et al. 2014), highly involved in leech macrophage recruitment (Schorn et al. 2015a, b) and used as DAMP, in the medicinal leech, *Hirudo verbana*, a species closely related to best-known *H. medicinalis*. This invertebrate model shows a very simple anatomy and has a parenchymatous body and a coelom reduced to small cavities (Mann and Kerkut 1962; Sawyer 1986). Underneath the epithelium is an avascular muscular layer formed by muscle fibers organized in fields and surrounded by a scant extracellular matrix in which a few resident immunocompetent cells, such as macrophages and granulocytes type I, are present. The muscular sac is separated from the inner digestive tube by a loose connective tissue containing the botryoidal and the vasofibrous tissue, two specific hematopoietic tissues from which most of the myeloid lineage-derived leucocytes arise (Grimaldi et al. 2006, 2011, 2018). The injection of LPS or r*Hm*AIF-1, a pro-inflammatorycytokine, in the leech body wall induces the activation of immunocompetent cells that migrate towards the challenged area, crossing the extracellular matrix located among muscle fibers (Schorn et al. 2015b; Grimaldi 2016; Baranzini et al. 2017). In this study, we found that in vivo treatment with LPS or r*Hm*AIF-1 induced the expression of a TLR4-like receptor and upregulation of a TNF-α-like molecule in immunocompetent cells recruited in the challenged areas of the leech. Using a specific and selective TLR4 antagonist, CyP, which was demonstrated to be active in human, mouse and porcine immune cells (Macagno et al. 2006; Thorgersen et al. 2008; Gemma et al. 2016), we found efficient inhibition of a TNF-α-like molecule expression induced by treatments, both in vivo and in vitro.

Material and methods

Animals and treatment

Adult leeches (*H. verbana*, Annelida, Hirudinea, from Ricarimpex, Eysines, France) measuring 10 cm were kept in water at 19–20 °C in aerated tanks. Animals were fed weekly with calf liver. Before injection and/or dissection, leeches were anesthetized with a 10% ethanol solution and all treatments were performed at the 20° metamere. Animals were sacrificed 6 h after each treatment and tissue samples were processed for light microscopy, electron microscopy, immunofluorescence and Western blot analysis. The animals were randomly divided into four experimental groups (three animals for each group):

Group 1 Unstimulated control leeches, to verify the normal and correct morphological information about the body organization.

Group 2 Animals injected 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), to confirm that PBS alone does not induce an immune response.

Group 3 Animals injected with 100 μL of PBS containing 20 ng of the recombinant medicinal leech AIF-1 protein r*Hm*AIF-1 (Schorn et al. 2015a), highly involved in the leech inflammatory response and used as DAMP.

Group 4 Animals injected with 100 μL of PBS containing 100 ng/ml of LPS (used as PAMP) obtained from *Escherichia coli* (Serotype O55:B5, Sigma, St. Louis, MO, USA) to evaluate the expression of TLR4 in the cells involved in the immune response.

Group 5 Animals injected 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 (Macagno et al. 2006).

Group 6 Animals injected with 100 μL of PBS containing 100 ng/ml of LTA obtained from *Bacillus subtilis* (Sigma, St. Louis, MO, USA) and specifically recognized by TLR2 receptor.

Light and electron microscopy

Leech tissues, dissected from the area of the injection, were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h at room temperature. Specimens were then washed in the same buffer and post fixed for 1 h in 1% osmium tetroxide in cacodylate buffer. Subsequently, samples were washed five times in 0.1 M cacodylate buffer and then dehydrated with ascending ethanol scale.

The preparations were then transferred into propylene oxide and Epon resin (1:1 ratio) for 1 h, left overnight in resin and finally included. The semi-thin sections (0.75 μm) for light microscopy were cut with Reichert Ultracut S ultratome (Leica, Nussloch, Germany), collected on slides and colored with crystal violet and basic fuchsine (Moore et al. 1960). The slides were observed with a Nikon Eclipse Ni light microscope (Nikon, Tokyo, Japan) and photographed with a Nikon Digital Sight DS-SM (Nikon, Tokyo, Japan) digital camera.

Ultrathin sections (80 nm) were placed on copper grids, stained 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 digital camera system (MORADA, Olympus, Tokyo, Japan).

In silico identification of TLR4 sequence in *Hirudo verbana* transcriptome

After opening the database using the reading program Notepad ++ https://notepad-plus-plus.org/, we carried out a search for the TLR4 sequence. Among the several sequences found, we selected the code TRINITY_DN36106_c0_g1_i4 (the transcribed RNA sequence). Subsequently, using the program ApE - A plasmid editor http://jorgensen.biology.utah.edu/wayned/ape/, we found the correct open reading frame and, using the same program, we then translated the nucleotide sequence into amino acids obtaining the sequence of TLR4 from *H. verbana.* This sequence was subsequently aligned with sequences of TLR4 form other protostomes or form some vertebrates (mammals and fish), using Blast https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Immunofluorescence

Samples were embedded in Polyfreeze tissue freezing medium (OCT, Tebu-Bio, Le Perray-en-Yvelines, France) and immediately frozen in liquid nitrogen. Cryosections were then obtained with a Leica CM 1850 cryotome. For immunofluorescence assays, sections were rehydrated with PBS for 5 min, preincubated for 30 min with blocking solution (2% bovine serum albumin and 0.1% Tween20 in PBS) and later incubated for 1 h at room temperature with the primary antibodies anti-TLR4 (rabbit, polyclonal, Santa Cruz Biotechnology, CA, USA, sc-10741, recognizing an epitope corresponding to amino acids 242–321 mapping to an internal region of TLR4 of human origin), anti-CD14 (mouse, monoclonal, Santa Cruz Biotechnology, sc-515785, which specifically stains monocytes and macrophages of leech) (de Eguileor et al. 2000a), and polyclonal rabbit anti-tumor necrosis factor-TNF-α (Abcam, Cambridge, UK, ab6671) diluted 1:200 in blocking solution. All specimens were washed before incubation respectively with anti-rabbit Cy3-conjugated or anti-mouse FITC-conjugated (Jackson Immuno Research Laboratories, West Grove, USA) secondary antibodies, for 1 h at room temperature.

Double-labelling experiments to detect CD11b/TLR4, CD14/TLR4, CD14/CD11b and CD14/*Hm*AIF-1 co-expressing cells were performed with the same method previously described (Grimaldi et al. 2011), using a different combination of primary antibodies raised in different species: anti-CD11b (goat, polyclonal, Santa Cruz Biotechnology, sc-28664, which specifically stains leech granulocytes type I) (de Eguileor et al. 2000b); anti-TLR4 (rabbit, polyclonal, Santa Cruz Biotechnology), anti-CD14 (mouse, monoclonal, Santa Cruz Biotechnology) and anti-*Hm*AIF-1 (rabbit anti-Hirudo AIF-1) (Drago et al. 2014), a specific marker for leech macrophages (Schorn et al. 2015b), kindly donated by Prof. Jacopo Vizioli, University of Lille 1, France.

Then, samples were incubated with a mix of the appropriate secondary antibodies: anti-rabbit FITC-conjugated (Abcam), anti-rabbit Cy3-conjugated, anti-goat Cy3-conjugated and anti-goat FITC-conjugated anti-mouse FITC-conjugated (Jackson Immuno Research).

Since the antibodies for the dual *Hm*AIF-1/TLR4 were produced in the same species, a different method was used as previously described (Grimaldi et al. 2011; Girardello et al. 2015b). After the first staining cycle, the sections were incubated with anti-rabbit IgG (Sigma) at 1:500 for 2 h, to prevent the secondary antibody of the second staining cycle binding to the primary antiserum applied in the first stage.

Nuclei were counterstained with 4.6-diamidino-2-phenylindole (DAPI, 0.1 mg/ml in PBS) for 3 min and slides were mounted with Cityfluor (Cityfluor Ltd., UK). In negative control experiments, primary antibodies were omitted or substituted with pre-immune serum (1:20,000) and sections were incubated only with the secondary antibodies.

Western blot analysis

The tissues extracted 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 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 min at 4 °C in a refrigerated Eppendorf Minispin microcentrifuge (Hamburg, Germany). Supernatants containing total proteins extracted were denaturated at 95 °C for 5 min (Laemmli 1970). Samples were assayed for protein concentration with the Coomassie Brilliant Blue G-250 (Pierce, Rockford, IL, USA) protein assay, using bovine serum albumin as standard. Equal amounts of protein (10 μl of a 2 mg/ml final concentration) of denatured protein were loaded on 8% acrylamide minigels for SDS-PAGE analyses. The proteins were separated by SDS-PAGE and transferred to a nitrocellulose filter by means of a gel transfer system by applying 400 mA for 1 h and 45 min. Subsequently, membranes were incubated in a blocking solution, 5% milk in Tris-buffered saline (TBS: Tris HCl 50 mM pH 7.5, NaCl 150 mM) for 2 h in continuous stirring. Membranes were then incubated O/N at 4 °C with the primary rabbit polyclonal anti-TLR4 or anti-TNF-α antibodies diluted 1:250 in blocking solution. For the anti-TNF-α immunoblot, the blocking solution was composed by 5% bovine serum albumin in TBS, according to the datasheet. After washing with TBST (0.1% Tween20 in TBS) three times of 10 min each, membranes were incubated with a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories) diluted 1:7500 in blocking solution for 1 h at room temperature. After further washing, the membranes were incubated with luminol LiteAblot® PLUS Enhanced Chemiluminescent Substrate (EuroClone S.p.A., Pero, Italy) to reveal the immunocomplexes and exposed to a Kodak X-Omat AR film. Then, the blots were stripped in the stripping solution (62.5mMTris–HCl pH 6.7, 2% (*w*/*v*) SDS and 100 mM β-mercaptoethanol) for 30 min at 50 °C. The blots were washed with Tris-buffered saline and after treatment with blocking solution, were incubated with a rabbit anti-human polyclonal antibody IgG recognizing the housekeeping protein D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) diluted 1:1000 (Proteintech, Chicago, USA). After intensive washes, the immunolabelled bands were detected using a peroxidase-conjugated anti-rabbit secondary antibody (Jackson Immuno Research Laboratories) diluted 1:7500 in blocking solution for 1 h at room temperature. The processed blots, before and after stripping, were scanned and, for quantification analysis, were subjected to densitometric 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. The densitometry data for each TLR4 and TNF-α band were normalized to that of GAPDH. The expression level of TLR4 and TNF-α were reported relatively to untreated animals.

Functional studies

For the in vivo study, animals were injected with 100 μL of PBS containing 1 μg/ml of CyP and/or 100 ng/ml of LPS. Lipoteichoic acid (LTA) and a combination of LTA/CyP were used as positive control. For the in vitro study, the same combinations of CyP and/or stimulants were added to the culture medium of primary macrophage cultures, isolated as previously described (Girardello et al. 2015a, 2017).

Image acquisition and recording

Slides were examined with the Nikon Eclipse Ni (Nikon, Japan) light and fluorescence microscope equipped with three different excitation/emission filters: 360/420 nm for DAPI nuclear staining, 488/525 nm for FITC signals and 550/580 nm for Cy3 signals. Images were acquired using Plan 20x, 0.40 NA and Plan 100X, 1.25 NA, oil objectives.

Data were recorded with a Nikon Digital Sight DS-SM (Nikon, Tokyo, Japan) digital camera and images were combined using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Statistical analysis

Experiments were performed in triplicate and data represent the mean values ± SD. Statistical significance was assessed by one-way or factorial ANOVA followed by Tukey's post hoc test and $p < 0.01$ was considered statistically significant (GraphPad Prism 7, GraphPad Software, La Jolla, CA, USA).

Fluorescence intensity was assessed by analyzing three different microphotographs for each time point/assay using the ImageJ software package. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Statistical differences were calculated by factorial ANOVA followed by Tukey's post hoc test and $p < 0.01$ was considered statistically significant.

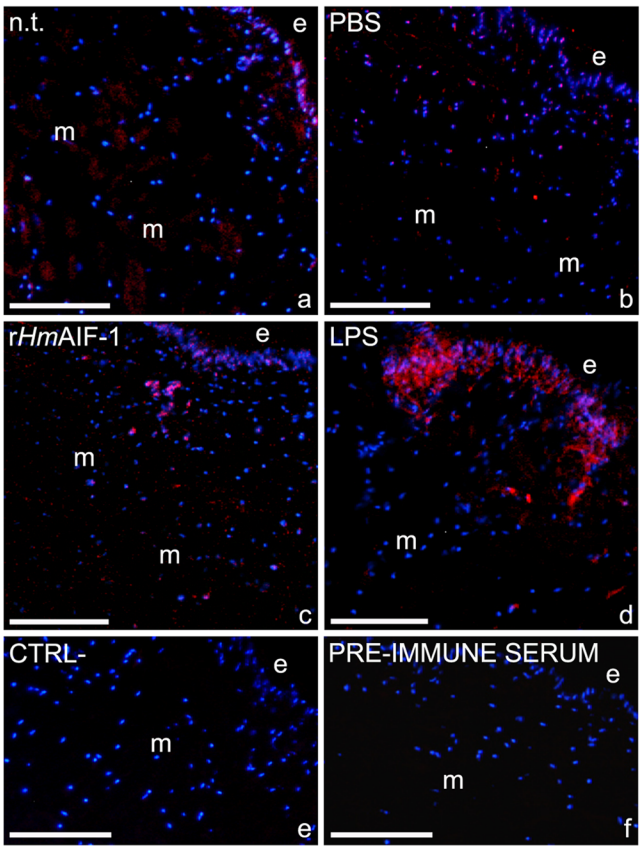
Results

A receptor closely related to TLR4 is constitutively expressed in unlesioned leeches and is highly induced by LPS challenge in vivo

First of all, we identified a TLR4-like sequence (Fig. a in supplementary information S1) in the *H. verbana*

transcriptome (Northcutt et al. 2018, GeneBank https://www.ncbi.nlm.nih.gov/genbank/, BioProject No. PRJNA435743, Sequence Read Archive (SRA) SRR6782848). This protein was characterized by the presence of the LRR and TIR specific domains of TLR4 (Fig. b in supplementary information S1) and showed a different grade of homology with TLR4 from other lophotrochozoan protostomes (i.e., 30% with molluscs *Octopus bimaculoides,* GenBank accession no. XP014770880.1 and *Crassostrea gigas*, GenBank accession no. XP011422119.1, 40% with annelids *Helobdella robusta,* GenBank accession no. XP009025691.1) and 26% with TLR4 from mouse (GenBank accession no. AAF05316.1) and from human (GenBank accession no. AAF05316.1). Moreover, the TLR4 sequence from *H. verbana* had an identity of 85% with TLR1 from the closely related species *H. medicinalis* (GenBank accession no. ADK94453.1, Fig. c in supplementary information S1) and of 23% with TLR22 of fish (*Sparus aurata*, GenBank accession no. CDK37745.1). Based on already published data (Schikorski et al. 2009) demonstrated a crucial role of *Hm*TLR1 in immune responses developed by the leech nervous CNS, here we investigated the direct relationship between *Hv*TLR4 and the peripheral leech immune response induced by bacterial infection or by stimulation with endogenous molecules, such cytokines. In particular, to assess the effects of LPS or the recombinant protein r*Hm*AIF-1 in inducing TLR4 expression in the peripheral leech immune cells, we injected LPS or r*Hm*AIF-1 in the leech body wall. Leeches treated with PBS were employed as controls. TLR4-like expression in the activated and migrating immune cells was assessed by experiments on cryosections from unlesioned, PBS, r*Hm*AIF-1 or LPS-injected 6 h post-treatment leeches using an anti-human polyclonal antibody recognizing an epitope corresponding to amino acids 242–321 mapping to an internal region of TLR4 of human origin (Fig. d in supplementary information S1).

Our data showed that the anti-TLR4 antibody positively reacted with a protein expressed at a low level in the body wall of unlesioned leeches (Fig. 1a). This factor was mainly



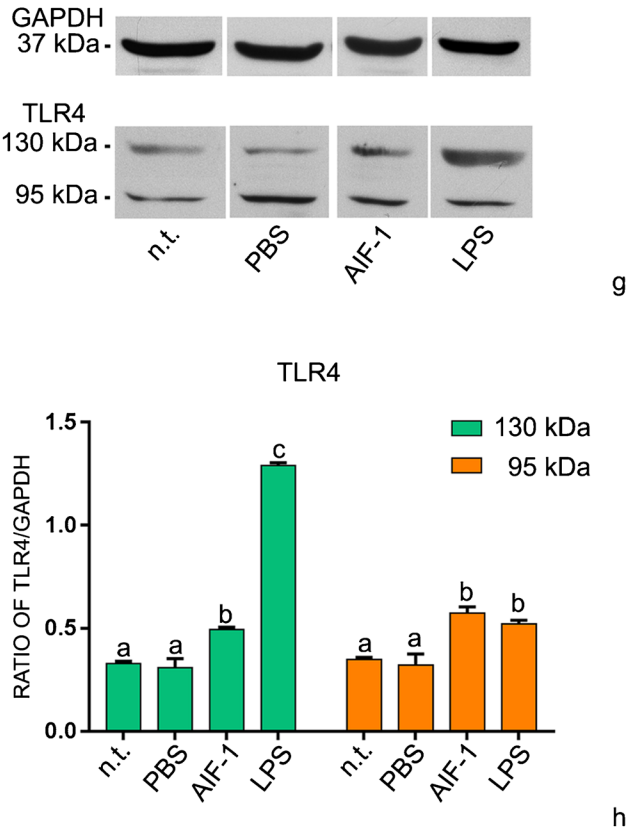


Fig. 1 TLR4 immunolocalization and Western blot assay. Only a few TLR4^{+} cells are detectable in untreated (**a**) and PBS injected samples (**b**) while the number of these cells increases in r*Hm*AIF-1 and LPS-challenged samples (**c**, **d**). No signal is detected in negative control experiments in which the primary antibody is omitted (**e**) or substituted by preimmune serum (**f**). Nuclei are counterstained in blue with DAPI. Bars in **a**–**f**, 100 μm. e epithelium, m muscle fibers. Western blot analysis shows the two immunoreactive products of 130 kDa and 95 kDa (**g**). n.t. untreated animals. GAPDH is used as internal reference. A representative Western blot from one of three independent experiments is shown for both antibodies. The individual signals from each lane have been cropped (square) from larger digital images, which are available as Supplementary Information (Fig. S2). The graph illustrates immunoblot quantification (**h**). Statistical differences have been calculated by factorial ANOVA followed by Tukey's post hoc test; vertical bars denote standard deviation; different letters indicate statistically significant differences ($p < 0.01$)

expressed in cells located in the connective tissue underlying the epithelium and surrounding the fields of muscle fibers.

A similar pattern was observed in samples analyzed 6 h after PBS treatment (Fig. 1b). Interestingly, 6 h after r*Hm*AIF-1 (Fig. 1c) or LPS (Fig. 1d) injection, numerous cells were clearly recognizable in the challenged area.

These migrating cells positively reacted with the anti-TLR4 antibody and were mainly localized under the epithelium and among the muscle fiber fields. No signal was detected in negative control experiments, where the primary antibody was omitted or substituted by serum preimmune and sections were incubated only with the secondary antibody (Fig. 1e, f).

The expression profile of TLR4-like was analyzed by Western blot assay. Immunoblot analysis on body wall protein extracts confirmed the presence of immunoreactive products at about 130 and 95 kDa (Fig. 1f). GAPDH was used as an internal reference and band intensity appeared to be homogeneously distributed in the loaded samples (Fig. 1f). In comparison with basal expression or after PBS injection, stimulation with r*Hm*AIF-1 or LPS significantly induced the amount of the 130 kDa TLR4-like form, more evidently, after LPS challenge (Fig. 1g).

TNF-α-like molecule expression in unlesioned and challenged leeches

Since one of the final targets of the TLR4 signaling pathway is TNF-α, this marker was used to evaluate the activation of the pro-inflammatory response. Immunocytochemical experiments with anti-TNF-α antibody showed no signal in untreated and PBS-treated leeches (Fig. 2a, b) while a spread signal of a TNF-α-like-molecule was detectable in r*Hm*AIF-1 and LPS injected samples (Fig. 2c, d). The negative controls, where the primary antibody was omitted or substituted by

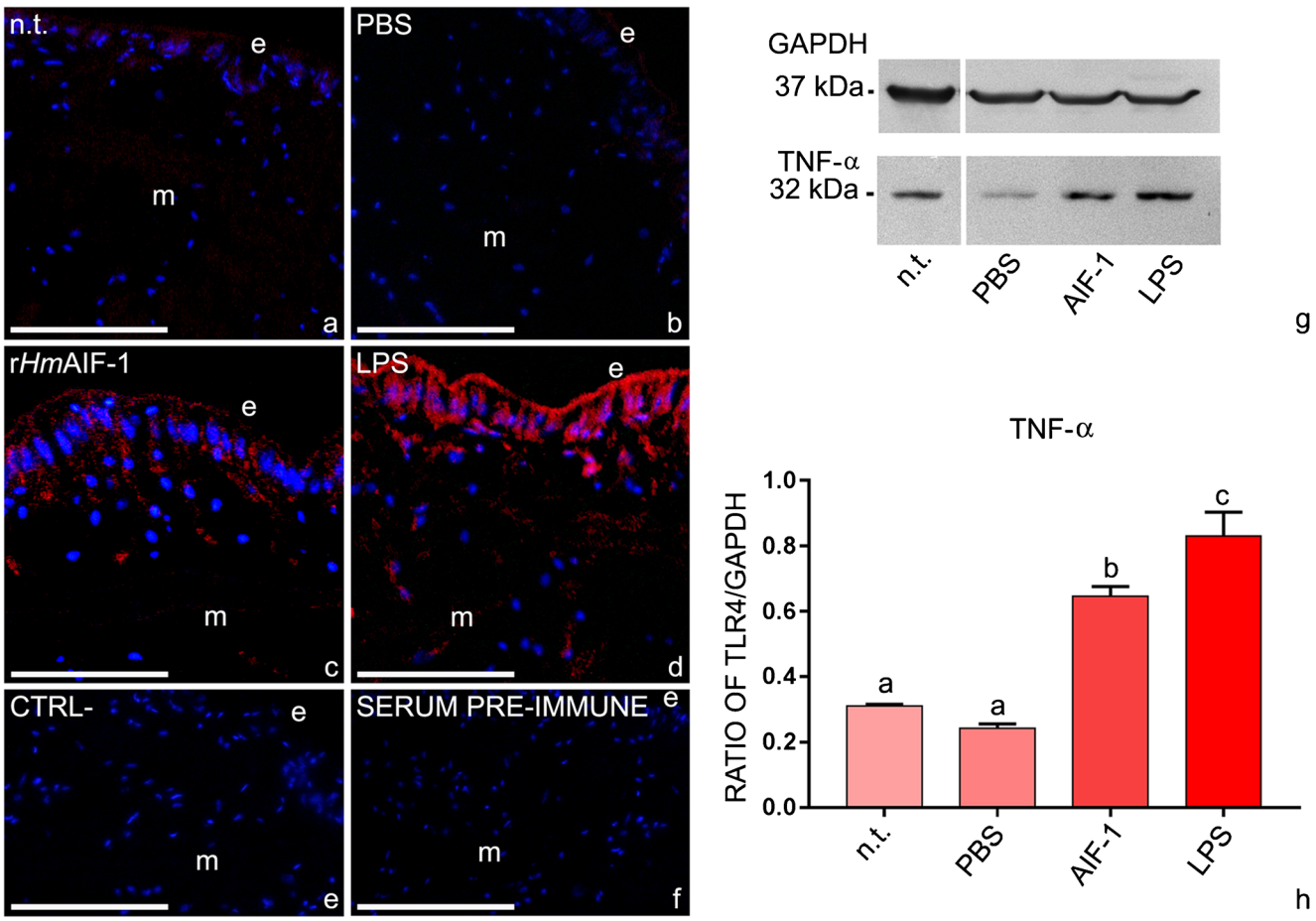


Fig. 2 TNF-α immunolocalization and Western blot assay. Compared to untreated (**a**) and PBS injected animals (**b**), where the TNF-α signal is practically absent, the positivity for this antibody is clearly observable in r*Hm*AIF-1 (**c**) and LPS injected samples (**d**). The control experiments, where the primary antibody is omitted (**e**) or substituted by preimmune serum (**f**), were negative. Nuclei are stained with DAPI (blue). Bars in **a**–**f**, 100 μm. e epithelium, m muscle fibers. In the Western blot assay (**g**) GAPDH is used as internal reference. A representative Western blot from one of three independent experiments is shown for both antibodies. The individual signals from each lane have been cropped (square) from larger digital images, which are available as Supplementary Information (Fig. S2). The graph illustrates TNF-α expression levels after the different treatments (**h**). n.t. untreated animals. Statistical differences have been calculated by One-way ANOVA followed by Tukey's post hoc test; vertical bars denote standard deviation; different letters indicate statistically significant differences ($p < 0.01$)

serum pre-immune, were completely negative (Fig. 2e, f). Western blot analysis evinced the presence of an immunoreactive product of approximately 30 kDa (corresponding to the expected molecular weights of the homologous 26 kDa isoform of human) that was compared to the internal reference GAPDH (Fig. 2f). The TNF-α-like molecule expression was notably increased after LPS challenge, thus confirming the pro-inflammatory effect of the bacterial product (Fig. 2g).

Characterization of the cell types recruited at the site of LPS injection and cross-reacting with anti-TLR4 antibodies

To characterize the cells expressing TLR4-like molecules, we performed both a morphological analysis with light and electron microscopes and immunofluorescence assays. Our morphological data confirmed that 6 h after r*Hm*AIF-1 or LPS injection, the leech body wall was infiltrated by cells showing the typical features of macrophage-like cells (Fig. 3), vasofibrous tissue and granulocytes of type I, the last deriving from the vasofibrous tissue (Grimaldi et al. 2011) (Figs. 4 and 5).

Macrophages (Fig. 3a–d) were characterized by a ruffled surface, due to the presence of pseudopodia and a high ratio of nucleus to cytoplasm. These cells, as demonstrated by double immunofluorescence staining, were *Hm*AIF-1$^+$/TLR4$^+$ (Fig. 3c), *Hm*AIF-1$^+$/CD14$^+$ (Fig. 3d) and TLR4$^+$/CD14$^+$ (Fig. 3e). No signal was detected in negative controls (Fig. 3f, g).

In unstimulated leeches, vasofibrous tissue was poorly represented in the loose connective tissue surrounding the muscle fibers (Fig. 4a, b). It was composed by vasocentral cells, with a cytoplasm containing a few large granules, surrounded by vasofibrous cells, showing a cytoplasm rich in small, highly electron-dense granules (Fig. 4b). In this tissue, only the vasofibrous cells were CD11b$^+$/ TLR4$^+$ (Fig. 4c), CD11b$^+$/ CD14$^+$ (Fig. 4d) and TRL4$^+$/CD14$^+$ (Fig. 4e), while the associated vasocentral cells did not express these markers. No signal was detected in negative controls (Figs. 4f, g).

After LPS stimulation, the vasofibrous cells detach from vasocentral cells and differentiate into type I granulocytes

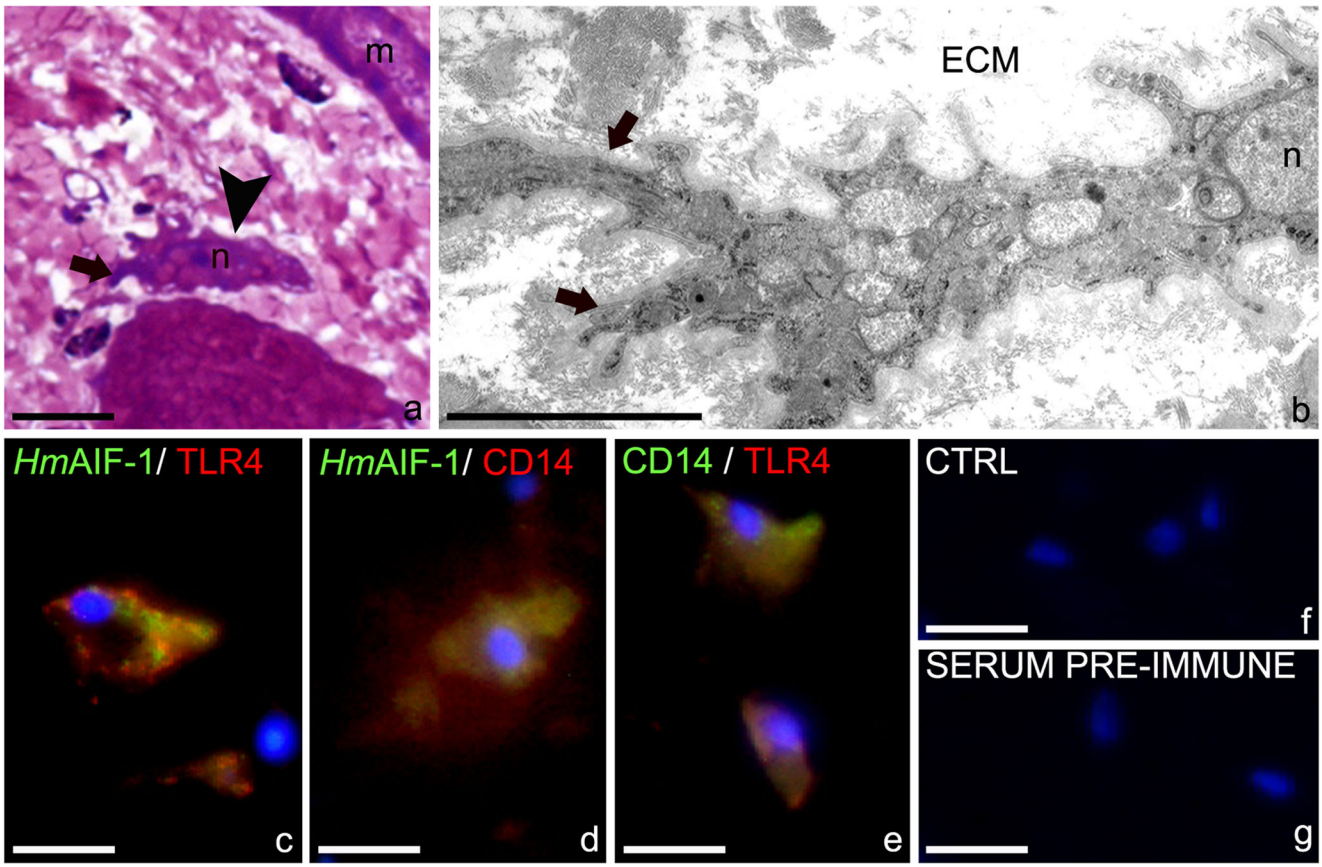


Fig. 3 Macrophages: morphological and immunocytochemical characterization. Leech macrophage-like cells at optical microscopy (arrowhead in **a**) and TEM (**b**) showing pseudopodia (arrows), typical of migrating cells. Double immunolocalization with r*Hm*AIF-1 (green), specific macrophagic marker, coupled with anti-TLR4 (red) (**c**) and anti-CD14 (red) (**d**) antibodies. Double labelling of TLR4 (red) and CD14 (green) (**e**). The merge results in yellow. The control experiments, in which the primary antibody is omitted (**f**) or substituted by preimmune serum (**g**), were negative. Nuclei are counterstained with DAPI (blue). Bars in **a**, **c**–**g**, 10 μm; bar in **b**, 2 μm. m muscle fiber, ECM extracellular matrix, n nuclei

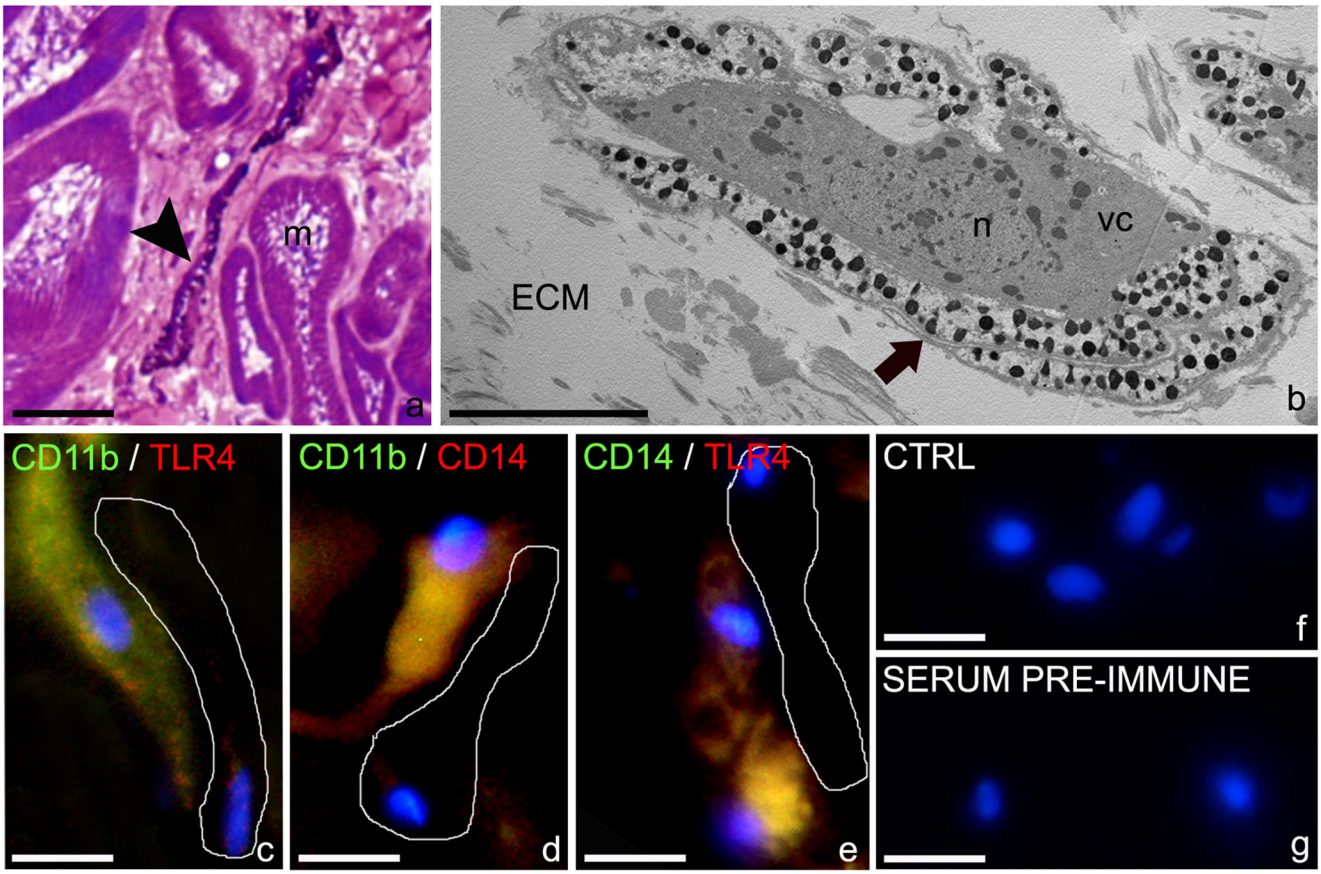


Fig. 4 Vasofibrous tissue cells: morphological and immunocytochemical characterization. Details of vasofibrous tissue at optical microscopy (arrowhead in **a**) localized in the extracellular matrix (ECM) surrounding muscle fibers (m). TEM image (**b**) showing a vasocentral cell (vc), with a cytoplasm containing a few large granules surrounded by a vasofibrous cell (arrow) characterized by small and highly electron-dense granules. Double labelling with CD11b (green) coupled with anti-TLR4 (red) (**c**) and anti-CD14 (red) (**d**). Double localization of TLR4 (red) and CD14 (green) (**e**). Only the vasofibrous cells (in yellow) expressed these markers, while the vasocentral cells (encircled) were completely negative. In the control experiment, where the primary antibody is omitted (**f**) or substituted by preimmune serum (**g**), no signal was detected. Nuclei are counterstained in blue with DAPI. Bars in **a**, **c**–**g**, 10 μm; bar in **b**, 5 μm. n nucleus

(Fig. 5a, b). Indeed, double-labelling experiments clearly demonstrated that mature type I granulocytes maintained the same markers observed for the vasofibrous cells. In fact, type I granulocytes were $CD11b^+/TLR4^+$ (Fig. 5c), $CD11b^+/CD14^+$ (Fig. 5d) and $TRL4^+/CD14^+$ (Fig. 5e). No signal was detected in negative controls (Fig. 5f, g).

Functional study

To elucidate whether the selective TLR4 antagonist CyP might antagonize activation of the pro-inflammatory response induced by LPS, we evaluated TNF-α-like-molecule expression in leeches treated with both LPS and CyP. As controls, we employed leeches treated with LTA, a gram-positive product known to induce a TLR4-independent pro-inflammatory response. In not treated as well as in CyP-injected leeches, a few cells expressing the TNF-α-like molecule were detectable underneath the epithelium, where the injection was performed (Fig. 6a, b). On the contrary, after LPS treatment, the signal was markedly higher (Fig. 6d) and numerous cells positive for the TNF-α-like-molecule and migrating towards the challenged area were detectable among the muscle fibers and underneath the epithelium. A similar result was obtained in positive control samples treated with LTA alone (Fig. 6f). In CyP/LPS-injected leeches, the TNF-α-like-molecule signal was significantly downregulated compared to those treated with LPS alone (Fig. 6c). Interestingly, leeches treated with CyP/LTA (Fig. 6e) did not show any inhibition of the TNF-α-like molecule signal, thus suggesting that the pro-inflammatory response induced by LPS and LTA involves different signaling pathways also in annelids. Western blot data (Fig. 6g) confirmed the trend of the TNF-α-like molecule expression (Fig. 6h).

The in vitro study confirmed the results obtained in vivo. In detail, a low positivity for the TNF-α-like molecule was found in not treated as well as for CyP or CyP/LPS-treated macrophages (Fig. 7a–s), while in LPS, CyP/LTA and LTA macrophages, the signal was much more evident (Fig. 7d–f) as confirmed by fluorescence quantification (Fig. 7g).

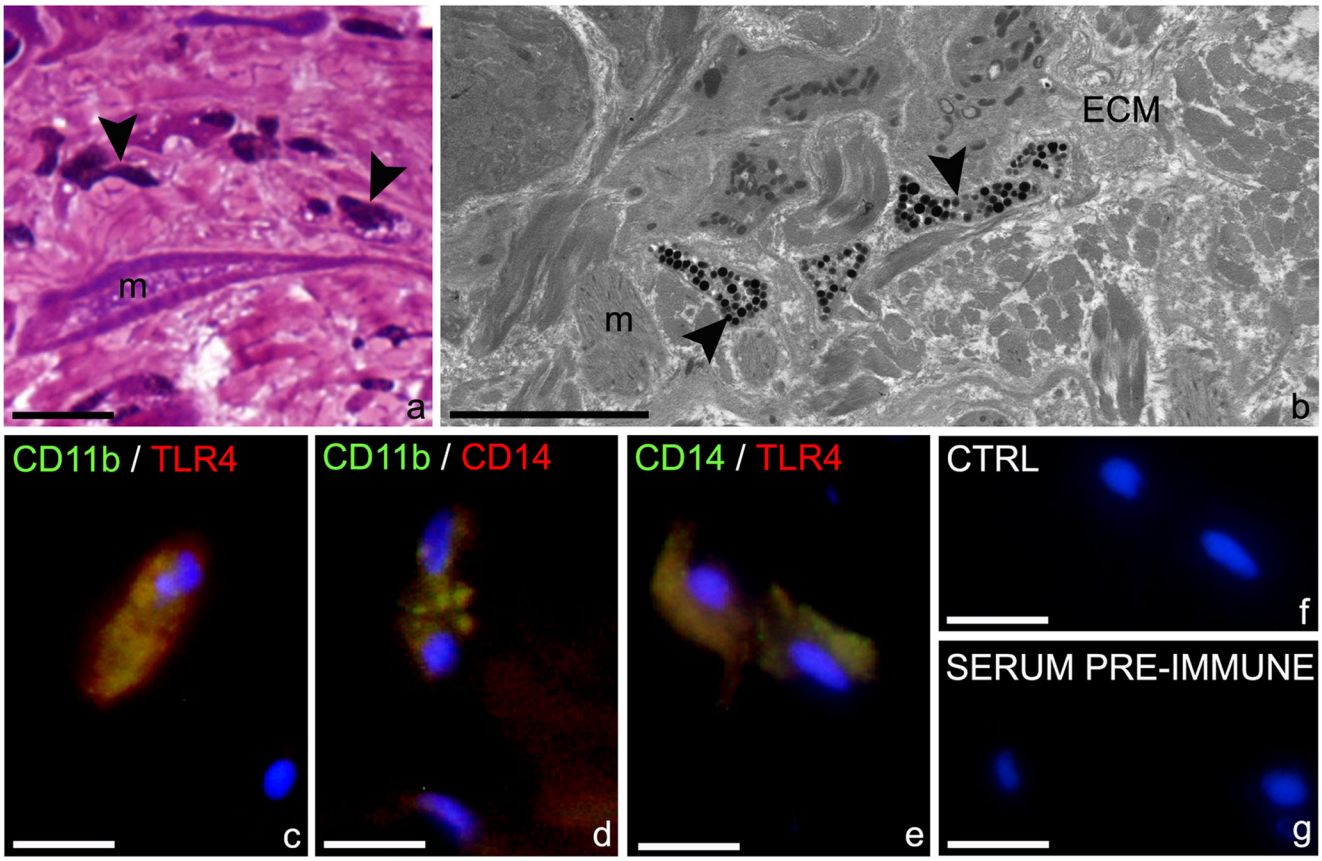


Fig. 5 Granulocytes: morphological and immunocytochemical characterization. Type I granulocytes at optical microscopy (arrowheads in **a**) and TEM (arrowheads in **b**) after LPS stimulation. As well as vasofibrous cells, CD11b^{+} (green) granulocytes also express TLR4 (red in **c**) and CD14 (red in **d**). Double labelling with TLR4 (red) and CD14 (green) (**e**). No signal was detected in the control experiment, in which the primary antibody is omitted (**f**) or substituted by preimmune serum (**g**). Nuclei are counterstained with DAPI (blue). Bars in **a**, **c**–**g** 10 μm; bar in **b** 5 μm. m muscle fiber, ECM extracellular matrix

Discussion

The primary goal of this study is to demonstrate that the perception of both PAMPs and DAMPs in leech implicates, like in mammals, the specific sensing receptor TLRs. Moreover, with our findings, we suggest the medicinal leech as a powerful and simpler model for better elucidating the function and regulation of TLRs in the peripheral innate immune response. Within this context, our previous observations showed that, in the medical leech, peripheral immune cells can discriminate the pathogenic components (Schorn et al. 2015a) and secrete antibacterial molecules (Baranzini et al. 2018). These molecules are involved in immune cells recruitment and activation, antimicrobial activity and in regenerative processes (Schorn et al. 2015a, b; Baranzini et al. 2017). Here, we provide further experimental evidence showing an upregulation of a TLR4-like molecule in the stimulated leech body wall and supporting the hypothesis of its critical role in orchestrating a response against pathogen infection. The sequence of TLR4 from *H. verbana* was retrieved by in silico analysis of all its transcriptome (Northcutt et al. 2018). Strikingly, phylogenetic studies show that although there is significant conservation between *Hv*TLR4 and TLR4 sequences from other protostomes and mammals, TLR4 from *H. verbana* is more closely related to TLR1 from its related species *H. medicinalis*. This observation seems to reinforce the hypothesis that, like *Hm*TLR1 in a neural context (Schikorski et al. 2009), *Hv*TLR4 as well could be involved in regulating the peripheral innate immune response. However, differently from *Hm*TLR1, mainly implicated in GRAM^{+} recognition, *Hv*TLR4 should be involved in GRAM^{-} recognition, like its mammalian counterpart. The possible functional homology between leech and mammal TLR4 was confirmed by analyzing *Hv*TLR4 expression levels by immunofluorescence and immunoblot assays in the body wall from LPS or r*Hm*AIF-1-injected leeches. These two molecules have been chosen since they are two well-known strong stimulators of leeches' innate immune response and promote a massive recruitment and accumulation of leukocytes at the microbial challenge or lesioned site (de Eguileor et al. 2000a; Schorn et al. 2015b). Subsequently, in vivo and in vitro functional experiments using the selective TLR4 antagonist CyP definitively confirmed *Hv*TLR4 specificity in LPS recognition and in activating the TNF-α expression, the final target of its signaling pathway.

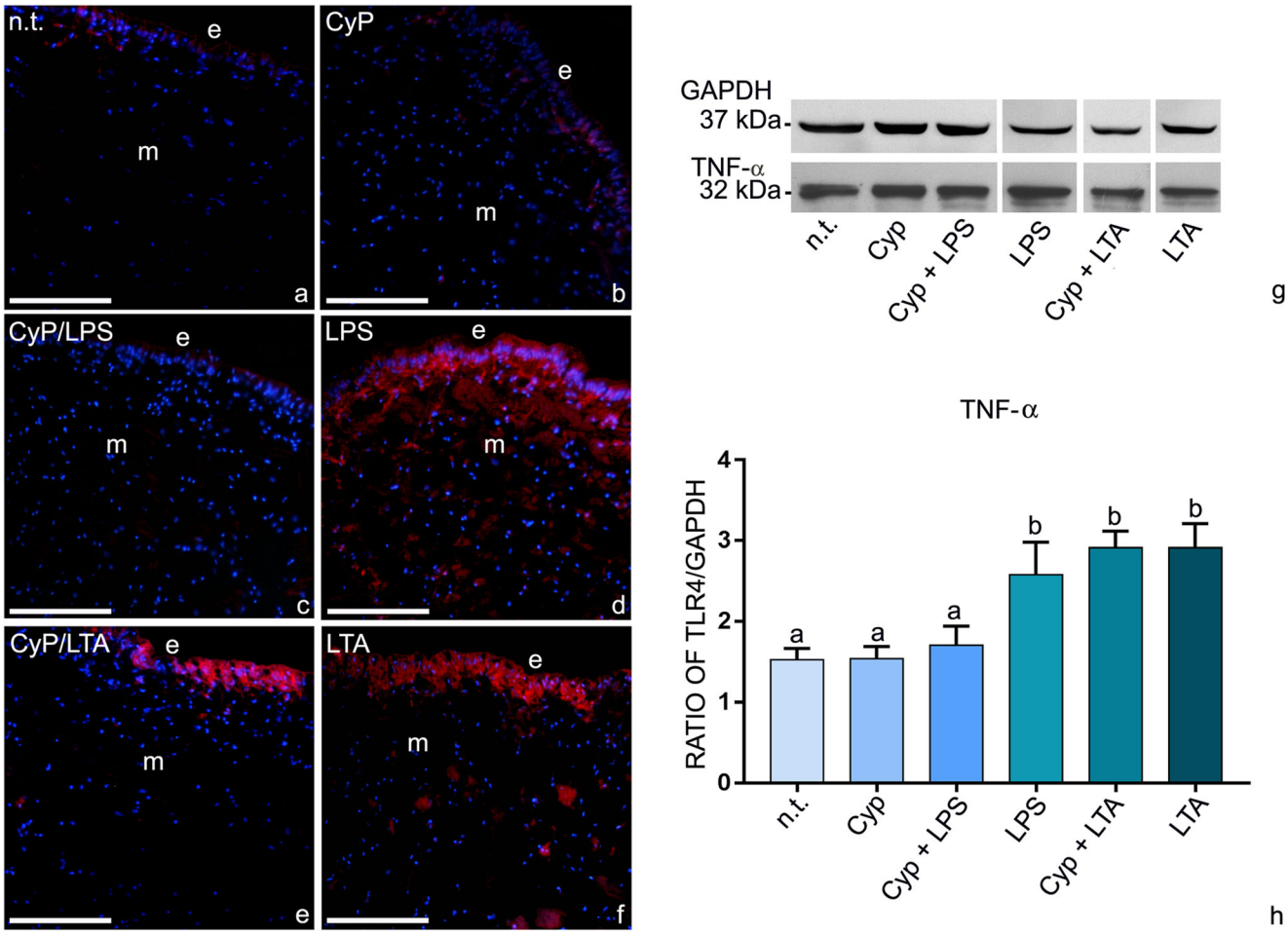


Fig. 6 Functional in vivo study. Immunofluorescence images showing anti-TNF-α labelling (red) of untreated (**a**) and animals injected with CyP (**b**), Cyp/LPS (**c**), LPS (**d**), Cyp/LTA (**e**) and LTA (**f**). Nuclei are counterstained with DAPI (blue). Bars in **a**–**f** 100 μm. Western blot assay showing a 30 kDa product for TNF-α and the internal reference used for normalization (GAPDH) (**g**). A representative Western blot from one of three independent experiments is shown for both antibodies. The individual signals from each lane have been cropped (square) from larger digital images that are available as Supplementary Information (S1). The graph represents immunoblot quantification (**h**). n.t. untreated animals. e epithelium, m muscle fibers. Statistical differences have been calculated by one-way ANOVA followed by Tukey's post hoc test; vertical bars denote standard deviation; different letters indicate statistically significant differences ($p < 0.01$)

TLR4-like receptor and TNF-α-like molecule expression in leech immune cells

First tissue localization of TLR4-like protein was evaluated by immunocytochemical techniques in leeches unlesioned and stimulated with LPS or with r*Hm*AIF-1. Our data suggest a constitutive expression of a TLR4-like protein in unlesioned leeches and a massive increase of this marker after challenge with r*Hm*AIF-1 or LPS. In PBS-injected leeches, the expression of this receptor remains at a basal level, indicating that the mechanical stress induced by the injection or the vehicle solution alone does not exert a significant effect. Interestingly, Western blot analysis highlights that the polyclonal antibody anti-TLR4 recognizes two different bands with molecular weights of about 130 and 95 kDa, respectively. Interestingly, two isoforms of 130 and 110 kDa have also been described in vertebrates (Ohnishi et al. 2003). The 130 kDa one corresponds to the glycosylate TLR4 mature form and it is expressed on the cell surface (da Silva Correia and Ulevitch 2001). N-linked glycosylation is a major form of co-translational modification in eukaryotic protein synthesis and this type of modification is necessary for TLR4 maturation and its translocation to the cell surface. Otherwise, the 110 kDa form, being partially glycosylated, is unable to translocate to the membrane surface. Our data suggest that in the medicinal leech, like in mammals, two forms of TLR4-like molecules exist. At present, we do not know whether the two TLR4-like forms in leech correspond to different glycosylation patterns and if the high molecular weight form is the only one expressed on the cell membrane. Further analyses are needed to clarify this point; indeed, the finding of polyclonal antibodies cross-reacting with TLR4-like molecules in leech, will be of great value to deepen the mechanism of action of these isoforms in leech. Following LPS or r*Hm*AIF-1

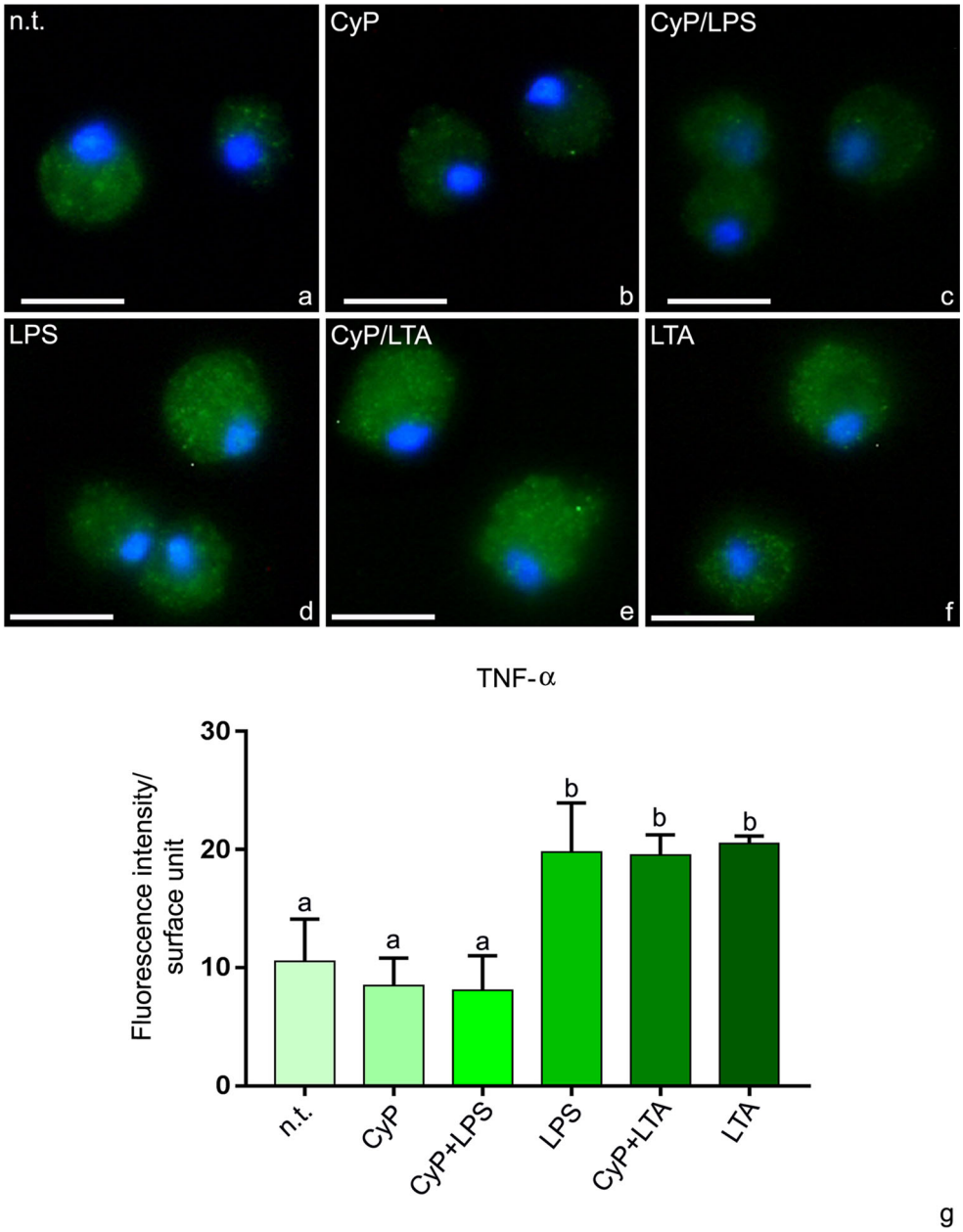


Fig. 7 Functional in vitro study. Fluorescence images showing anti-TNF-α labelling of not treated (**a**) and macrophages treated for 1 h with CyP (**b**), CyP/LPS (**c**), LPS (**d**), CyP/LTA (**e**) and LTA (**f**). Nuclei are counterstained with DAPI (blue). Objective × 100, bars in **a**–**f** 10 μm. Graph showing fluorescence intensity for surface unit (**g**). n.t. untreated animals. Statistical differences have been calculated by one-way ANOVA followed by Tukey's post hoc test; vertical bars denote standard deviation; different letters indicate statistically significant differences ($p < 0.01$)

stimulation, we also found an increase of a TNF-α-like-molecule, the typical pro-inflammatory cytokine released after TLR4 challenge by LPS. In addition, double immunolocalization experiments using different markers, such as the antibody anti *Hm*AIF-1 for macrophages and the antibody anti-CD11b for granulocytes (Girardello et al. 2015a; Schorn et al. 2015b; Grimaldi 2016) confirmed that TLR4-like and its co-receptor CD14 are expressed in these innate immune cells. These results and previous observations, indicating that annelids possess a homolog of LBP (lipopolysaccharide-binding protein) (Macagno et al. 2010), suggest that a machinery for the transduction of LPS signaling similar to that observed in vertebrates is active in leech. Interestingly, we also found a co-expression of CD11b and TLR4-like molecules in granulocytes. These data are in accordance with other authors' findings in vertebrates (Ling et al. 2014) and suggest a possible role of CD11b in TLR4-induced signaling regulation.

Functional in vitro and in vivo studies: TLR4 pathway was blocked by using the selective TLR4 antagonist CyP

Functional analyses performed both in vivo and in vitro confirmed that in leech the cyanobacterial TLR4 antagonist CyP could significantly inhibit the LPS-activated pro-inflammatory response. Indeed, the effect of CyP was highly specific. A significant inhibition of the TNF-α-like molecule expression was observed in leeches or cell cultures treated with LPS in the

presence of CyP. Conversely, no effect was observed in experiments employing LTA as a pro-inflammatory stimulus. It is well known that LTA, a product of gram-positive bacteria, activates a pro-inflammatory response mediated by TLR2 in vertebrates (Trianiafilou et al. 2004). Our results suggest that two independent activation pathways involving recognition respectively of LPS and LTA are active in leech and similar to what is observed in mammals (Macagno et al. 2006; Gemma et al. 2016), CyP is capable of downregulating the LPS pathway.

In conclusion, our data suggest that a TLR4-like molecule is expressed by macrophages and granulocytes of leeches and is up-regulated by LPS. After LPS triggering, there is an induction of a pro-inflammatory response in innate immune cells, as evidenced by the increase of a TNF-α-like molecule expression, which can be specifically inhibited by the presence of TLR4 antagonist CyP. These results support the data obtained by other investigators showing the existence of TLRs in the genome of medicinal leech and highlight the presence of a functional TLR4-like receptor mediating pro-inflammatory response. Moreover, our data clearly showed for the first time that not only LPS but also endogenous DAMPs (i.e., the cytokine AIF-1), mainly produced after injury and grafts (Schorn et al. 2015b), are linked to the activation of a TLR4 pathway. These data not only confirm the existence in leech of a link between TLRs and chemotaxis factors both in peripheral and in neuroimmune systems (Schikorski et al. 2009) but also suggest that TLR1 and TLR4 could play a conserved functional role in regulating the inflammatory response.

Taken together, our results confirm once again that the medicinal leech, well known as one of the few invertebrates highly employed in surgical replantations and therapeutic applications for human disease (Adams 1988; Heckmann et al. 2005; Porshinsky et al. 2011), could represent an interesting and simple new alternative model to deciphering several physiological and cellular mechanisms in more complex animals including humans.

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Author contributions AG, MM and CR conceived and designed the experiments. RG and NB performed the experiments. AG and RG analyzed the data and wrote the manuscript. GT and MdeE provided expertise for TEM and imaging and contributed reagents/materials/analysis tools. All authors critically reviewed the manuscript.

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Compliance with ethical standards

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

References

Adams SL (1988) The medicinal leech. A page from the annelids of internal medicine. Ann Intern Med 109:399–405

Baranzini N, Pedrini E, Girardello R et al (2017) Human recombinant RNASET2-induced inflammatory response and connective tissue remodeling in the medicinal leech. Cell Tissue Res 368:337–351. https://doi.org/10.1007/s00441-016-2557-9

Baranzini N, Monti L, Vanotti M et al (2018) AIF-1 and RNASET2 play complementary roles in the innate immune response of medicinal leech. J Innate Immun:1–18. https://doi.org/10.1159/000493804

Chen G, Zhuchenko O, Kuspa A (2007) Immune-like phagocyte activity in the social amoeba. Science 317:678–681. https://doi.org/10.1126/science.1143991

Coscia M, Giacomelli S, Oreste U (2011) Toll-like receptors: an overview from invertebrates to vertebrates. ISJ 8:210–226

da Silva Correia J, Ulevitch RJ (2001) MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor*. https://doi.org/10.1074/jbc.M109910200

Davidson CR, Best NM, Francis JW et al (2008) Toll-like receptor genes (TLRs) from Capitella capitata and Helobdella robusta (Annelida). Dev Comp Immunol 32:608–612. https://doi.org/10.1016/j.dci.2007.11.004

de Eguileor M, Grimaldi A, Tettamanti G et al (2000a) Different types of response to foreign antigens by leech leukocytes. Tissue Cell 32:40–48

de Eguileor M, Grimaldi A, Tettamanti G, et al (2000b) Lipopolysaccharide-dependent induction of leech leukocytes that cross-react with vertebrate cellular differentiation markers. doi: https://doi.org/10.1054/tice.2000.0132

Drago F, Sautière PE, Le Marrec-Croq F et al (2014) Microglia of medicinal leech (Hirudo medicinalis) express a specific activation marker homologous to vertebrate ionized calcium-binding adapter molecule 1 (Iba1/alias aif-1). Dev Neurobiol 74:987–1001. https://doi.org/10.1002/dneu.22179

Gemma S, Molteni M, Rossetti C (2016) Lipopolysaccharides in cyanobacteria: a brief overview. Adv Microbiol 06:391–397. https://doi.org/10.4236/aim.2016.65038

Girardello R, Drago F, De Eguileor M et al (2015a) Cytokine impregnated biomatrix: a new tool to study multi-wall carbon nanotubes effects on invertebrate immune cells. J Nanomed Nanotechnol 6. https://doi.org/10.4172/2157-7439.1000323

Girardello R, Tasselli S, Baranzini N et al (2015b) Effects of carbon nanotube environmental dispersion on an aquatic invertebrate, Hirudo medicinalis. PLoS One 10:e0144361. https://doi.org/10.1371/journal.pone.0144361

Girardello R, Baranzini N, Tettamanti G et al (2017) Cellular responses induced by multi-walled carbon nanotubes: in vivo and in vitro studies on the medicinal leech macrophages. Sci Rep 7:8871. https://doi.org/10.1038/s41598-017-09011-9

Grimaldi A (2016) Origin and fate of hematopoietic stem precursor cells in the leech Hirudo medicinalis. Invertebr Surviv J 13:257–268

Grimaldi A, Tettamanti G, Perletti G et al (2006) Hematopoietic cell formation in leech wound healing. Curr Pharm Des 12:3033–3041

Grimaldi A, Banfi S, Vizioli J et al (2011) Cytokine loaded biopolymers as a novel strategy to study stem cells during wound-healing processes. Macromol Biosci 11:1008–1019. https://doi.org/10.1002/mabi.201000452

Grimaldi A, Tettamanti G, de Eguileor M (2018) Annelida: Hirudinea (leeches): heterogeneity in leech immune responses. In: Advances in comparative immunology. Springer International Publishing, Cham, pp 173–191

Heckmann JG, Dutsch M, Neundorfer B et al (2005) Leech therapy in the treatment of median nerve compression due to forearm haematoma. J Neurol Neurosurg Psychiatry 76:1465

Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat Immunol 11:373–384. https://doi.org/10.1038/ni.1863

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685

Ling GS, Bennett J, Woollard KJ et al (2014) Integrin CD11b positively regulates TLR4-induced signalling pathways in dendritic cells but not in macrophages. Nat Commun 5:1–12. https://doi.org/10.1038/ncomms4039

Macagno A, Molteni M, Rinaldi A et al (2006) A cyanobacterial LPS antagonist prevents endotoxin shock and blocks sustained TLR4 stimulation required for cytokine expression. J Exp Med 203:1481–1492. https://doi.org/10.1084/jem.20060136

Macagno ER, Gaasterland T, Edsall L et al (2010) Construction of a medicinal leech transcriptome database and its application to the identification of leech homologs of neural and innate immune genes. BMC Genomics 11:407. https://doi.org/10.1186/1471-2164-11-407

Mahla RS, Reddy MC, Prasad DVR, Kumar H (2013) Sweeten PAMPs: role of sugar complexed PAMPs in innate immunity and vaccine biology. Front Immunol 4:248. https://doi.org/10.3389/fimmu.2013.00248

Mann KH, Kerkut GA (1962) Leeches (Hirudinea) : their structure, physiology, ecology and embryology, with an appendix on the systematics of marine leeches. Elsevier Science

Molteni M, Gemma S, Rossetti C (2016) The role of toll-like receptor 4 in infectious and noninfectious inflammation. Mediat Inflamm 2016:1–9. https://doi.org/10.1155/2016/6978936

Moore RD, Mumaw V, Schoenberg MD (1960) Optical microscopy of ultrathin tissue sections. J Ultrastruct Res 4:113–116. https://doi.org/10.1016/S0022-5320(60)90047-2

Northcutt AJ, Fischer EK, Puhl JG et al (2018) An annotated CNS transcriptome of the medicinal leech, Hirudo verbana: De novo sequencing to characterize genes associated with nervous system activity. PLoS One 13:e0201206. https://doi.org/10.1371/journal.pone.0201206

Ohnishi T, Muroi M, Tanamoto K-I (2003) MD-2 is necessary for the toll-like receptor 4 protein to undergo glycosylation essential for its translocation to the cell surface. Clin Diagn Lab Immunol 10:405–410. https://doi.org/10.1128/CDLI.10.3.405-410.2003

Porshinsky BS, Saha S, Grossman MD et al (2011) Clinical uses of the medicinal leech: a practical review. J Postgrad Med 57:65–71. https://doi.org/10.4103/0022-3859.74297

Rast JP, Smith LC, Loza-Coll M et al (2006) Genomic insights into the immune system of the sea urchin. Science 314:952–956. https://doi.org/10.1126/science.1134301

Rodet F, Tasiemski A, Boidin-Wichlacz C et al (2015) Hm-MyD88 and Hm-SARM: two key regulators of the neuroimmune system and neural repair in the medicinal leech. Sci Rep 5:1–13. https://doi.org/10.1038/srep09624

Sawyer RT (1986) Leech biology and behaviour. Clarendon Press

Schikorski D, Cuvillier-hot V, Slomianny C et al (2009) Deciphering the immune function and regulation by a TLR of the cytokine EMAPII in the lesioned central nervous system using a leech model. J Immunol 183:7119–7128. https://doi.org/10.4049/jimmunol.0900538

Schorn T, Drago F, de Eguileor M et al (2015a) The allograft inflammatory Factor-1 (AIF-1) homologous in Hirudo medicinalis (medicinal leech) is involved in immune response during wound healing and graft rejection processes. Invertebr Surviv J 1:129–141

Schorn T, Drago F, Tettamanti G et al (2015b) Homolog of allograft inflammatory factor-1 induces macrophage migration during innate immune response in leech. Cell Tissue Res 359:853–864. https://doi.org/10.1007/s00441-014-2054-y

Tasiemski A, Salzet M (2017) Neuro-immune lessons from an annelid: the medicinal leech. Dev Comp Immunol 66:33–42. https://doi.org/10.1016/j.dci.2016.06.026

Thorgersen EB, Macagno A, Rossetti C, Mollnes TE (2008) Cyanobacterial LPS antagonist (CyP)—a novel and efficient inhibitor of Escherichia coli LPS-induced cytokine response in the pig. Mol Immunol 45:3553–3557. https://doi.org/10.1016/j.molimm.2008.05.005

Trianiafilou M, Manukyan M, Mackie A et al (2004) Lipoteichoic acid and Toll-like receptor 2 internalization and targeting to the Golgi are lipid raft-dependent. J Biol Chem 279:40882–40889. https://doi.org/10.1074/jbc.M400466200

Wakabayashi Y, Kobayashi M, Akashi-Takamura S et al (2006) A protein associated with toll-like receptor 4 (PRAT4A) regulates cell surface expression of TLR4. J Immunol 177:1772–1779. https://doi.org/10.4049/jimmunol.177.3.1772