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RESEARCH ARTICLE

Morphological and functional characterization of leech circulating blood cells: role in immunity and neural repair

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Abstract Unlike most invertebrates, annelids possess a closed vascular system distinct from the coelomic liquid. The morphology and the function of leech blood cells are reported here. We have demonstrated the presence of a unique cell type which participates in various immune processes. In contrast to the mammalian spinal cord, the leech CNS is able to regenerate and restore function after injury. The close contact of the blood with the nerve cord also led us to explore the participation of blood in neural repair. Our data evidenced that, in addition to exerting peripheral immune functions, leech blood optimizes CNS neural repair through the release of neurotrophic substances. Circulating blood cells also appeared able to infiltrate the injured CNS where, in conjunction with microglia, they limit the formation of a scar. In mammals, CNS injury leads to the generation of a glial scar that blocks the mechanism of regeneration by preventing axonal regrowth. The results presented here constitute the first description of neuroimmune functions of invertebrate blood cells. Understanding the basic function of the peripheral circulating cells and their interactions with lesioned CNS in the leech would allow us to acquire insights into the complexity of the neuroimmune response of the injured mammalian brain.

Keywords Blood · Annelid · Invertebrate · Immunity · Neural repair · Central nervous system · Antimicrobial peptide

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Introduction

Most invertebrates such as the abundantly studied arthropods and molluscs present an open circulatory system. In these species, cells of the coelome called coelomocytes migrate into the blood vessel lumen and, reciprocally, cells of the vessel lumen called hemocytes migrate into the coelome [1]. It has been suggested that hemocytes and coelomocytes represent a single class of cell [2]. Among invertebrates, annelids are exceptions, as they possess a closed vascular system distinct from the fluid of the coelomic cavities. In ringed worms, the participation of the coelomic fluid in various aspects of the cellular (phagocytosis, encapsulation and cytotoxicity) and humoral (antimicrobial, haemolytic and clotting properties) immunity is abundantly documented [3–6]. However, in contrast to coelomocytes, the morphology and immune functions of annelid blood cells has yet to be described.

Among annelids, the medicinal leech, *Hirudo medicinalis*, presents the original characteristic of having a nerve cord enclosed within the ventral blood sinus. Interestingly,

one of the most striking features of *Hirudo* resides in its ability to regenerate and restore normal CNS functions in response to injury. Indeed, if its nerve cord is cut, axons grow across the lesion and conduction of signals through the damaged region is restored within a few days [7, 8]. Our group has recently evidenced that restoration of CNS functions subsequent to CNS transsection was critically dependent on the co-initiation of an antibacterial response [9]. This immune response is based, amongst other factors, on the production of antimicrobial peptides (AMPs), namely *Hm*-lumbricin and neuromacin. These antibiotic molecules produced by nervous cells (microglia and neurons), in addition to exerting immune properties, appeared to promote neural repair [9].

In mammals, the participation of circulating blood cells in brain immunity is well described even if data remain controversial. For example, neutrophile macrophages or T cells have been suggested to exacerbate axonal injury, demyelination and functional loss of the CNS [10–14]. Conversely, macrophages and T cells have been demonstrated to secrete factors that promote neuroprotection and/or neuroregeneration after spinal cord injury [15–17]. Because of these contrasted immune effects, it is difficult to distinguish the beneficial effects from the deleterious effects associated with the infiltration of blood cells into the mammalian brain.

In this report, we examine the blood cells of *H. medicinalis*. We first provide a morphological characterization of these cells before examining their roles in peripheral and neural immunity. The direct contact between the blood and the CNS also led us to consider a possible implication of this body fluid in the regenerative process of the CNS. Our goal is to acquire insights into the complexity of the neuroimmune response of the mammalian brain by using a simple organism such as the medicinal leech. To our knowledge, this is the first report describing the neuroimmune function of the blood in an invertebrate.

Materials and methods

Animals

Adult *H. medicinalis* were purchased from Ricarimpex (Bordeaux-France) and maintained in autoclaved 1% Instant Ocean (Aquarium Systems), changed daily, for 1 week before starting any experimental procedure.

Collection and treatments of leech nerve cords and blood cells

Leeches were anaesthetized by immersion in 10% ethanol-spring water for 20 min at 4°C. The nerve cords were removed as described previously [9]. Protocols to deplete microglial cells from nerve cords were also reported previously [18]. Blood was collected from the lateral sinus. Collected fluid was centrifuged at 4,000 rpm for 8 min at 4°C. Supernatant (plasma) was then separated from the pellet containing the cells.

For treatment purposes, blood was diluted in vitro in L-15 medium containing a mixture of killed bacteria (Gram-positive *Aeromonas hydrophila* and -negative *Micrococcus nishinomyaensis* 3×10^7 CFU/ml) for different times ($T = 0$, 6, and 24 h) at room temperature. In vivo, bacteria were injected into the blood sinus. Incubations without bacteria were performed in the same conditions as controls. All the steps were performed under sterile conditions.

Microorganisms/parasites

The Gram-positive and Gram-negative bacteria, respectively *M. nishinomiyaenis* and *A. hydrophila*, were isolated from the natural environment of *H. medicinalis*, as previously described [9]. Leeches are sometimes infected by a parasite, which is localized in the muscular tissue, and this parasite is used for the encapsulation observation as described below.

Morphology of the leech blood cells

Fixation and preparation for electron microscopy

The blood cells were collected, and immediately fixed according to the protocol previously described [19]. Cells were dehydrated with ethanol and embedded in Epon (polymerization at 60°C for 48 h). Ultrathin sections (80–90 nm) were cut from the Epon blocks, placed on 200-mesh copper grids, and counterstained routinely with uranyl acetate and lead citrate. Specimens were observed on a Hitachi H 600 electron microscope.

Flow cytometric analysis

Cellular samples were analyzed with a flow cytometer (EPICS XL-MCL4; Beckman Coulter). During analytical experiments, 10,000 threshold events per sample were collected, with side scatter (for cell complexity/granularity) and forward scatter (for cell size). The results were analysed using the Expo 32 software (Cytometry systems).

Biological activities

Melanisation assay

Cysticercoids were extracted from leech muscles and were then incubated with blood in a 4-well culture dish (NUNC)

containing 500 µl of L-15 (L15; Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 0.6% glucose and 10 mg/ml gentamicin. The encapsulation phenomenon was observed under photonic microscope (Nikon Eclipse TS100). Some samples were fixed for electron microscopy, as described before.

Phagocytic activity

Blood cells were collected as described before and were incubated at room temperature with killed bacteria, Gram-positive *M. nihinomiyaensis* or -negative *A. hydrophila* at 3×10^7 CFU/ml bacteria. After 1 h incubation, cells were fixed and treated for ultrastructural analyses.

Lyso-plate assay

The enzymatic activity was measured using the *M. luteus* lytic (*Micrococcuslysodeikticus* Sigma) assay as described by Selsted [20]. Measures were performed on pools of plasma collected from three animals. In this assay, the diameter of the cleared zones is proportional to the concentration of lysozyme. The diameters can be measured directly and compared to the diameters obtained with standard solutions of lysozyme for quantification.

cDNA cloning and gene expression analysis

RNA from purified cells were extracted (Qiazol; Qiagen) and used for cDNA synthesis with oligo dT (SuperScript II; Invitrogen) to avoid any genomic amplification. One-quarter of the RT reaction was amplified by PCR using an oligo(dT) primer and a specific sense oligonucleotide. PCRs were performed as follows: 94°C for 2 min before 40 cycles at 94°C for 1 min, 50°C 1 min and 72°C for 1 min using Taq polymerase (Go Taq, Promega) in presence of 1.5 mM $MgCl_2$).

Actin primer F: 5′-AGAGGAACACCCAGTCCTCCTGAC-3′
Hm-lumbricin F primer: 5′-AGATGGAGGAGGAAATTGAAGAACT-3′
Hm-theromacin F primer: 5′-TGTTCGAAGATTGGAGTCGTTGTTCG-3′
Hm-theromyzin F primer: 5′-GACCATCACCACGACCATGGGCACG-3′
Oligo (dT) primer: 5′-CGAGTCGACATCGATCGTTTTTTTTTTTTTTT-3′
Destabilase: 5′-CCTACTGGATTGACTGTGGA-3′

In situ hybridization (ISH)

Nerve cords were fixed with 4% paraformaldehyde at 4°C overnight. Plasmids containing the coding region of destabilase probes were used as templates for the synthesis of the probes. Digoxigenin-UTP-labeled antisense and sense riboprobes were generated from linearized cDNA plasmids by in vitro transcription using a RNA-labeling kit (Roche). Digoxigenin-labeled riboprobes (40–100 ng/slide) were hybridized as previously described [21]. Slides were observed under a Zeiss Axioskop microscope. As a control, antisense riboprobes were replaced by sense riboprobes.

Purification and identification of the peptides

Peptidic extractions were performed from the plasma of blood stimulated or not by bacteria as described above. Extraction and purification followed the protocol described previously [9]. Purification steps were carried out on a Perkin Elmer HPLC system. Fractions corresponding to absorbance peaks were collected in polypropylene tubes, lyophilized, reconstituted in water and tested for antimicrobial activity, as described below.

The purity assessment and the molecular mass determination of the peptides were carried out by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) on a DE STR PRO (Applied Biosystems).

Antimicrobial activity

The antibacterial activity was tested by liquid growth inhibition assay, as described in previous studies [22].

Immunocytochemistry

A rabbit anti-*Hm*-lumbricin antibody was produced in the laboratory according to the protocol previously described [18]. Theromacin and Theromyzin antisera were described previously [21]. After collection, blood cells were incubated for 6 h with living bacteria (*A. hydrophila* or *M. nishinomyaensis*). Cells were fixed for 45 min at 4°C in 4% paraformaldehyde. The SHANDON Cytospin 3 was used to spin blood cells suspension onto poly-lysine slides (8 min, 2,000 rpm). Membranes were permeabilized, and nonspecific background staining was blocked with 3% normal goat serum (NGS) and 1% ovalbumin in PBS 0.1% Triton X-100 for 4 h at room temperature. Cells were incubated overnight at 4°C with rabbit anti-*Hm*-lumbricin (1/100) or anti-theromacin (1/100) and mouse anti-theromyzin (1/100) in PBS containing 0.1% Triton X-100, 1% NGS and 1% ovalbumin. Cells were then incubated for 4 h at room temperature with FITC-conjugated anti-rabbit or Texas Red-conjugated anti-mouse secondary antibodies (1:100; Jackson Immunoresearch Laboratories). Samples were examined using a confocal microscope (Zeiss LSM 510).

Time-lapse movies on axotomized nerve cords

The time lapse movies on axotomized nerve cords [section of the faivre's nerve (FN)] are performed as described [9]. Nerve cords depleted of microglia cells were obtained 6 h after having opened the capsule surrounding the ganglia with fine scissors. The blood effect on the regenerative process was evaluated by adding the blood collected from one animal to one cultured nerve cord. Photographs were taken every 24 h for 1 week (objective ×5) using a Leica inverted microscope DMIRE2. Images were taken using the Bioposition version 3.0 software developed on the Matrox MIL 7.5 Base Library. Nerve cords were fixed in 4% paraformaldehyde and fluorescent conjugates of phalloidin (50 µg/ml; Sigma) were used to label actin filament and follow the reconnection process of the FN. The green fluorescence was examined by confocal microscopy (Zeiss LSM 510).

Fig. 1 Ultrastructure of leech blood cells. **a** Schematic cross-section of *H. medicinalis*' body representing the vascular structures. The CNS is enclosed within the ventral sinus. *Inset* represents the picture of the lateral sinus. **b** Flow cytometry analysis of leech blood cells. *Gate 1* represents heterogeneous population of blood cells. **c** On paraffin sections, blood cells are visible (*arrow*) within the ventral sinus (*vs*). The blood cells are located between a ganglion of the CNS (*g*) and myoendothelial cells (*me*). **d** Leech blood cells in culture. These cells rapidly adopt a round shape after collection resulting in a mix of ovoid and spheric cells (*arrow*). **e**, **f** Electron microscope images showing a blood vessel (**e**) or in culture (**f**), ×15,000 (*n* nucleus, *c* cytoplasm, *v* vacuole, *bv* blood vessel, *e* endothelial cell). These cells are (5 µm) and possess a central nucleus (*n*) and a thick cell coat (*cc*). The cytoplasm contains a small amount of rough endoplasmic reticulum (*rer*), few mitochondria (*m*), extensive microfilaments (*mf*), and vacuoles (*v*). Long and thin pseudopodia (*ps*) are also a prominent feature of these cells, ×15,000. **g** Vacuoles (*v*) containing a dark flocculent material are abundant in the cytoplasm. The perinuclear endoplasm shelters a lot of free ribosomes (*r*), ×60,000. **h** The cytoplasm contains mitochondria (*m*), centriole (*asterisk*) and microtubules (*mf*), ×80,000

Results

Morphology of the leech blood cells

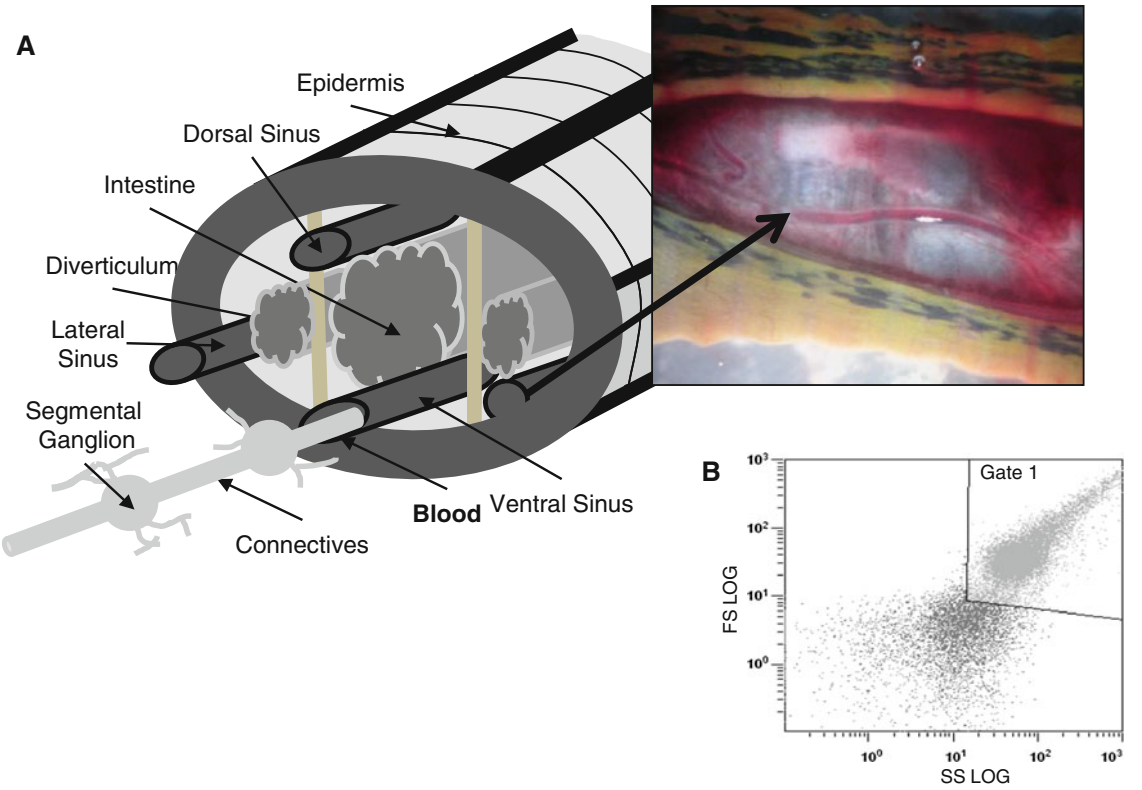
Because no data were available on annelid blood cells, we initiated the morphological characterization of blood cells collected from the lateral sinus of *H. medicinalis*. The vascular system of this leech is organized around four longitudinal sinuses (Fig. 1a). Close observation of paraffin-embedded sections confirmed the presence of blood cells within the sinuses (Fig. 1c). Upon collection, these cells appear slightly ovoid but rapidly become spheric when maintained in culture (Fig. 1d). This technical artifact caused by the extreme sensitivity of the cells to the preparation occurs frequently in invertebrates [23]. Flow cytometry analyses of freshly collected blood confirmed the presence of a unique cell population in various morphology states (Fig. 1b).

Ultrastructural features of leech blood cells were then examined by electron microscopy to further characterize their morphology in situ (Fig. 1e) and ex vivo (Fig. 1f). These cells appear small (5 µm), with a central nucleus and a low nucleocytoplasmic ratio. The cytoplasm contains small amounts of rough endoplasmic reticulum, few mitochondria, extensive microfilaments and vacuoles (Fig. 1g, h). In addition to their round shape, cells present long and thin pseudopodia suggesting an ability of these circulating cells to migrate. The relative transparency of the cytoplasm and the presence of pseudopodia are characteristics of hyaline cells [2].

Implication of the blood cells in cellular immunity

Cellular immunity includes various mechanisms such as cytotoxicity, phagocytosis and the encapsulation process which leads to the elimination of foreign bodies bigger than bacteria. Cytotoxic properties of the leech blood cells were evaluated by incubating them with the coelomic cells of another annelid species, i.e., *Eisenia fetida*. No cytotoxicity was observed under these conditions (data not shown). The ability of the leech blood cells to phagocyte bacteria known to be living in the aquatic environment of the medicinal leech was also explored (Fig. 2). As evidenced in Fig. 2a, b, leech blood cells are able to engulf alive bacteria by projecting small filopodia around their targets independently of their Gram status (Gram-positive *M. nishinomiyaensis* or -negative *A. hydrophila*) suggesting the absence of specificity of this cellular defense.

To evaluate the cellular immune functions upon parasitic infection, leech cells were incubated with the cystic form of a cestode (Fig. 2c, d). This plathyhelminth that normally accumulates into the muscles of our model is currently being identified by our group. Interestingly, a high parasitic load appears lethal for the annelid, therefore identifying the first natural pathogen of the medicinal leech. Cells incubated with the cysticercoids attach to them and then release their dark granule contents which accumulate at the surface of the parasite (Fig. 2d, e). The production of the black substance is not triggered by the presence of the parasite and also occurs when cells are incubated with Sephadex beads (data not shown). In contrast, attachment of the cells seems to follow a more specific process since blood cells attach to the parasite but not to Sephadex beads. The parasite is neither surrounded by blood cells nor covered by black substance in the muscle of infested leeches suggesting that the cestode is able to escape the immune surveillance of the leech in some tissues.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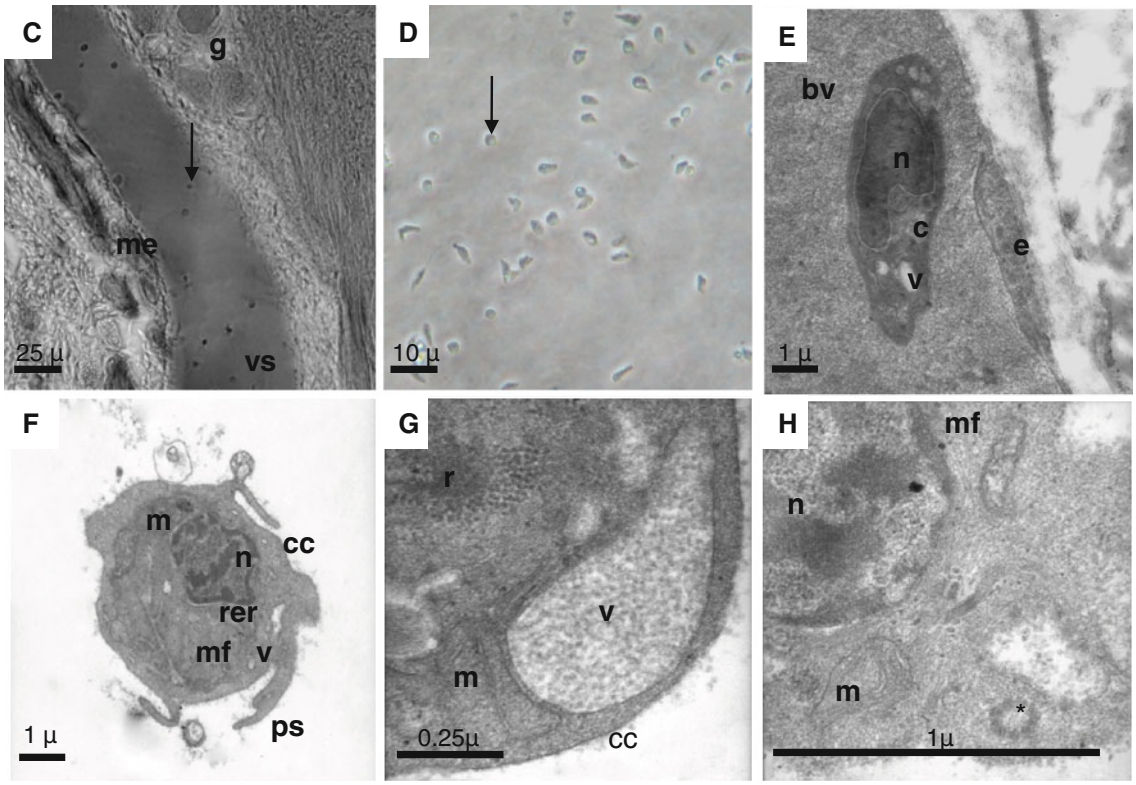
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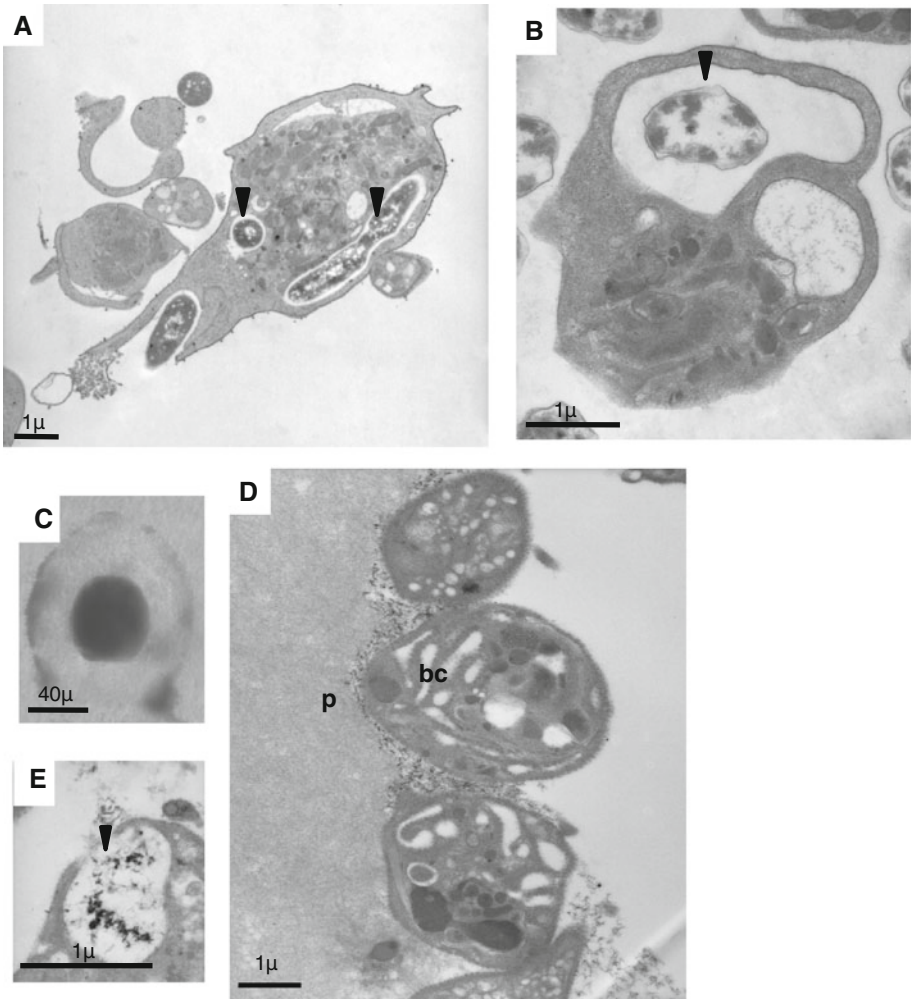

Fig. 2 Phagocytosis activity and melanine production by blood cells. **a** After 1 h incubation with Gram-negative, bacteria-containing phagolysozomes (*arrowhead*) become visible, ×15,000. **b** After 1 h incubation with Gram-positive, bacteria-containing phagolysozomes (*arrowhead*) become visible ×20,000. **c** Cells can be seen on the surface of a natural parasite. **d** Electron microscope image showing highly vacuolized blood cells (*bc*) surrounding a natural parasite (*p*) and producing melanin, ×8,000. **e** The liberation of the melanin pigment (*arrow*) is detected by electron microscope, ×35,000

Implication of the blood in the humoral immunity

Humoral immunity in invertebrates includes the production/secretion of active molecules such as AMPs and lysozyme-like by immune cells [24–26]. AMP and lysozyme are important immune effectors widely distributed in many organisms.

The presence of these molecules within the cell-free plasma was investigated by reversed phase HPLC (Fig. 3). Plasmas from blood incubated for 6 h with or without bacteria, were acidified and subjected to Sep Pak C18 cartridges. The 60% ACN (acetonitrile) Sep Pak fractions were loaded onto RP HPLC and each fraction was individually tested for its antimicrobial property. Compared to the controls (Fig. 3; $T = 0$ and 6 h), an antimicrobial activity in material eluted between 24 and 32% ACN, and between 43 and 48% ACN was observed in the plasma of blood incubated with Gram positive (Fig. 3; $T = 6$ h, Gram-positive) but not with Gram-negative bacteria (Fig. 3; $T = 6$ h, Gram-negative). This material was submitted to mass spectrometry analysis and gave molecular masses of 8,447, 6,422 and 9,965 m/z. These masses were not detected in the HPLC fractions of the control. These elution percentages range and molecular masses correspond to those of *Hm*-theromacin, *Hm*-lumbricin and *Hm*-theromyzin, respectively, three leech AMPs already characterized by our group [27]. In all blood treatments, the 27% eluted fraction presented a lysozyme activity suggesting a constitutive release of the molecule into the blood. Mass spectrometry analyses allowed us to identify the peptide as being destabilase (data not shown). Destabilase first isolated from the salivary gland of the leech is an enzyme presenting both a peptidase and a lysozyme activity [28, 29].

In order to determine the origin of the plasmastic AMPs and destabilase, messengers were amplified by RT PCR using RNAs of leech blood cells incubated in presence or not of bacteria for 6 h (Fig. 4a). Whereas no AMP transcripts were detected under basal conditions ($T = 0$ h), microbial challenge of the cells led to a gene induction of the leech AMPs. Stress generated by the cell culture itself appears as a slight inductor of the expression of these three

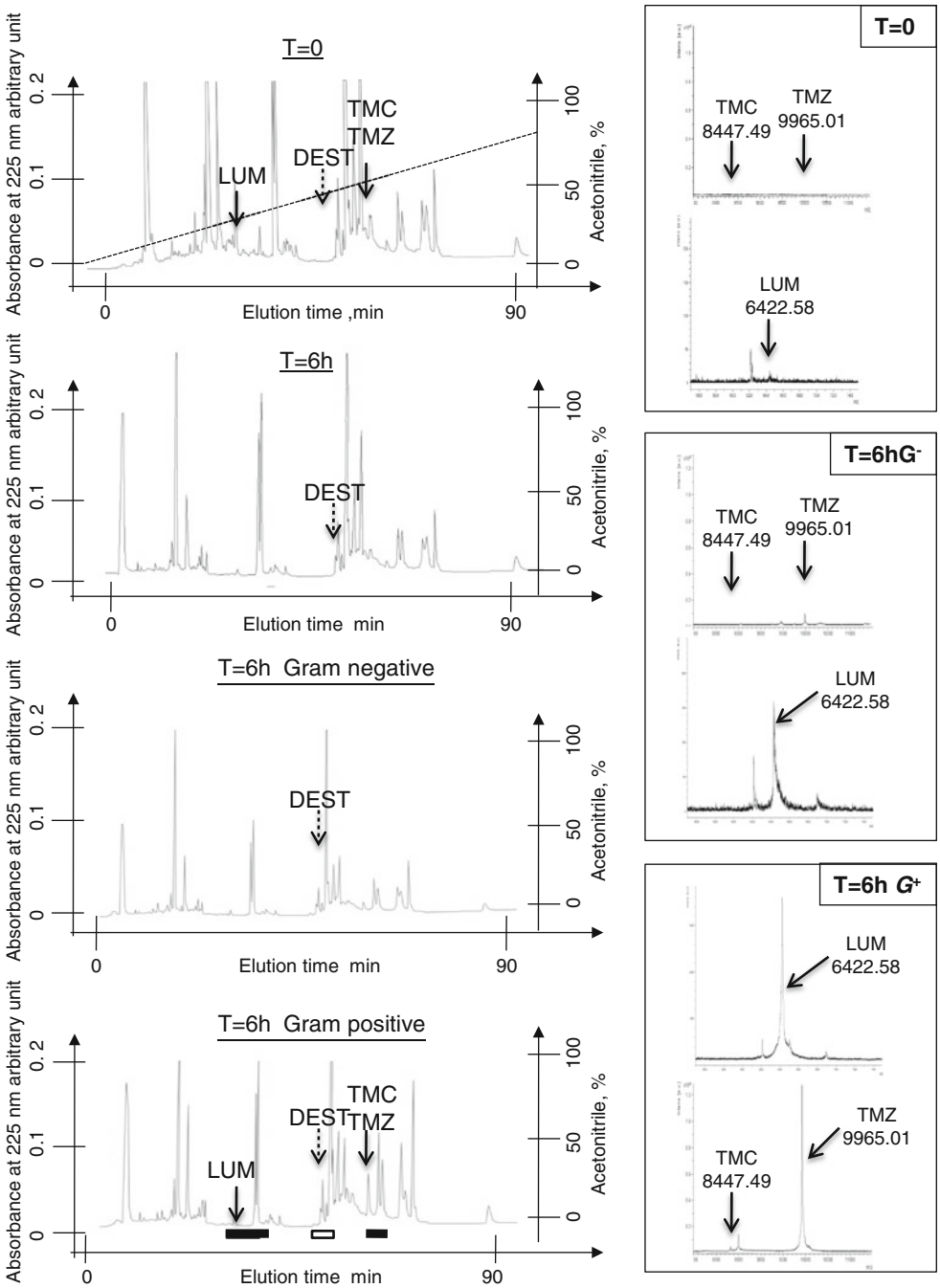

Fig. 3 RP-HPLC of acidic extract obtained from leech plasma challenged or not with bacteria. After prepurification by solid-phase extraction, the material eluted with 60% ACN was loaded onto C18 column (250 × 4 mm^2; Vydac). Elution was performed with a linear gradient of ACN in acidified water and absorbance was monitored at 225 nm. Each individually collected peak was a test for its antimicrobial and lysozyme activities. The antibacterial and the lysozyme fractions are identified by a *black* and a *white rectangle* respectively. *Hm*-lumbricin (*LUM*) (*arrow*), *Hm*-theromacin (*TMC*) and *Hm*-theromyzin (*TMZ*) (*arrow*) and destabilase (*DEST*) were identified by MALDI TOF-MS

genes ($T = 6$ h). So, the synthesis of AMPs by blood cells critically depends upon the presence of bacteria. To verify whether the peptides are immediately released after their synthesis, as suggested by the peptidomic analysis of the plasmatic extracts, *Hm*-theromacin, *Hm*-theromyzin and *Hm*-lumbricin immune reactivity distributions in unchallenged versus challenged blood cells were compared by immunofluorescence (Fig. 5). Unexpectedly, although no AMP transcripts were detected under basal conditions ($T = 0$), the three peptides were immunodetected in the blood cells, suggesting a storage of these active compounds after synthesis. Interestingly, post-bacterial challenge, the immune staining is not observed in the cells any more, but rather accumulates at the periphery (LUM/TMZ) or inside the bacteria (TMC) (Fig. 5a3, b3, c3), corroborating (1) the antimicrobial property of these AMPs and (2) their secretion upon microbial challenge.

RT PCR results excluded blood cells as potential production sites for destabilase (Ds) (Fig. 4a). However, destabilase transcripts were detected by RT PCR and by in situ hybridization in the endothelial cells delimiting the ventral sinus (Fig. 4b, c). Bacteria were directly injected

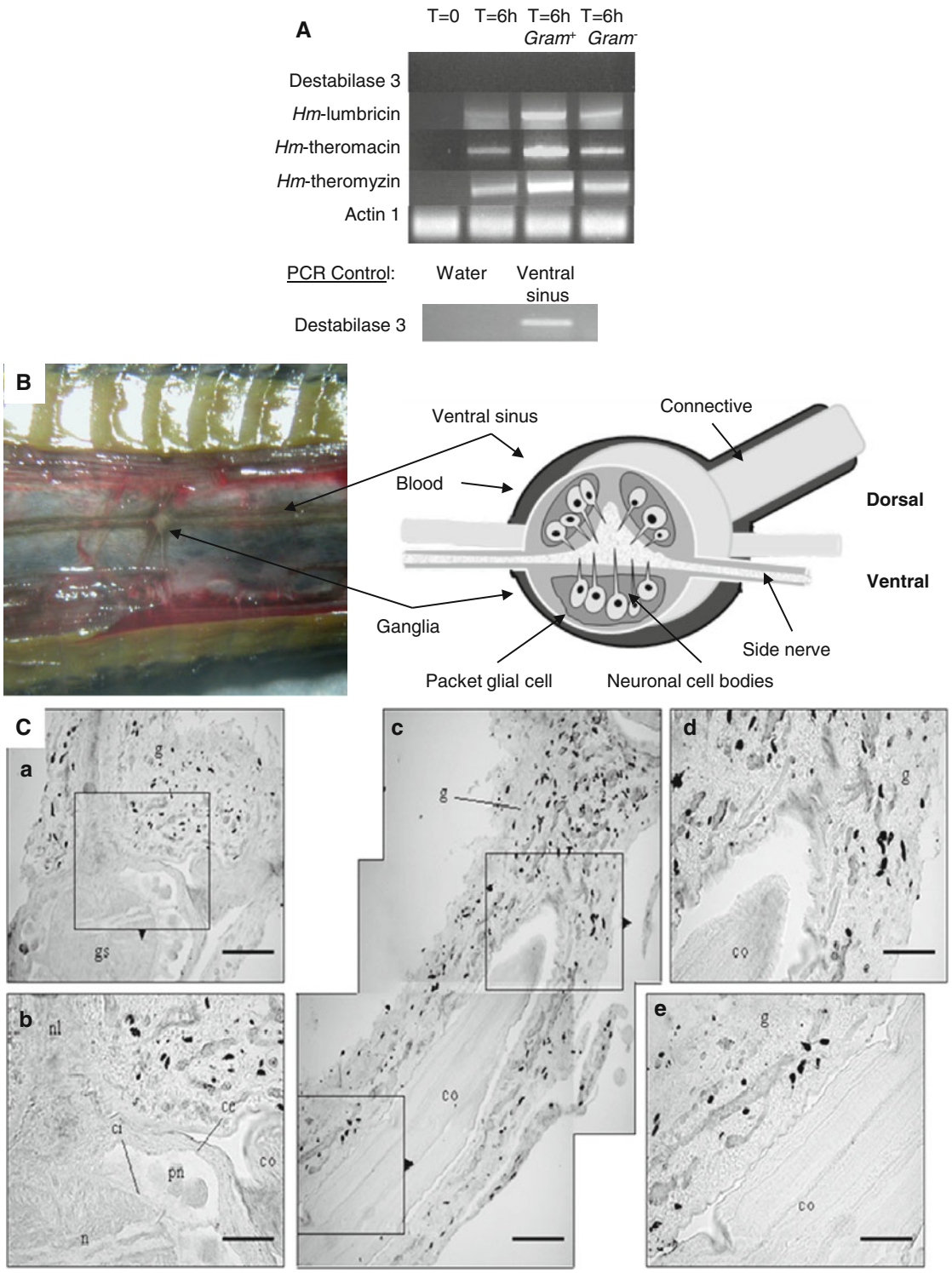

Fig. 4 Analysis of destabilase and AMPs gene expression sites in isolated cells and in ventral sinus. **a** Analysis of destabilase, *Hm*-lumbricin, *Hm*-theromacin, and *Hm*-theromyzin gene expression in isolated blood cells. AMPs and destabilase cDNAs were amplified by RT-PCR from blood cells just after collection ($T = 0$), after 6 h of culture in sterile condition ($T = 6$ h), after 6 h of culture in presence of bacteria ($T = 6$ h $GRAM^+$ or $T = 6$ h $GRAM^-$). PCR control of destabilase gene expression sites in ventral sinus. **b** Picture and diagram illustrating the close contact between the blood and the CNS. **c** Detection of destabilase mRNA in the endothelial cells delimiting the ventral sinus by in situ hybridization: *a*, *b* detection with an antisense probe on sections from the level of segmental ganglia (*gs*), and *c*–*e* this marking by antisense probe on sections through a connective. Detection with sense probe shows no markings on these cuts. *co* connective, *g* endothelial cells, *n* neuropil, *nl* nerve and lateral, *pn* a group of neurons. *Scale bars* 40 μm (*a*, *c*), 20 μm (*b*, *d*, *e*)

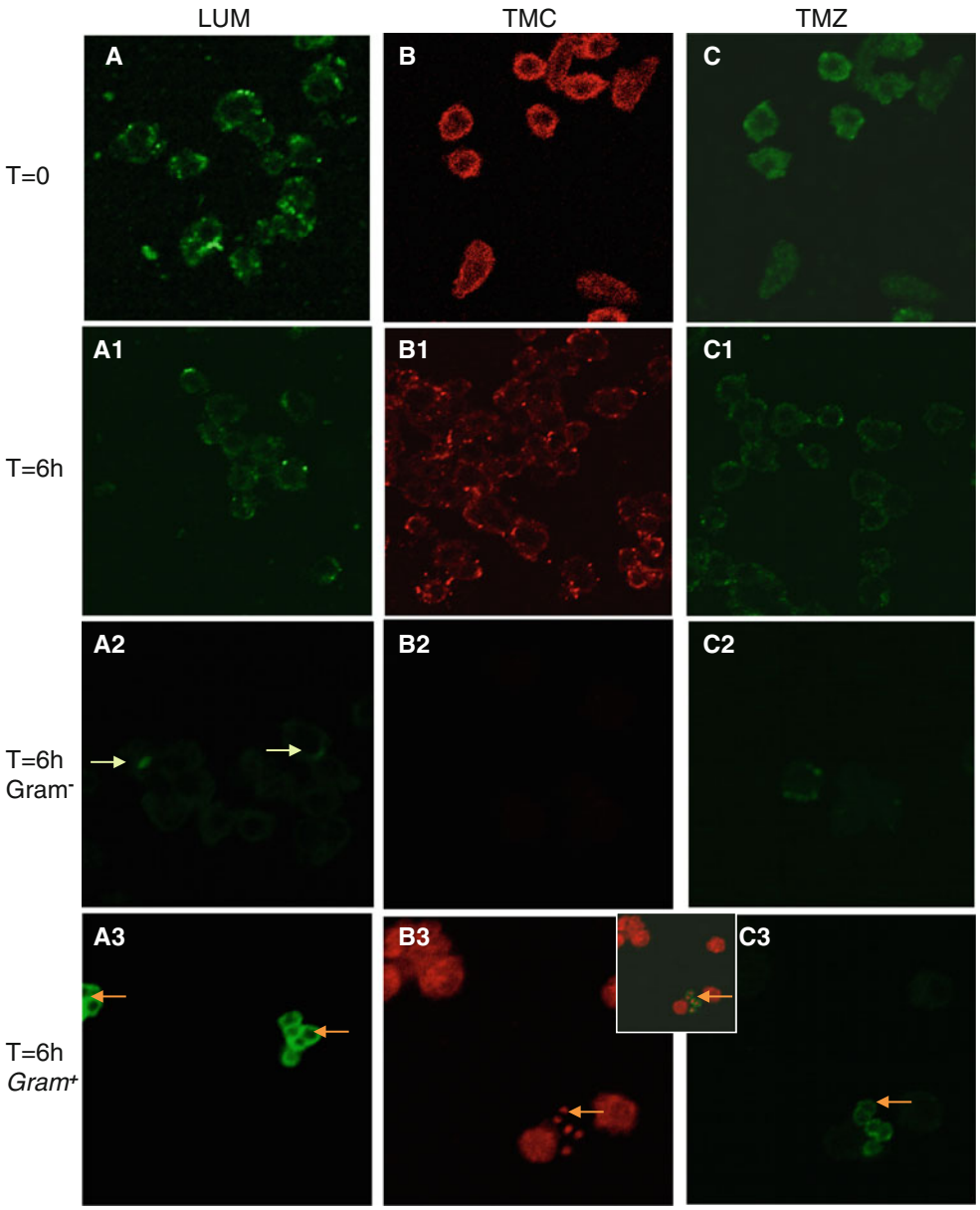

Fig. 5 Detection by immunofluorescence of AMPs in the blood cells incubated with killed bacteria for 6 h. **a–c** Immunohistochemistry performed on the blood cells with: **a** the anti-*Hm*-lumbricin antibody, **b** the anti-*Hm*-theromacin antibody, or **c** the anti-*Hm*-theromyzin. The blood cells incubated for 6 h with killed bacteria: (**a3, b3, c3**) *A. hydrophila*, (**a4**, **b4**, **c4**) *M. nishinomyaensis*. **a**, **c** The immunodetection was performed using FITC labeled secondary, or **b** Texas red-labeled secondary antibody. Controls were performed with preimmune sera

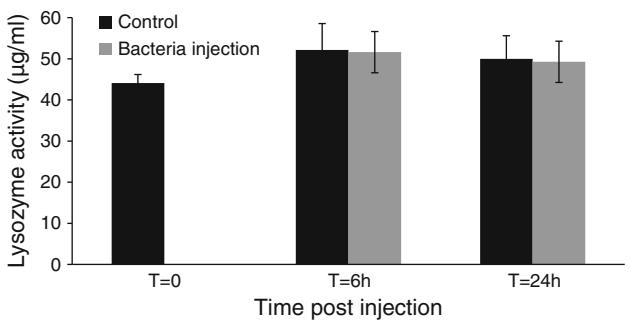

Fig. 6 Determination of lysozyme activity in plasma using the lysoplate assay. A mix of environmental bacteria was injected directly into the lateral sinus of the leech. The control condition corresponds to L15 medium, without bacteria

into the vascular blood system of the leech to determine whether the secretion of Ds by the endothelial cells could be regulated by the presence of microorganisms (Fig. 6). Under this in vivo condition, the level of lysozyme activity remains constant, even upon immune stimulation confirming the constitutive presence of lysozyme-like substances into the leech blood, as suggested by the RP HPLC data.

Function of the leech blood in neural repair

The plasmatic release of AMPs previously described as exerting neurotrophic activities [9] and the direct contact between the blood and the CNS led us to address the question of a potential effect of the blood on neural repair.

FITC-conjugated phalloidin was used to visualize the nervous reconnection at neurofilament level. The success of the regeneration is well-observable for the FN, the median connective which contains 97 axons. Since the leech CNS lies within a blood vessel, it can be easily removed from the source of blood cells. Under these conditions, restoration of the connective nerve across the cut began 4 days after axotomy and was complete 4 days later (Fig. 7a). These data are in line with the observations reported by Muller et al. [30] who showed that the normal functions of axotomized leech neurons were restored after 8 days.

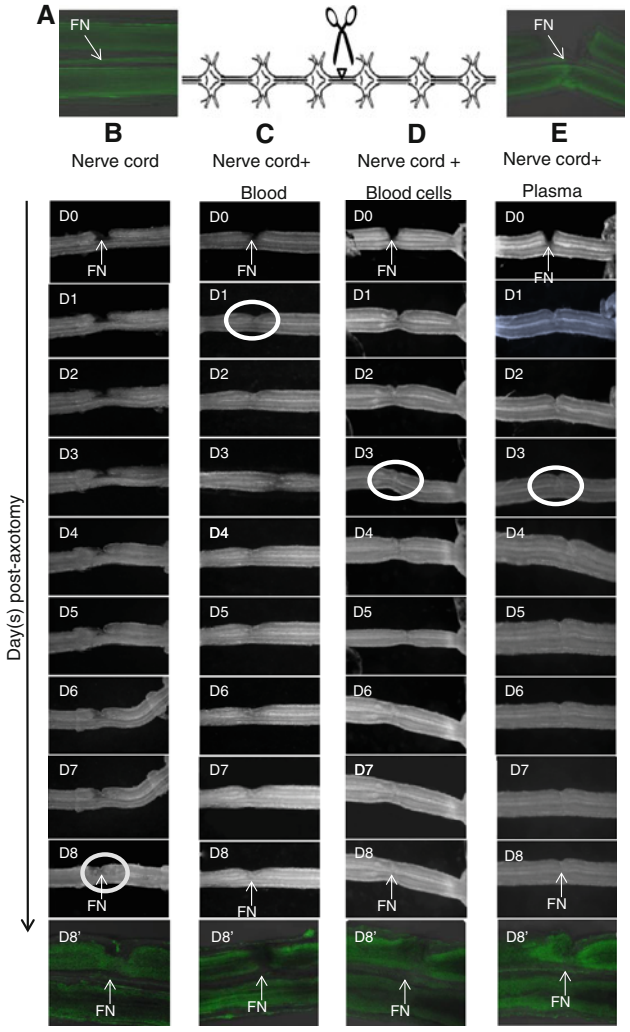

Fig. 7 Effects of blood components on regeneration of axotomized leech CNS. **a–d** Sequential micrographs, were taken 24 h apart, from 1 (**D0**) to 8 days (**D8**) after axotomy, documenting the regeneration of the severed connective nerve. **a** Preparation in sterile culture medium, **b** incubated with blood, **c** incubated with blood cells, and **d** incubated with plasma. Structural regeneration was complete after 8 days in control conditions, 1 day in presence of blood, and 3 days in presence of either blood cells or plasma. *Top* and *bottom*: fluorescent conjugates of phalloidin were used to label actin filament and follow the reconnection process of the FN. The *green* fluorescence was examined by confocal microscopy

The same experiment was performed in presence of blood in the cell culture medium of axotomized nerve cords. The presence of blood accelerated the regeneration which was then complete 24 h after axotomy (Fig. 7b). When adding either blood cells or cell-free plasma, the regenerative effect was also observed, although it was slower than in presence of complete blood (Fig. 7c, d). These observations suggested that cells and plasma are equally important for optimal neurotrophic effects of blood on the regenerative process.

The ability of the blood cells to directly interact with the leech CNS was then investigated by electron microscopy (Fig. 8). As demonstrated in Fig. 8b, under normal conditions (without any axotomy), no blood cells adhere to the fibrous capsule delimiting the nerve cord. In contrast, sectioning one side of the paired connective nerve linking adjacent ganglia led to the recruitment and an accumulation of blood cells at proximity of the lesion site (Fig. 8c, d). At the lesion site, cells adopt the morphology of migrating cells within 12 h post-axotomy and clearly infiltrate the CNS on the following days (Fig. 8e, f, i, j). Interestingly, a fibrous substance reminding collagen is observed all over the infiltrated cells (Fig. 8g). Collagen represents not only a structural component but also plays a major role in the modulation of several cell functions, including adhesion [31].

In order to determine whether AMPs are responsible for the neural repair, neurotrophic assays were performed with blood cells or with plasma issued from blood incubated with bacteria (Fig. 9). Microbial challenge leads to the secretion of the three AMPs into the extracellular medium, as evidenced by RP HPLC and by confocal microscopy (Figs. 3 and 5). Under these immune challenges, cells become impoverished in *Hm*-theromacin, *Hm*-theromyzin and *Hm*-lumbricin peptides as the plasma gets enriched. As demonstrated in Fig. 9, only the plasma from challenged blood has a positive effect on the regenerative process of the leech injured CNS, whereas plasma from untreated cells does not have any effect. Thus, it seems that components secreted from blood cells challenged with bacteria exert a positive impact on the regenerative process of the injured CNS of the leech.

Although blood cells are still in a good shape after a 1-week culture, microglial cells die rapidly (in less than 24 h). Microglial cells are resident macrophages in mammals [32, 33], arthropods [34], and leeches [8] which respond rapidly to brain injury by moving to the lesion and accumulating there. Whether they subsequently divide, as in mammals and arthropods, or not, as in leeches, recruited microglia phagocytose cellular debris [35]. We wondered if the presence of microglia was primordial

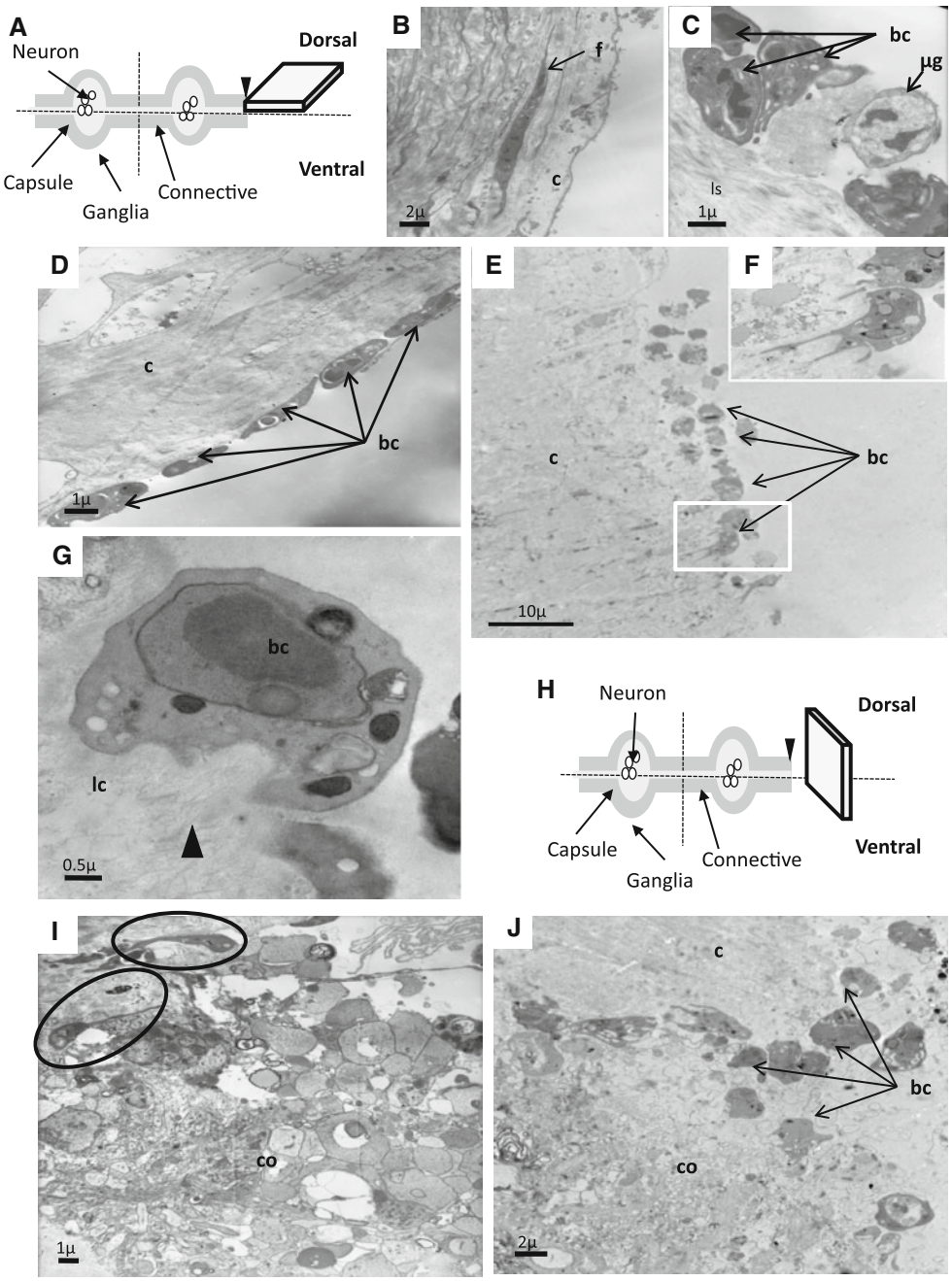

Fig. 8 Function of the leech blood cells in neural repair. **a** Diagram of the leech CNS. Neuron cell bodies within ganglia project axons into connectives toward adjacent ganglia. *V-symbol* indicates the location of the cut of one of the two connectives linking two segmental ganglia. *Black rectangle* indicates a longitudinal section through the regeneration connective. **b** Electron microscope of capsule structure (**c**), which is encapsulated by a tough fibrous sheath that is composing of muscular cells and fibroblast (**f**), ×4,000. **c–g** Electron microscope images of axotomized nerve cords incubated in presence of blood. **c** The presence of blood cells (*bc*) beside microglia (*µg*) (*arrows*) at proximity of the lesion site (*ls*) 24 h post-axotomy, ×10,000. **d** After 24 h, blood cells (*bc*) adhere to connective (*c*), ×2,500. **e**, **f** At the lesion site regeneration, blood cells (*bc*) directly in contact with connective (c), after a week, **g** The regenerating lesion site (*ls*) is characterized by longitudinally sectioned fibrils (*arrowhead*) similar to the structural pattern of collagen. **h** Diagram of the leech CNS. *Black rectangle* indicates a transversal section through connective (*c*) and connective (*co*). **i**, **j** Electron microscopy observation showing the presence of blood cells (*in circle*) in nerve cord after 7 days of regeneration, ×5,000

to promote the regenerative process of the injured nerve cord, or if the blood by itself could be sufficient. To answer this question, nerve cords were depleted in microglial cells according to the procedure established previously [9], and blood was added to the culture medium. Absence of microglial cells led to the generation of a scar which inhibits the mechanism of repair (Fig. 10b). The presence of blood did not prevent the formation of this scar (Fig. 10c) suggesting that an optimal regeneration require microglial cells for initiation and blood cells to facilitate and accelerate the process.

Discussion

Annelids are the only protostomian to possess a closed vascular system in which the blood circulates separately from the coelomic liquid. Coelomic cells have already been described in various segmented worms including leeches [4, 19, 36, 37]. As for arthropods and molluscs, four major cell types, prohemocytes, hyaline hemocytes, granular hemocytes, and eleocytes, have been structurally characterized in the coelomic liquid of annelids [37]. The clear differentiation in the structure of annelid coelomocytes is

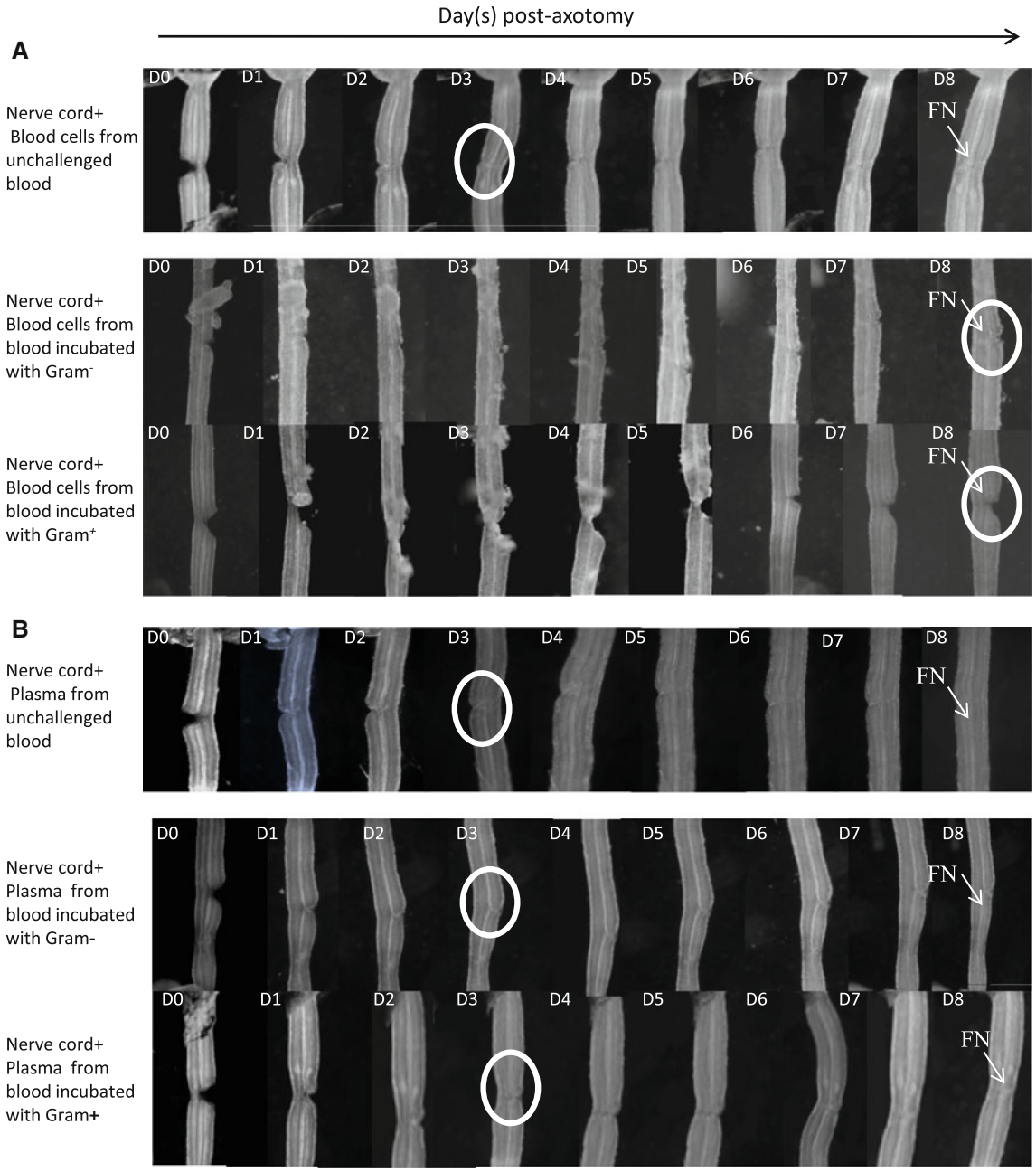

Fig. 9 CNS regeneration in presence of blood components previously challenged or not with bacteria. Blood cells (**a**) or plasma (**b**) previously incubated with $Gram^+$ or $Gram^-$ bacteria (*second and third rows* in each *panel*) or unchallenged (*first row*) were added to axotomized leech CNS. Bacteria challenged blood cells affect the regenerative process unlike challenged plasma. *fn* Faivre's nerve

associated with their function, except for prohemocytes which constitute immature cells. Hyaline hemocytes participate in melanization/encapsulation processes. Granular coelomocytes exert phagocytosis activities although eleocytes constitute the functional equivalent of the fat body of the insect.

This work was aimed at characterizing, both morphologically and functionally, circulating blood cells of annelids. Unlike coelomic cavities of annelids, our data evidenced the presence of a unique type of blood cell in the vascular system of the medicinal leech. These cells appear to share morphological characteristics with hyaline cells, also called plasmatocytes, which represent the most prevalent type of differentiated circulating cells in invertebrates. Hyaline cells have been compared to cells of the macrophage lineage of vertebrates [1]. Because of lack of conservation between mammalian macrophage markers, such as CD14 and CD61, and leech orthologs, well-described macrophage markers could not be detected in these cells. However, our data described a clear involvement of these

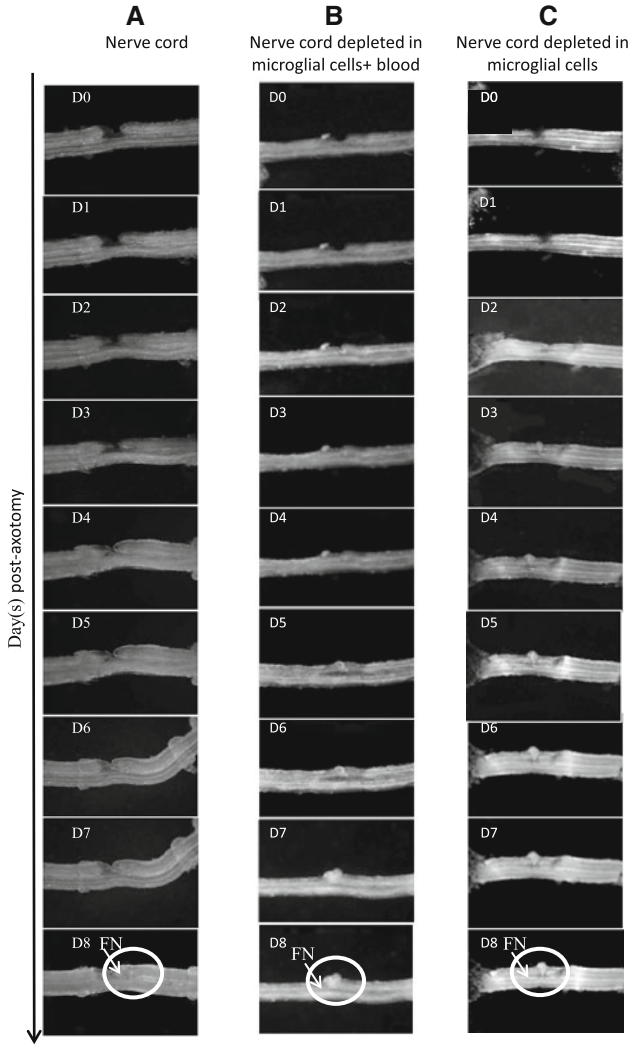

Fig. 10 Role of microglial cells in CNS regeneration. Regeneration process of axotomized CNS in presence of microglial cells (**a**), in absence of microglial cells (**b**), and in absence of microglial cells and presence of blood (**c**). Absence of microglial cells does not impact the reconnection process but induces the formation of a scar which is also formed in presence of blood. *fn* Faivre's nerve

cells in both cellular and humoral immune response involved in antibacterial and antiparasitic defense mechanisms. These roles appear comparable to the responses established by immune cells of vertebrates.

The response of leech blood cells is similar when challenged with either Gram-positive or -negative bacteria, suggesting the absence of specificity in the phagocytic process. By this function, leech blood cells contribute to the sterilization of the blood by eliminating bacteria.

The proteolytic cascade of melanisation is a common response to parasite entry in invertebrates [38, 39], vertebrates [40], and plants [41]. Melanin formation is the result of a phenolic oxidation. In most invertebrates, a redox enzyme, commonly called phenoloxydase (PO), catalyses the reaction. This enzyme is often synthesized by circulating cells and is secreted upon immunostimulation [38]. In vertebrates, the enzyme carrying the activity, tyrosinase, is membrane-bound and is localized in a specialized organelle, the melanosome. In the present work, we have observed the release of a melanin-like substance by the blood cells incubated with a foreign body (natural parasite or Sephadex bead). Further investigations should be done in order to determine whether leech blood cells are able to produce and release PO into the plasma, as commonly done in invertebrates including other annelids [42]. An attachment of the blood cells together with an accumulation of the black substance produced by the cells at the surface of the parasite was also observed when introducing the cystic form of the parasite into the blood. Interestingly, this phenomenon does not occur for the parasites accumulated into the muscles of infested leeches, suggesting that the mobile stage of the cestode is able to escape the immune surveillance of our model. In addition to their immune properties, melanins produced by nervous cells (neuromelanins) have also been evidenced to scavenge reactive oxygen species, and to bind and sequester toxic metals in stable complexes that prevent neuronal toxicity in vertebrates [43]. In human neurodegenerative conditions, such as Parkinson disease, the rate of loss of neuromelanin appeared to be enhanced by a loss of neurons containing this pigment and by a reduced content of melanotic components in surviving neurons [43]. We hypothesize that the formation of melanin-like substances by the blood cells provides double advantages to the medicinal leech (1) by participating in the exclusion of pathogens and (2) by playing a neuroprotective role. Moreover, this last function could explain the accumulation of melanin that we have observed inside the neurons of the leech (data not shown). The production of melanin by nervous cells has also been reported in the earthworm, *Lumbricus terrestris*, and is reminiscent of what is observed in the neurons of the human gray matter [44].

Leech blood cells also participate in the elimination of bacteria by producing and releasing three AMPs, *Hm*-theromacin, *Hm*-theromyzin, and *Hm*-lumbricin. These antibiotic molecules have already been described in the leech [21]. By contrast with *Hm*-theromacin and *Hm*-theromyzin, *Hm*-lumbricin is also produced by leech neurons. As for neuromacin, a close relative of *Hm*-theromacin synthesized by neurons, *Hm*-lumbricin presents neurotrophic properties. The synthesis of AMPs by circulating cells is a widespread mechanism found in both invertebrates and vertebrates meant to deal with septic conditions. Unlike the phagocytic activity, the gene induction, as well as the secretion, appears to be specific to the pathogens, suggesting that blood cells are able to discriminate microbial components, as neural cells of the

leech CNS do [9]. The data presented here also suggest that an antimicrobial protein named destabilase also contributes to "the sterilisation" of the blood. However, destabilase is not expressed by the blood cells but rather by the endothelial cells delimiting the sinus. The constitutive gene expression is correlated with a constant lysozyme activity in the leech plasma. With this systemic action, destabilase may provide a permanent protection which may be reinforced by a pathogen-specific production of AMPs by the blood cells during a bacterial challenge.

Interestingly, leech destabilase and *Hm*-lumbricin have also been shown to exhibit neurotrophic properties [44, 45]. The close contact between the blood and the nerve cord of the leech, together with the presence of such plasmatic active molecules, led us to consider the participation of the body fluid in neural repair of injured CNS. It appeared that the regenerative process of the axotomized CNS is favored by the presence of blood through a synergistic mode of action implicating both plasma and cells. The positive impact of the plasma could be explained by the presence of destabilase. Interestingly, plasma enriched in AMPs appeared more efficient than the basal one, without being as efficient as the blood itself. The concomitant importance of the cells and their AMP production in leech neural repair is illustrated by their ability to rapidly migrate and infiltrate the lesion site and by the loss of their regenerative capacity after immune activation when they have been discharged in AMPs.

In this context, we wondered whether blood cells could favor nerve cord repair in the absence of microglia. In leeches, as in mammals, microglial cells, considered as the resident phagocytic cells of the CNS, have been demonstrated to respond rapidly for neural protection or healing after CNS injury [46–48]. Despite their capacity described by Muller et al. [8] to phagocyte cellular debris, we have not observed any ability of the leech microglial cells to phagocyte alive or dead Gram-positive or -negative bacteria (data not shown). This observation, together with their inability to proliferate, makes them different from their vertebrate counterparts. Our data demonstrated that leech nerve cords depleted in microglial cells do not recover from injury. The formation of an obstructive scar clearly visible in our preparations appears as an explanation of the failure of regeneration. Addition of blood cells does not reverse the phenomenon. This suggests that microglial cells, and/or the method used to take them from the nerve cord (mechanical disruption of the capsule, a fibrous membrane surrounding the leech nerve cord), are involved in inhibiting the scar formation. Further investigations will be performed in order to understand this process. In contrast, the formation of a scar is frequently observed in mammals and remains one of the most studied but poorly understood barriers to regeneration of CNS axons [48].

Altogether, the data presented here constitute the first morphofunctional characterization of blood cells in an annelid and the first evidence of neuroimmune function of blood cells in invertebrates. Understanding the basic functions of the peripheral circulating cells and their interactions with injured neurons in the leech CNS would allow us to better understand mechanisms and actors that can promote regeneration after brain injury in mammals.

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