CHAPTER 5

LEECH IMMUNITY: From Brain to Peripheral Responses

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Abstract: In the present chapter, we will emphasize the immune response in two compartments (Central nervous system and peripheral system) in two blood sucking leeches i.e., the medicinal leech and the bird leech *Theromyzon tessulatum*. In the medicinal leech, the neuroimmune response has been described in the context of septic trauma at the cellular and humoral levels through microglia, Toll-like, cannabinoids and chemoattractant factors activation and modulation. In the bird leech, the antimicrobial responses have been dissected at the cellular and molecular levels. Altogether, this chapter presents a complete integrate immune response from the brain and the systemic compartments with high similarity to the vertebrates one. These points that the neuroimmune and immune responses evolved sooner than can be expected.

INTRODUCTION

Leeches are derived from their cousin's earthworm in the class of annelids called hirudinea. Leeches evolved during the Cambrian Explosion, a time of rapid biological development 540 million years ago.[1-6] There are now approximately 700 species of leeches distributed throughout freshwater, marine and terrestrial ecosystems worldwide. Phylogenetic studies assess that the common leech ancestor was probably a bloodsucking leech with a proboscis rather than an unspecialized ectocommensal.[7] During the course of leech evolution, a reduction of the proboscis could have taken place in predatory arhynchobdellid ancestors to enable swallowing of larger prey. A second gain of sanguivory by the jawed Hirudiniforms could have been facilitated

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by pre-adaptations to ectoparasitic blood feeding.[7] Recently, leech EST data from the jaw leech *hirudo medicinalis* have been obtained[8] and it can be seen high sequence homologies between the medicinal leech and mammals[8] suggesting a co-evolution between the parasite and the host. Thus, taking into account such evolutive data, we will present in this manuscript, data obtained from two haematophageous leeches, the jawed leech *Hirudo medicinalis* and the gut leech *Theromyzon tessulatum* focused on the immune responses angle. Due to their anatomic differences, *Hirudo medicinalis* appears as a good model for studying the immune response of the nervous system although the easy access to the body fluids of *Theromyzon tessulatum* makes this animal interesting for understanding the systemic response. Taken together, we will try to show that immune response from these blood sucking animals evolve closely to their vertebrate host, pointing the fact that their use currently in hospital never trigger patient immune responses. Host immune tolerance is due to co-evolution and molecular mimicry.[9]

THE MEDICINAL LEECH AS A MODEL FOR STUDYING THE IMMUNE RESPONSE OF THE CNS

The central nervous system (CNS) of the leech has a fixed number of bilateral neuromeres, 32. The 4 anterior-most neuromeres fuse to form the sub-esophageal ganglion and the 7 posterior-most fuse to form the tail ganglion; single bilateral neuromeres comprise the individual ganglia found in each of the corresponding body segments. A supra-esophageal ring of nonsegmental origin, together with the sub-esophageal ganglion, comprises the head ganglion. The central ganglia are connected to each other by a bilateral pair of nerves (the lateral "connectives") and a single small medial nerve (Faivre's) and to the periphery by two bilateral pairs of nerves (the "roots") that branch in a stereotypic pattern that allows the identification of branches up to fourth-order and even to fifth-order in some cases (Fig. 1). In hirudinid leeches, each segmental ganglionic primordium gives rise to about 400 neurons.[10] Most of these are bilateral pairs (~180-190 pairs), but perhaps 5-8% are unpaired, with at least some becoming unpaired through cell death.[10,11] Thus, understanding how a leech segmental ganglion functions requires, in principle, detailed knowledge of the function and connectivity of only ~200-220 individual neurons. Moreover, since each segmental ganglion is a variation on a theme (with the exception of the "sex" ganglia of body segments 5 and 6, which have additional complements of neurosecretory cells), the leech has one of the most accessible nervous systems from a systems analysis point of view.

An important property of leeches is their capacity to regenerate neurites and synaptic connections in the adult CNS. Neurites that have been damaged or severed can sprout, establish de novo growth cones and extend and reconnect specifically with normal targets.[12] Early stages of leech CNS regeneration following a mechanical lesion are characterized by two events that appear to be crucial for successful repair: one is the increased activity of epithelial nitric oxide synthase (NOS) in the area of the lesion and the generation and diffusion of nitric oxide (NO) and the second is the induced migration of microglia towards and their accumulation at, the injury site.[13,14] Microglial cells are considered as the brain immune cells.

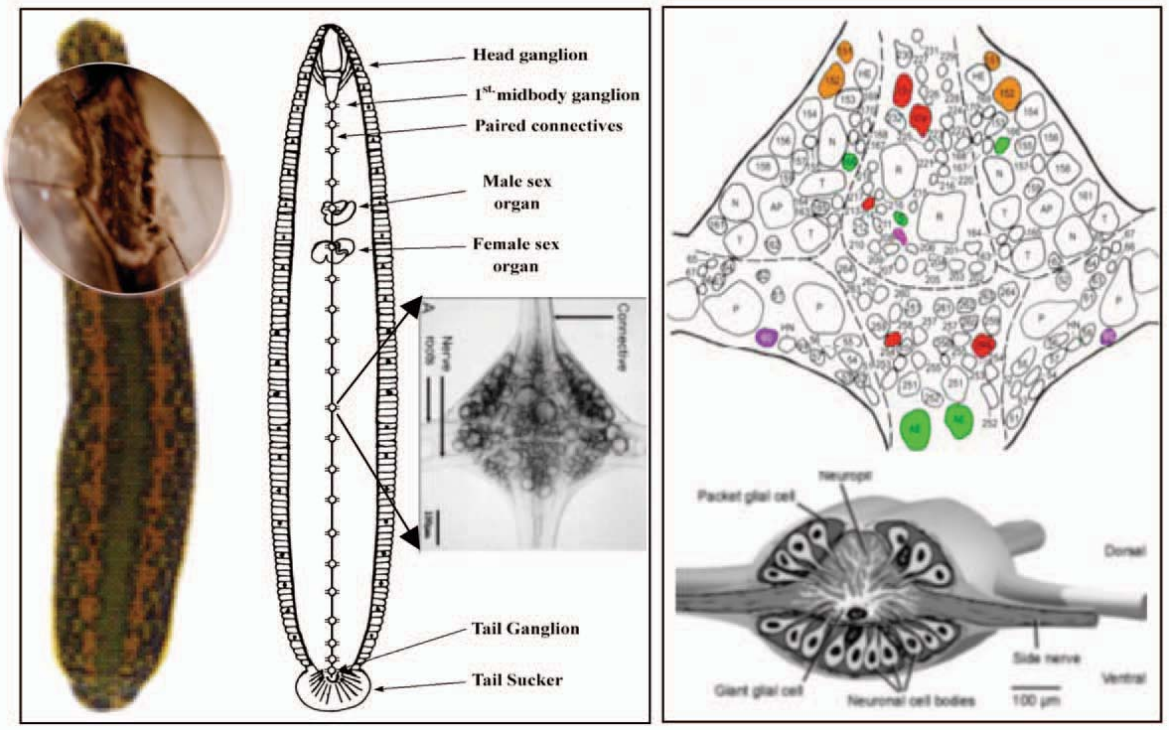

Figure 1. The central nervous system (CNS) of the leech has a fixed number of 32 bilateral neuromeres. The four anterior-most neuromeres fuse to form the sub-esophageal ganglion and the seven posterior-most fuse to form the tail ganglion; single bilateral neuromeres comprise individual ganglia found in each of the corresponding mid-body segments. The central ganglia (insert, inset photography) are connected to each other by a bilateral pair of nerves (lateral "connectives") and a single small medial nerve (Faivre's) and to the periphery by two bilateral pairs of nerves ("roots") that branch in the body wall and carry motor activity centrifugally and sensory signals centrally. B) (Top) Diagram of a leech ganglion with some well-known neurons labeled. One of our goals is to create protein profiles for all of these neurons in order to understand the molecular mechanisms that give rise to their different functions. (Bottom) Drawing showing the typical organization of a leech ganglion: a rind of cell bodies and a central neuropil containing processes and synapses.

NO and Cannabinoids in Leech Microglia Chemotaxis Involvement

To assay directly for a role of NO on microglial accumulation at the injury site, Chen et al[15] modulated NO levels in several ways. As demonstrated by NOS immunoreactivity, a large increase in NOS occurs at the crush site within 5 min of injury and this high level persists for at least 24 hrs. Microglial accumulation at the lesion, however, is not detectable at 5 min but is quite strong after a few hours and peaks at ~24 hrs. Inhibition of NO synthesis by the prior application of the NOS inhibitor L-NAME effectively blocks microglial accumulation, while the presence of its inactive enantiomer D-NAME has little or no effect. Interestingly, increasing NO levels with the NO donor spermine NOate (SPNO) also inhibits accumulation of microglia at the crush, but not in the presence of the NO scavenger cPTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide).Examination of microglial kinetics in living nerve cords shows that the effect of SPNO application occurs by the reduction of average microglial migratory speeds, even to no movement. Thus, NO is clearly implicated as a modulator of microglial movement and indeed appears to function as a stop signal at high levels, leading to the higher density of these cells at the injury site. Moreover, we recently found that the NO time-course profile in the injured leech brain is partially under the control of endocannabinoids, namely, anandamide (AEA) and 2-arachidonylglycerol (2AG), which affect the NO time-course through their modulation of cannabinoid-like receptors.[16] AEA blocked microglial cell accumulation before their arrival to the lesion site in a concentration dependent-manner and this effect of AEA on microglial cell recruitment was also demonstrated in vitro. Moreover, stimulation with a concentration of AEA determined elsewhere by (Matrix assisted laser desorption ionization time of flight/ time of flight (MALDI TOF/TOF)mass spectrometry[17] enhanced the release of NO at the lesion site of the harmed connective. This NO release may be related to the AEA-activated CB1-like receptor carried by neurons of the leech present in the injury site. This same report suggested that this pathway leads to a microglia stop signal. In a complementary mode of action, the second most described endocannabinoid 2AG has been shown to enhance and drive the chemotaxis of microglial cells in a dose-dependent manner. Moreover, a better result in chemotaxis was obtained using a concentration of 2AG detected 30 min after injury of the leech nerve cords.[17] This result is reinforced by Cabral et al who suggested that 2AG could act through the autocrine/paracrine system to chemoattract microglial cells after brain insult.[18] In leech brain, AEA level declines[17] and in contrast the 2AG climbs during the first 4 h following a lesion of the leech CNS.[16]

Knowing that these two endocannabinoids are able to act as chemoattractant factors on leech microglia in a dose-dependent manner in combination with NO release, the role of their receptors in the control of NO release and chemotaxis towards the lesion site has been investigated.[16] Blocking the CB1-like receptor in the leech with a specific antagonist of the mammalian CB1 receptor (AM-251) failed to block the accumulation of microglial cells at a distance from the lesion site. This latter result underscores the role of the CB1-like receptor in microglial chemotaxis. On the other hand, blocking the CB2-like receptor in the leech with the specific antagonist AM-630 completely abolished the recruitment of microglial cells at the lesion site and costimulation of a crush with 2AG and AM-630 failed to reverse the accumulation of microglia as compared to the 2AG treatment alone.[16] This latter result suggests that the CB2-like receptor in the leech is triggered by 2AG in order to promote chemotaxis and the direct recruitment of microglial cells to the site of injury. In parallel, the treatment of injured connectives with a physiological concentration of 2AG released upon CNS injury in the leech provoked a

specific response in NO production at the lesion site just after the stimulation.[16] Blockade of the CB2-like receptor by AM-630 revealed an inhibition of NO secretion and the time-course of NO production was significantly delayed in comparison with the control condition. In this way, the whole of these data demonstrated that at least part of the NO produced by the injured leech was related to the 2AG-activated CB2-like receptor, as was described in experiments using Guinea pig mast cells.[19]

Even if 2AG and AEA are both able to produce NO during a lesion of the leech brain, only 2AG seems to play a major role in the chemotaxis of microglia and its capacity to do so is closely related to a putative functional CB2-like receptor expressed by activated microglia. Previous pharmacological studies have named the CB2 receptor as playing a crucial role in the early inflammatory process, thereby implicating microglia in this process as well in mammals. The CB2 receptor is expressed very early in the different activation steps of microglia, thus describing a "window" of functional relevance for the expression of the CB2 receptor in microglial cells.[20] The delayed time of the initiation of microglial cell activation is linked to changes of their morphology from resting to responsive and allows them to acquire CB2 receptors in correlation with chemotaxis and phagocytosis. The next steps of activation, named "primed" and "responsive," were discarded from the initial step of CB2 receptor expression. As a diffusible molecule, NO has been demonstrated to take part in the migration of microglia and their accumulation at lesions of the leech CNS in a dose-dependent manner. The mechanisms by which the resting microglia becomes responsive are not yet fully understood. However, we hypothesize that the NO immediately released by the damaged neurons might stimulate the resting microglial cells present at the lesion site and allow them to be responsive for CB2-like receptor expression.

Thus, the cannabinoid system becomes activated at the lesion site and produces the two major endocannabinoids (AEA and 2AG) from membrane precursors in an opposite concentration time-course of,[17] consistent with our previous work where the 2AG concentration was found to progressively increase in opposition with the AEA concentration after the lesion. It has been hypothesized that when the diffusible lipid 2AG increases, the microglia become responsive and start to accumulate at the lesion site via changes in cell morphology related to the activation of the CB2-like receptor expressed in the lamellipodia of responsive microglia.[21] This hypothesis is reinforced by our ex vivo results regarding the stimulation of crushed connectives with 2AG (30 μM) 1 h after the lesion.[16] In comparison with the connectives simultaneously crushed and treated with 30 μM 2AG, the same stimulation 1h after the crush is stimulated NO production more rapidly. This observation suggests that microglial cells are more responsive to 2AG 1h after the lesion than at the instant of the lesion, at which time microglia might be resting. During the responsive step, the leech microglia might express functional CB2 receptors at the cell surface and when these receptors are activated by the endocannabinoid 2AG, NO might be produced and participate in cell recruitment as a chemical gradient from the lesion site towards the periphery. On the other hand, the AEA released at the lesion site decreased in concentration after the lesion and it can activate NO release by targeting the CB1-like receptor and inhibiting the accumulation of microglia at the lesion site. This pathway has been described as leading to a microglia stop signal.[22] The dual activity of these two endocannabinoids working in apparent opposition might be a means of controlling microglial cell recruitment to the lesion site. However, both molecules control the long-term release of NO, as shown in our time-course measurements of NO after stimulation of injured connectives with cannabinoids.[16] This can be explained by the immunosuppressive response of microglial cells described both in vertebrate[23] and invertebrate models.[22]

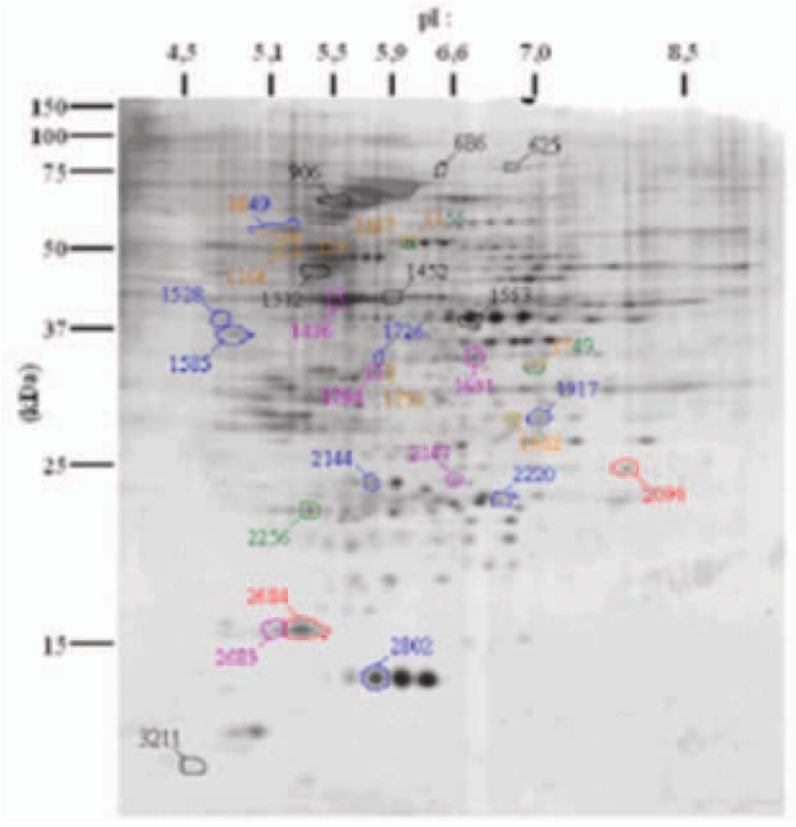

Figure 2. 2D gel between control and immune challenged leech nervous systems. Differentially represented spots are highlighted by arrows and depends on the variation times (variations at 1 h, 6 h, 12 h and 24 h) from reference 26.

Bacterial Infection and Leech Brain Regeneration

Differential display proteomic analyses using 2D gel electrophoresis (Fig. 2), coupled to mass spectrometry, yielded evidence that the leech CNS responds to bacterial infection by modulating the expression of at least sixteen proteins. These proteins appear between 1 and 24h after bacterial challenge and have been assigned to the immune response because they are not induced by exposure to control sterile medium. These immune-response induced proteins include cytoskeletal and metabolic proteins, foldases, calcium sensors, kinases and neurohemerythrin, reflecting specific cytoskeletal rearrangements linked to cell migration, vesicular trafficking and/or phagocytosis, as well as the modulation of synaptic activity. Interestingly, several of these up-regulated proteins, such as gliarin[24] and neurohemerythrin[25] are expressed specifically in glial and microglial cells, suggesting a key role for these cells in the immune response of the leech nervous system, similar to what has been observed in vertebrates.[26] Gliarin up-regulation, in particular, could thus serve as a new marker of proliferation and maturation of leech glial cells, it's up-regulation reflecting glial activation in response to the immune challenge. The accompanying

cytoskeletal rearrangements might result from morphological changes associated with phagocytosis or with the migration of cells. Indeed, it has been shown that leech microglial cells are able to migrate within the ganglionic chain to the site of a lesion and that they can play a phagocytic role.[27] By contrast, the expected properties of neurohemerythrin have led to the proposal of several putative functions for this protein in the responses of leech nervous tissue: (i) a role as an oxygen supplier for metabolism, (ii) a role as a trap for reactive oxygen species and NO, protecting cells from cell death and (iii) a role as an antibacterial factor depriving bacteria of iron.[28]

The data from these initial studies of the effects of bacterial toxins show that the leech CNS is able to respond in an intrinsic manner to a septic stimulus, mounting a "neuroimmune" response. Careful study of potential roles of the identified proteins will be essential to fully understand the mechanisms involved, but some candidates can be proposed on the basis of the proteins identified in this study (ref. 26): (i) cytoskeletal rearrangements potentially responsible for morphological changes, cell migration, vesicular trafficking and/or phagocytosis, (ii) modulation of synaptic activity, (iii) calcium signalling and (iv) unfolded protein response controlling the functionality of proteins affected by the stress generated by the sepsis. The involvement in innate immunity of some proteins or protein families we identified here as previously been described by transcriptomic studies, but at the peripheral level and not within the nervous system.[29,30] Moreover, the protein families involved in the immune response of the medicinal leech nervous system appear to also be involved in nerve regeneration, as shown by Blackshaw *et al*, also in the medicinal leech[31] (discussed above) and by Perlson *et al* in *Lymnaea stagnalis*[32] (Table 1).

These observations suggest certain parallelism between CNS defence mechanisms and CNS regeneration leading the possibility of the involvement of neuroinflammation in such phenomena in leeches. Modulation of neuroinflammation after a crush seems to be necessary but highly regulated in the CNS during regeneration through cannabinoids as we previously demonstrated.[22,33-36] Interestingly, evidences that microbial infection triggers the leech brain regeneration (Fig. 3) has led us to perform some biochemical studies focused on antimicrobial peptides present in the leech CNS and expressed in course of infection or trauma. Two novel antimicrobial peptides, *Hm*-lumbricin and neuromacin have been fully characterized.[37] Neuromacin and *Hm*-lumbricin exert bactericidal activities against Gram positive bacteria without any haemolytic properties.[37] We have observed that in addition to exert antimicrobial activities, *Hm*-lumbricin and neuromacin have regenerative effects on the leech CNS.[37] The capacity of both peptides to promote the regeneration of the leech nerve cord was tested ex vivo by adding the neuromacin and/or *Hm*-lumbricin antibody(ies) to axotomized nerve cords in presence of killed bacteria.[37] Due to the presence of bacteria, the reconnection process should have started two days post-axotomy. It appeared that the presence of antibodies in the culture medium blocked the regeneration process since no reconnection was observed even seven days post-axotomy.[37] These observations were corroborated by the data obtained by adding native neuromacin to axotomized nerve cords under aseptic conditions. Nerve repair was evident sooner in the presence of neuromacin, reconnection starting in less than one day instead of four without an exogenous contribution in neuromacin.The participation of endogenous neuromacin and *Hm*-lumbricin in the neural repair is sustained by the accumulation of both peptides at the lesion site upon bacterial challenge of injured nerve cords.[37] Further investigations based on single cell RT-PCR analysis and on immunohistochemical analysis of a model, developed by our group, of leech CNS almost completely devoid of microglial cells allowed

Table 1. Similarities between proteins involved in innate immunity within invertebrates and those involved in central nervous system regeneration within Lophotrochozoaria, all identified by proteomic or transcriptomic approaches

Immunity		Leech CNS	Leech Periphery	Insect Periphery
Cytosk.	Microfilaments IF	Tropomyosin gliarin	Tropomyosin-2 Actin-2	Myosin II reg. light-chain Actin 5C
Calcium	Calcium sensor	NCS-2/Neurocalcin		Calmodulin, sarc. CaBP1
Metabolism	AA/nt metabolism Energy Others	AA dehydrogenase ATP synthase β subunit Acetyl transferase	Aldehyde dehydr.	Aldehyde dehydr. ATP synth. β subunit
Hsp and chaperones	Cyclophilin/PPI PDI	Cyclophilin PDI	PPI	FK506-BP-PPI, Cyclophylin PDI (ERp60), CaBP1-PDI
Metal-oxidation	Resp. molecule Others	Neurohemerythrin		Hemocyanin Thioredox., Transf., ferritin
Regeneration		**Leech CNS**	**Mollusc CNS**	
Cytosk.	Microfilaments IF Microtubules	Protein 4.1 Synapsin α- and β-tubulin	Tropomyosin Intermediate filament Tubulin	
Calcium	Calcium sensor Others	Calmodulin-like	Calmodulin, Calbindin Calpain	
Metabolism	AA/nt metabolism Energy	ATPase inhibitor	Glutamine Synthase ATP Synthase	
Hsp and chaperones	Cyclophilin/PPI PDI Others	 Hsp90	Cyclophilin PDI Hsp60, 14-3-3	
Metal-oxidation	Resp. molecule Others	Myohemerythrin COX I	Peroxiredoxin, Ferritin	

AA, aminoacyl; ATP synth., ATP synthase; calcin., calcineurin; CNS, central nervous system; COX I, Cyclooxygenase I; Cycloph., cyclophilin; dehydr., dehydrogenase; IF, intermediate filaments; nt, neurotransmitters; phosph., phosphatase; PK, protein kinase; PPI, peptidyl-prolyl cis-trans isomerase; sarc., sarcoplasmic; reg, regulatory; resp. molecule, respiratory molecule; Transf, transferring.

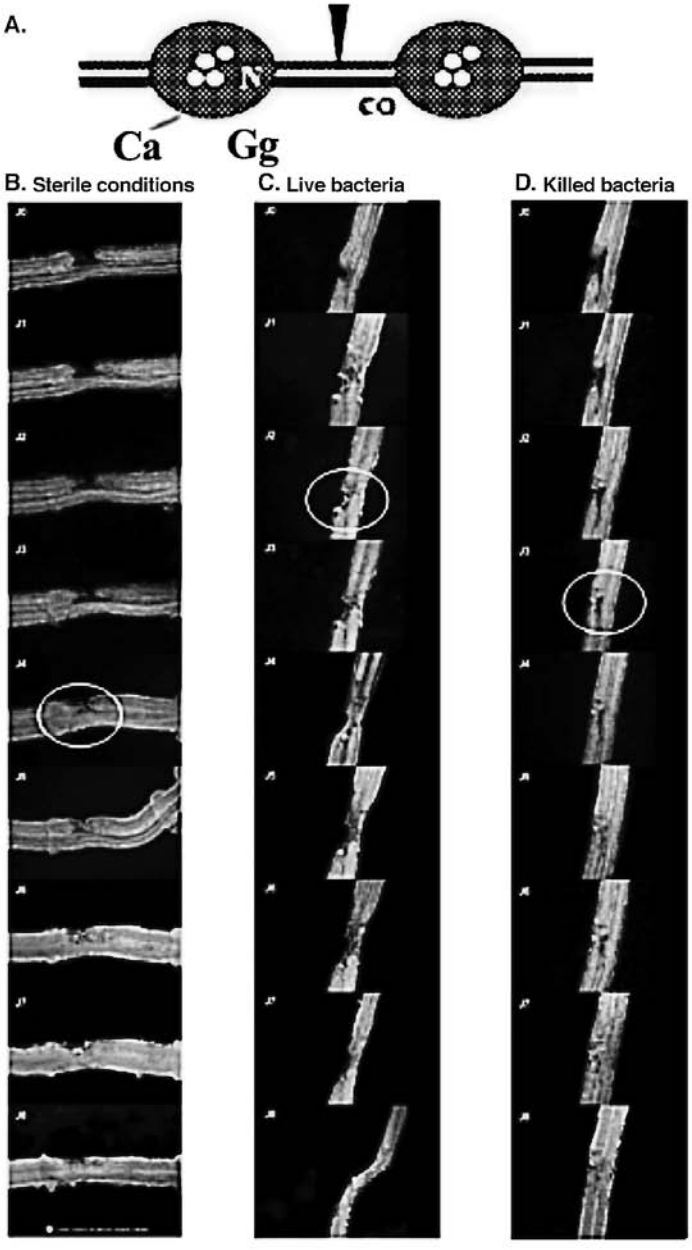

Figure 3. Effects on nerve regeneration of exposure of excised leech CNS to live or heat-killed bacteria (A) Diagram of the leech CNS in culture preparation. Neuron cell bodies (N) within ganglia (Gg) project axons into connectives (co) towards adjacent ganglia. V indicates the location of the cut of one of the two connectives linking two segmental ganglia. Microglial cells, evenly distributed in the nerve cord, are represented by dots. The nervous system is protected by a fibrous capsule (Ca). B-D) Sequential micrographs, taken 24 hr apart, from one (J1) to eight days (J8) post-axotomy, documenting the regeneration of the severed connective nerve. B) Preparation in sterile culture medium, (C) incubated with live bacteria and (D) incubated with killed bacteria.

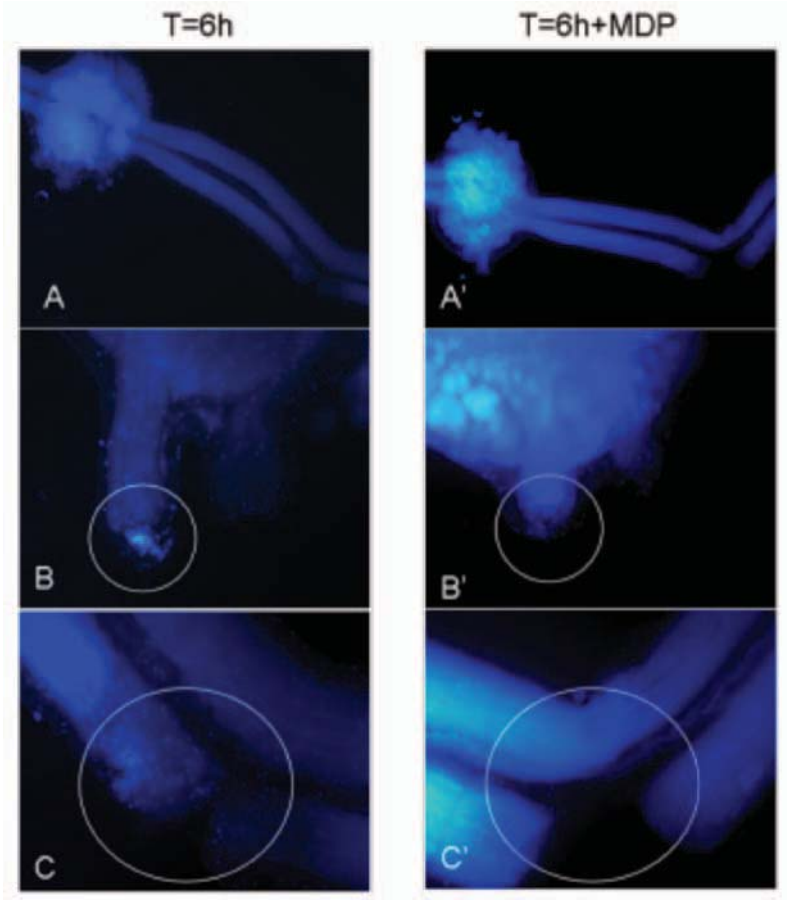

Figure 4. Effects on the recruitment of microglial cells of exposure of injured leech CNS to Muramyl DiPeptide (MDP). A) without addition of MDP to the medium culture, an accumulation of microglial cells is observed by nuclear staining at the injured site of both lesioned lateral (B) and interganglionary connective (C). (A′, B′, C′)This cell accumulation is not visible anymore post treatment with MDP.

us to conclude that the presence of both *Hm*-lumbricin and neuromacin at the axotomized site implicates peptide production by neurons and by the microglial cells recruited at the lesion site.[37] However silencing studies have not allowed connecting such antimicrobial peptides with *Hm*TLR1 receptor. Moreover, although bacteria cocktail enhance leech brain regeneration, Muramyl DiPeptide (MDP) blocks microglia migration (Fig. 4) which is line with the data obtained on macrophages migration, also inhibited by MDP.[38,39]

Taken together, these data confirm the immune role of the leech microglia and the presence of specific sensing receptor in these cells. The next step is the characterization of these receptors and effectors contained in leech brain. For this purpose, we started by *in silico* analyses through medicinal leech adult EST obtained by a consortium between Professsor E. Macagno, Professor Terry Gaasterland and Professor Michel Salzet. 91,233 transcripts were obtained from Genoscope (France) and JGI (USA) before annotated and 31,232 sequences were obtained.[8]

Leech Brain Immune Receptors and Effectors

Medicinal Leech Toll-Like Receptors (HmTLRs)

Five medicinal leech Toll-like receptors (TLRs) have been detected, one has already been fully characterized and data from the other ones are due soon. The first *Hm*TLR characterized is presented in Figure 5.[40] TLRs in general, share similarities in their extracellular Leucine Rich Repeat (LRR) and their intracellular Toll/IL-1 Receptor (TIR) domains. The TIR domain plays a central role in TLR signaling. All TLRs contain a cytoplasmic TIR domain, which, upon activation, acts as a scaffold to recruit adaptor proteins. It is well established that the differential recruitment of adaptors to TLRs provides a significant amount of specificity

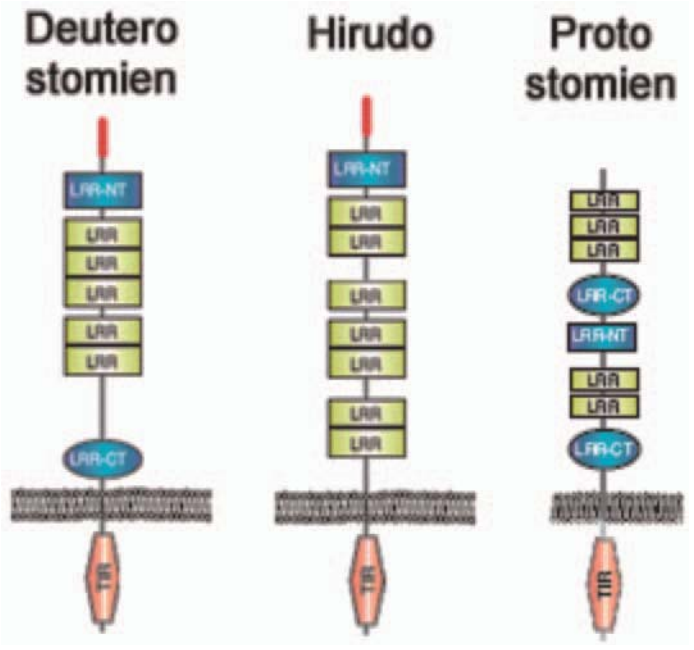

Figure 5. Structure comparison of *Hm*TLR1 and Protostomian and Deurterostomian TLRs.[40] SMART™ sequence analysis of the Nterminal part of *Hm*TLR1 revealed the presence of one LRRNT followed by six LRRs. *Hm*TLR1 presents the originality to exhibit an array of LRRs capped by one LRRNT only. By contrast to most TLRs described in invertebrates and vertebrates species, no LRRCT domains were identified from the analysis of the ectodomain of *Hm*TLR1. Blastp analysis was realized on the entire amino acid sequence of *Hm*TLR1. Data reveal great homologies mainly with TLR13s characterized in vertebrate species such as the mouse *Mus musculus* and the opossum *Monodelphis domestica* and in a lesser extent with TLRs of animals living in freshwater such as the zebra fish *Danio rerio*, the goldfish *Carassius auratus*, the salmon *Salmo salar* or again the rainbow trout *Oncorhynchus mykiss*. No homology with molecules identified in other lophotrochozoa was noticed. A second step, Blastp analysis of the LRR and the TIR domains of *Hm*TLR1 were performed separately, in order to get information on the function and the signalling pathway associated with this receptor respectively. The LRR domain of *Hm*TLR1 significantly (e-values < e-12) matches with the sequences of LRRs implicated in (i) pathogen recognition such as the LRR domain of the TLR3 and those of some Variable Lymphocyte Receptors (VLRs) and (ii) with the LRRs of vasorin, decorin and netrin known to participate in tissue remodelling and/or axonal guidance in vertebrates. Thus, both regenerative and immune functions could be attributable to this receptor. Concerning the TIR domain of *Hm*TLR1, Blastp analyses evidence a great percentage of homology with the TLR13.

to the TLR-signalling pathways. Among these adapter proteins MyD88 and TRIF are now considered as the signalling ones and hence the TLR pathways can be categorized as MyD88-dependent and TRIF-dependent. The LRR domain is an extracellular domain implicated in the detection of pathogens. Based on the organization of the extracellular LRR array, two types of TLRs have been described. Vertebrate TLRs have an array of LRRs capped by cysteine-rich domains located at the N- and C-terminal LRR domains (LRRNT and LRRCT, respectively). By contrast, most of invertebrate TLRs also contain LRRNT and LRRCT domains, but instead of capping the LRR array, these are located within the array in a tandem orientation. Interestingly, leech *HmTLR*1 presents the originality to exhibit an array of LRRs capped by one LRRNT only sharing sequence similarity with mouse TLR3. Based on EST medicinal leech sequence and Helobdella genome, four other HmTLR has been detected and their complete characterization is now in progress.

We recently demonstrate that *Hm*TLR1[40] is localized in both neurons and microglia and expressed upon septic trauma (Fig. 6). *Hm*TLR1 is co-expressed with a cytokine

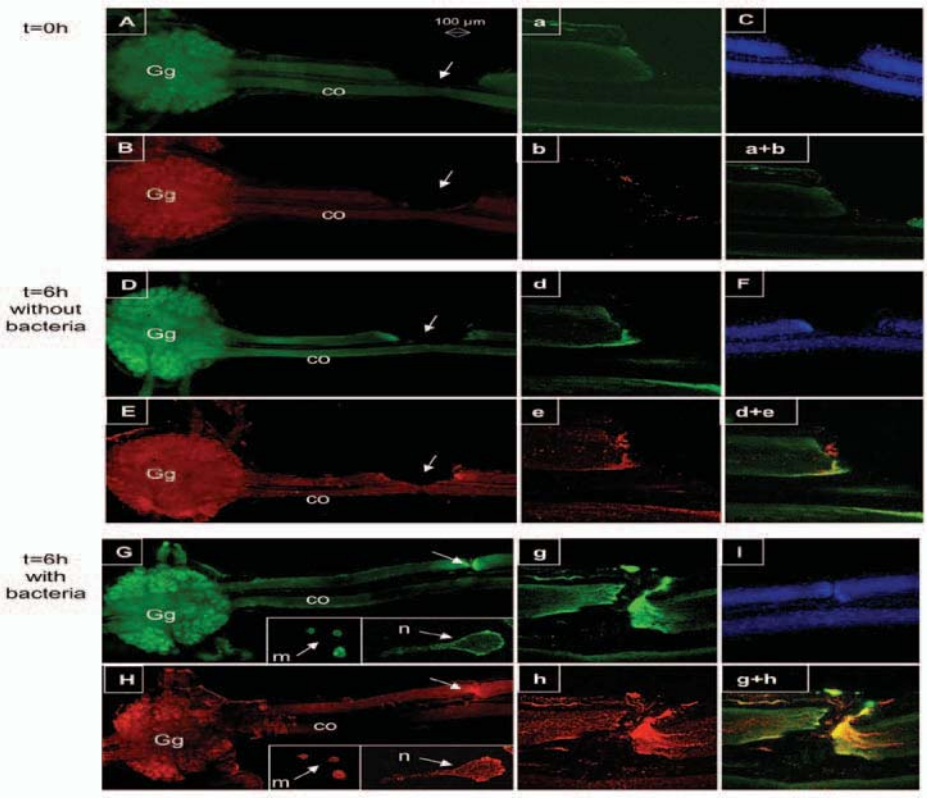

Figure 6. Co-appearance of *Hm*p43/EMAPII (Green) and *HmTLR*1 (Red) in the injured CNS incubated or not for 6 h with killed bacteria. Double staining of injured nerve cords was performed at t = 0 and t = 6 h post-axotomy using the fluorescent nuclear dye Hoechst 33258 (C, F and I) with both the anti-p43/EMAPII (A, D and G) and the anti-*HmTLR*1 (B, E and H) polyclonal Ab. Immunodetection was performed using green-labelled anti-EMAPII and red labeled anti-*HmTLR*1 secondary Ab. The results demonstrate an accumulation of *Hm*-EMAPII (G, g) and *HmTLR*1 (H, h) at the lesion site 6 h after axotomy in the presence of bacteria from reference 40. A color version of this figure is available at www.landesbioscience.com/curie.

related to the Endothelial Monocyte Activating Polypeptide (*Hm*EMAPII) sharing chemoattractive activity (Fig. 6). The complete intracellular trafficking upon pathogen challenges has to be undertaken to confirm the functionnal similarity between *Hm*TLR1 and mammalian TLR3.

*Hm*TLR1 and the Leech Cytokine (EMAPII)

Functional studies using silencing studies have not allowed connecting such antimicrobial peptides with *Hm*TLR1 receptor. By contrast, a cytokine sharing microglia chemoattactant activity recently characterized by our group in the medecinal leech e.g., *Hm*EMAPII,[40] shown based on RNAi silencing qPCR, western blot experiments (Fig. 7) and biological tests an association to *Hm*TLR1.[40] *Hm*EMAPII is processed from *Hm*p43 like mammals EMAP II. We hypothesized that *Hm*EMAPII could exert a chemoattractant effect on microglial cells as mammalian EMAPII does on monocytes. The chemoattractive effect of *Hm*EMAPII is blocked when an antihuman EMAPII antibody underscoring for the first time the ability of EMAPII to exert chemotactic effect toward microglial cells through CXCR3.[40] Leech CXCR3-related receptor cloning is on the way. These data points out that *Hm*TLR1 is linked to leech EMAPII and gives for the first time an immune function to a TLR in a nonecdysozoan model *i.e.,* in an invertebrate model different from *Caenorhabditis elegans* and *Drosophila melanogaster*.

All together, these data reflect that the medicinal leech express *Hm*TLR related to mammalian TLR. The first receptor characterized showed an intracellular localization and *Hm*TLR1 is linked to the cytokine related to EMAPII which exerts chemottractive effect after brain trauma or upon pathogen challenge. All these data confirm for the first time the presence of a complete TLR-signalling-Cytokine pathway implicated in immune response in medicinal leech nervous system. Such complex is also present in mammals reflecting again a co-evolution between the medicinal leech and its host mammals. It has also to be noted that such mechanism conservation is in line with a common origin of nervous system centralization between annelids and vertebrates as shown in the polychaete, *Platylnereis dumeriliis*.[41-43] Thus, the data presented above indicate that major players in innate immune response like danger sensing receptors coupling to cytokines or antimicrobial peptides and microglia are present in leech and strongly resemble that in vertebrates.

THEROMYZON TESSULATUM AS A MODEL FOR STUDYING THE PERIPHERAL IMMUNE RESPONSE

T. tessulatum is an ectoparasite of aquatic birds. Its life cycle was arbitrarily subdivided in stages (these are not larval stages) defined by taking, as indicators, the three blood meals. The third stage which corresponds to the gametogenesis phase is characterized by an important water uptake making the collection of the body fluid easy (Fig. 8). For this reason, *T. tessulatum* constitutes a convenient model for studying the antimicrobial response which takes place at the systemic level in coelomic fluid.[44] As a comparison, the medicinal leech has a parenchymatous body, coelomic cavities are reduced and the botryoidally tissue is immersed in a connective tissue. This makes the collection of the body fluid impossible. The antimicrobial response of *T. tessulatum* was investigated at the molecular level by focusing on the antimicrobial peptides (AMPs) released in the coelomic fluid and at the cellular level by determining the immune functions of the coelomocytes.

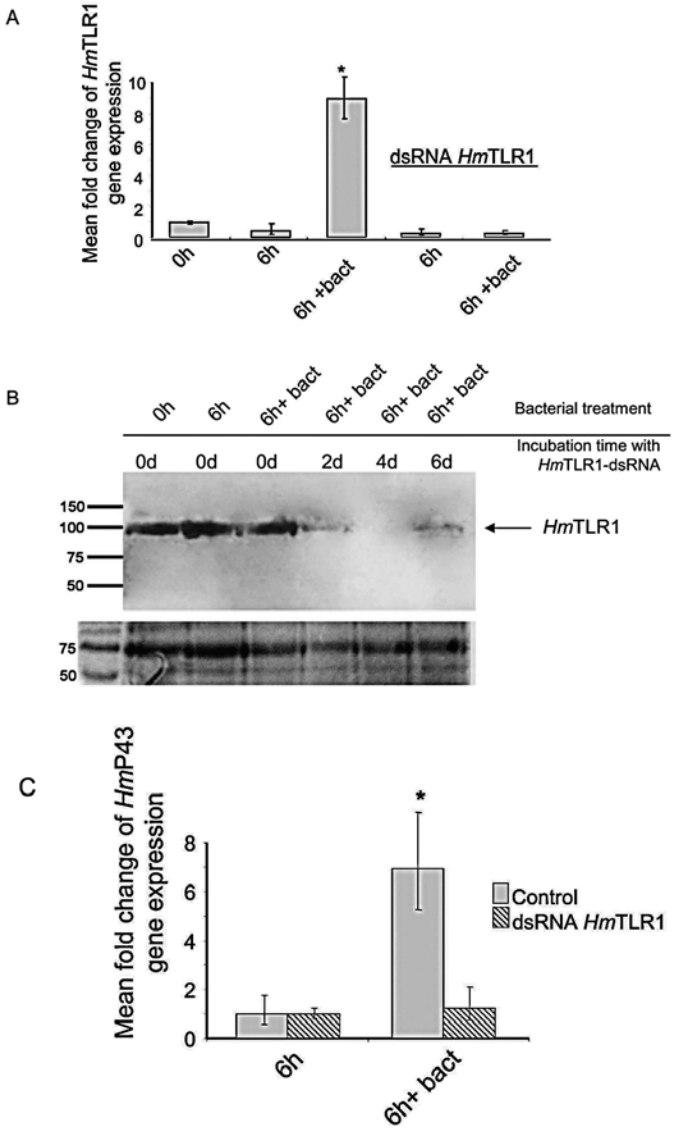

Figure 7. Impact of the *Hm*TLR1 gene silencing on induction of the HmP43/EMAPII gene in the leech CNS incubated with bacteria. A) The efficiency of the knock-down was quantified by measuring the level of *HmTLR*1 expression in nerve cords incubated with or without dsRNA. Data are expressed as relative levels comparatively to the basal level of expression measured in nerve cords processed immediately after sampling (0 h). *HmTLR*1 expression was quantified after 6 h of culture without bacteria (6 h) or with a mix of heat-killed Gram+ and Gram- bacteria (6 h + bact), revealing an induction of *HmTLR*1 gene under septic conditions. The bacterial induction of *HmTLR*1 is significantly reduced when the CNS is incubated for 4 days with *HmTLR*1-dsRNA (B) Western blot analyses of *HmTLR*1 protein level in the same conditions as for Figure 1. Best protein extinction is observed at 4 days (4d) of incubation with specific dsRNA confirming the efficiency of the knock down and the specificity of the anti *HmTLR*1 antibody. d, day (C) *HmTLR*1 gene silencing abolished the bacterial gene induction of *Hm*EMAPII observed in the control (without dsRNA *HmTLR*1), indicating a role of the *HmTLR*1 in the gene regulation of this cytokine in the leech CNS under septic conditions.[40]

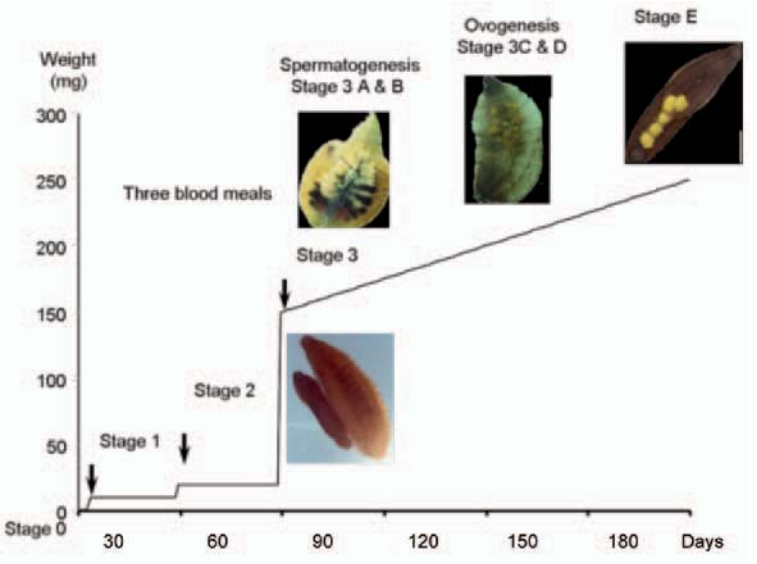

Figure 8. Life cycle of *Theromyzon tessulatum* from reference 61.

AMPs of *T. tessulatum*

Three antimicrobial peptides (AMPs) were isolated and fully characterized from the body fluid of *T. tessulatum*. These are theromacin, a cysteine rich AMP exhibiting bactericidal activities, theromyzin an anionic peptide with bacteriostatic properties[44] and peptide B an anionic peptide matured from a neuropeptide precursor, proenkephalin A (PEA).[45] They all present an activity directed against Gram positive bacteria. Recently, a cDNA encoding a peptide presenting high percentage homologies with lumbricin-1, an AMP firstly characterized from the earthworm *Lumbricus rubellus* was cloned in *T. tessulatum* (Fig. 9).

Theromacin belongs to the cysteine rich AMP family. In invertebrates, most of them share the disulfide array of the insect/arthropod defensin.[46] In addition to having ten cysteine residues instead of six, theromacin does not harbor this consensus sequence. Theromacin by contrast with neuromacin (see before) has never been evidenced in other lophotrochozoan models or in ecdysozoan and thus seems to be restricted to leeches.

Lumbricin is a linear peptide without any posttranslational modifications. As the majority of AMPs described in the literature, theromacin and lumbricin possess a global positive charge presumably allowing their interaction with the negatively charged bacterial membrane (Fig. 10).

Theromyzin and peptide B, in contrast to theromacin and lumbricin, are anionic molecules. The mode of action of anionic AMPs is still unknown even if several hypotheses have been advanced. In vertebrates, AMPs with anionic properties were evidenced in the human and the sheep lung[47] AMPs are anionic because of homopolymeric regions of aspartate and require zinc as a cofactor for bactericidal activity.[48] Histatins, a family of histidin rich AMPs found in human saliva, also need the presence of zinc ions for bactericidal activities. Circular dichroism studies showed that the antimicrobial activities of histatin-5 require a conformational change that results from the interaction

Figure 9. A) Nucleotide sequence of *Theromyzon tessulatum* lumbricin cDNA. The deduced amino acid sequence of the open reading frame is presented under the nucleotide sequence. B) Alignment of the *Tt* lumbricin (Lum T.t.) with the lumbricins characterized from the earthworm *Lumbricus rubellus* (Lum L.r.) and from the medicinal leech *Hirudo medicinalis* (Lum H.m.).

of the peptide with both zinc ions and negatively charged membranes.[49] The abundance of histidine residues at the N-terminal part of theromyzin could argue in favor of some common structures between the leech antibacterial peptide and histatins.

Consequently, it clearly appears that leeches present a relatively large variety of AMPs. It is interesting to remark that at present no defensins have been isolated from leeches and from annelids in general. Defensins which are considered as the most widespread family of invertebrate AMPs have not been found neither in the genomes of the leech

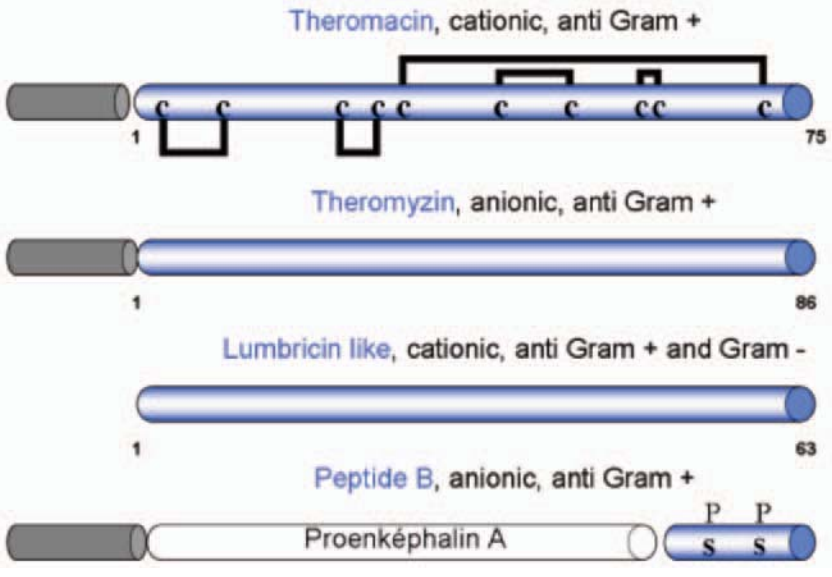

Figure 10. AMPs characterized in *T. tessulatum* from the coelomic liquid.[57]

Helobdella robusta ant the polychaeta *Capitella* nor in the EST libraries of the earthworm *Lumbricus terrestris*, *Eiseinia fetida* and *Hirudo medicinalis*. Reciprocally most AMPs described in annelids have not been found in the genomes of ecdysozoan invertebrate such as *C. elegans* and *Drosophila melanogaster*.

Expression Site and Regulation of the AMPs Synthesis in *T. tessulatum*

Theromacin, *theromyzin* and *Tt lumbricin* genes are preferentially expressed in large fat cells (LFC) evenly distributed in the leech and in contact with the coelomic liquid from which the AMPs were purified. Their transcriptional level is enhanced after bacteria challenge evidencing a regulation of the leech AMPs similar to that of the insect antimicrobial peptides genes. Indeed, in the fruit fly, genes encoding antibiotic peptides are rapidly induced following a septic injury.[50] The similarity between the antibacterial response of the leech and those of holometabol insects is also supported by the functional resemblance between the leech LFC and the insect fat body which possess the common capacity to produce egg-yolk proteins.[51]

Moreover, based on our first investigations, no difference in gene expression was observed after Gram positive or Gram negative injection suggesting that the antibacterial response of *Theromyzon* is aspecific. This nonspecificity has also been assumed in *Drosophila* until the work of Lemaitre *et al* demonstrated that the humoral antimicrobial response of the fruit fly discriminates between various classes of microorganisms and mounts a response that is adapted to the infection.[52] That suggested that in a more natural mode of infection the leech could also adapt its antimicrobial response, what was recently confirmed in our annelid models by using bacteria living in the environment of the leech.[37]

The peptide sequences deduced from the *theromacin* and *theromyzin* genes contain putative signal peptides, indicating that mature peptides correspond to secreted molecules. As for the lumbricin like characterized in the medicinal leech, the *Tt* lumbricin precursor lacks the typical signal peptide. We assume that *Tt* lumbricin as demonstrated in *Hirudo* could be secreted through a nonconventional mechanism already observed but still unexplained for several molecules in mammals also.

Peptide B is not produced by the LFC although it was also isolated from the body fluid of the leech. Its precursor, PEA, was immunodetected into circulating coelomocytes suggesting that peptide B could be released from these cells. In contrast to the other leech AMPs, the production of peptide B seems to be more regulated at the translational level by the enzymes implicated in the PEA processing than at the transcriptional level. Of equal importance is the finding that enkephalin such as methionin enkephalins (ME) and peptide B are simultaneously released from PEA. Invertebrate and vertebrate immunocytes contain delta 2 opioid receptors that appear to mediate activation of these cells. In this regard, ME can be envisioned to activate immunocytes and provide a chemotactic signal to further stimulate cell recruitment.

However, since this process may take many minutes to accomplish, the bactericidal peptide B may cover this activation latency period. In this scenario, peptide B is broken down with time, it could release during this time, the heptapeptide MERF. Since we demonstrated that this peptide was able to interact with delta 2 opioid receptors, MERF could keep the rate of immunocyte activity.[45] This hypothesis is supported by previous studies demonstrating an immune activating role for MERF in human and invertebrate immunocytes. MERF as well as ME was shown to induce rounded invertebrate immunocytes to become mobile and amoeboid as well as to initiate chemotaxis. (Fig. 11).

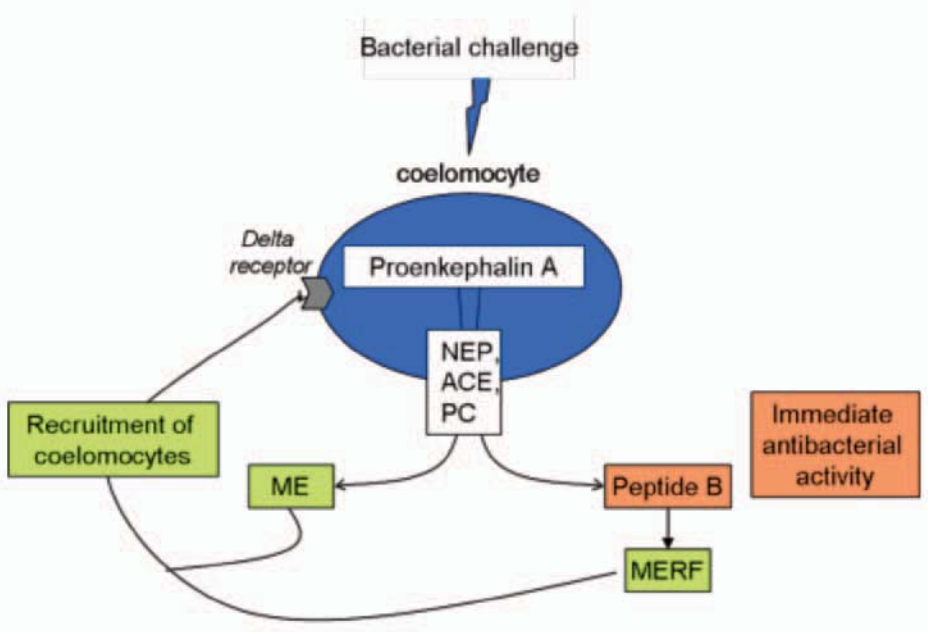

Figure 11. Illustration for the involvement of PEA derived peptides in the immune response of *T. tessulatum.* Upon an initial stimulus, i.e., bacterial challenge, smaller bioactive peptides Met enkephalin (ME) and peptide B processed by different enzymes _PC, NEP, ACE are released into the coelomic fluid of the leech. Peptide B plays its antibacterial role and is cleaved in a second time into Met enkephalin arg phe (MERF) which as ME does, can recruit coelomocytes.

Whatever the mode of regulation is, the accumulation of AMPs into the coelomic fluid after septic injury suggests that these peptides play their antimicrobial activities through a systemic action. Moreover the presence of theromacin and theromyzin in the intestinal epithelial cells and at the epidermis level also evokes participation in epithelial defense and/or in the control of the symbionts. The localization of antibiotic molecules in gastrointestinal tract has also been reported in insects and in vertebrates where they provide a rapid local immune response against exogenous pathogens brought in during feeding.[53] Theromacin, lumbricin and theromyzin were detected in the mucous covering the animal. That reminds the local defensive response reported in frogs in which antibacterial peptides secreted in the mucous prevent bacteria colonization and/or subsequent infection.[54]

As for lumbricin-1 in *L. rubellus*, physiological events occurring during gametogenesis phase appeared to be inducers of the AMPs gene expression in *T. tessulatum*. These data suggest that several hormonal factors implicated in sexual maturation may participate in the induction of genes encoded AMPs in annelids as described in *Drosophila* by Meister et al.[55] Interestingly, leech AMPs were detected in the eggs by mass spectrometry analysis whereas the transcripts were not amplified by RT-PCR (Fig. 12).

These results suggest a vertical transmission of the PAMs which could exert a protective role against bacteria during eggs development. AMPs may accumulate into

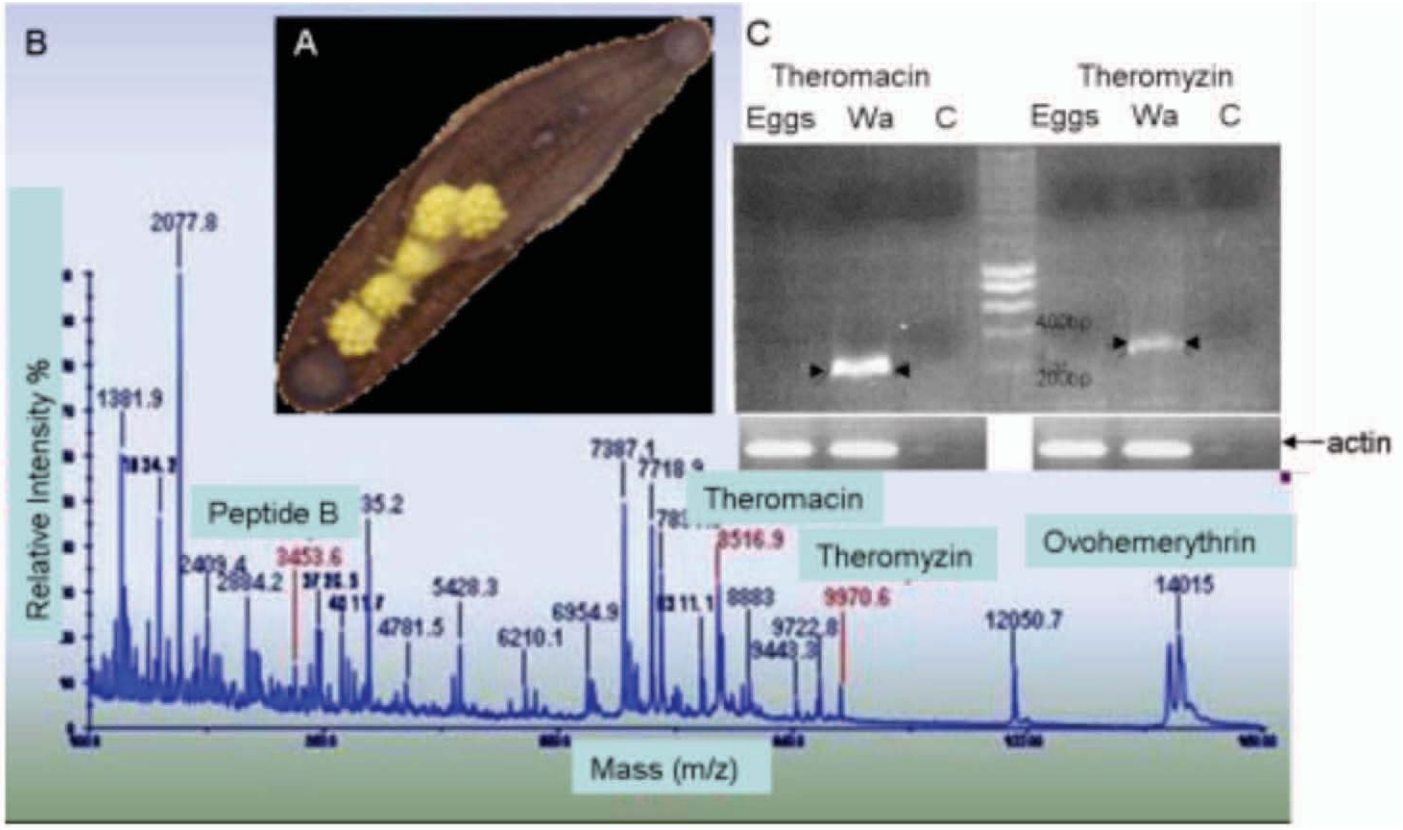

Figure 12. A) Ventral view of a Stage 3 *Theromyzon tessulatum* showing eggs (yellow balls) regrouped within five cocoons. B) Detection by mass spectrometry of molecular masses corresponding to theromyzin and theromacin in eggs extracts. C) Transcripts encoding theromacin and theromyzin are amplified by RT-PCR in whole animals (Wa) but not in eggs. C corresponds to the water control. A color version of this figure is available at www.landesbioscience.com/curie.

A

	Theromacin		Theromyzin	
	Unchallenged	Challenged	Unchallenged	Challenged
Stage 0	0	0	0	0
Stage 1	0	+	0	+
Stage 2	0	+	0	+
Stage 3	+	++	+	++

B

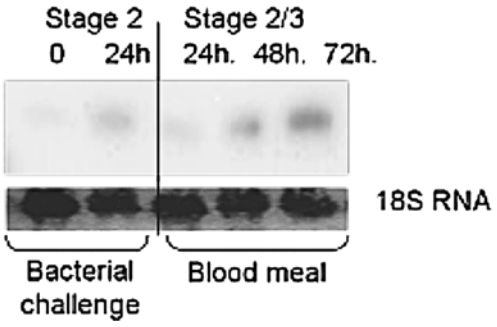

Figure 13. A) Table presenting the detection of transcripts coding for theromacin and theromyzin in leeches pricked or not with a mix of bacteria, at different stages of their life cycle. B) Northern blot showing that the theromacin gene expression is inducible upon bacteria challenge in Stage 2 leeches and after the last blood meal which marks the transition between the Stage 2 and 3.

the eggs by being captured from the body fluid of the mother though a pinocytosis mechanism before laying as described for egg yolks proteins and/or from the mucous covering the eggs after laying. A mass corresponding to ovohemerythrin, an egg yolk protein abundantly presents in the leech body fluid was detected by mass spectrometry analysis of eggs extracts supporting the first hypothesis without excluding the second one since AMPs were also detected in the mucous covering the leech. The study of the gene expression in course of the post-embryonic development demonstrated that *T. tessulatum* starts to synthesize its own AMPs from the Stage 1 of its life cycle and that a bacterial challenge is necessary for observing this synthesis. Indeed, a basal level of transcripts encoding theromacin and theromyzin was detected in unchallenged Stage 3 leeches only. We presume that AMPs are expressed at a basal level in adults for providing an immune protection to the eggs by a vertical transmission of the antibiotic molecules. By contrast, a bacterially inducible response is observable from the Stage 1 to the Stage 3 suggesting that leeches acquire the ability to establish an immune response after their first blood meal (Fig. 13).

Consequently, *T. tessulatum* is an original invertebrate model which has developed two modes of fighting infections by AMPs: (i) storage of antibacterial peptide derived from PEA and release of the peptide into the coelomic fluid after immune challenge (ii) induction after septic injury of gene coding for more classical AMPs, mainly in LFC and rapid release into the body fluid of the antibiotic peptides. Data collected from our group (unpublished) suggest that the same AMPs participate to the systemic antimicrobial

response of the medicinal leech, *Hirudo medicinalis*. Interestingly, the PEA processing appeared to be very well conserved in course of evolution since we have demonstrated that the same mechanism is observable during surgery in human patients undergoing cardiopulmonary bypass.[56,45]

The Cellular Immune Response

Annelids are primitive coelomates known to possess specially developed cellular immunity against microorganisms including phagocytosis, encapsulation and spontaneous cytotoxicity against allogenic or xenogenic cells.[57,58] In leeches, our group has characterized three distinct populations of coelomocytes in *T. tessulatum* (Fig. 14).

These are constituted by chloragocytes, granular amoebocytes and small coelomocytes.[30] Leech chloragocytes are the only cells expressing both the *Tt* cathepsin L and the *Tt* cystatin B. Granular amoebocytes present an immunoreactivity to the anti *Tt* cathepsin L antibody (Ab) although the small coelomocytes are not recognized by neither the anti *Tt* cathepsin L Ab nor the anti *Tt* cystatin B Ab. The immune functions

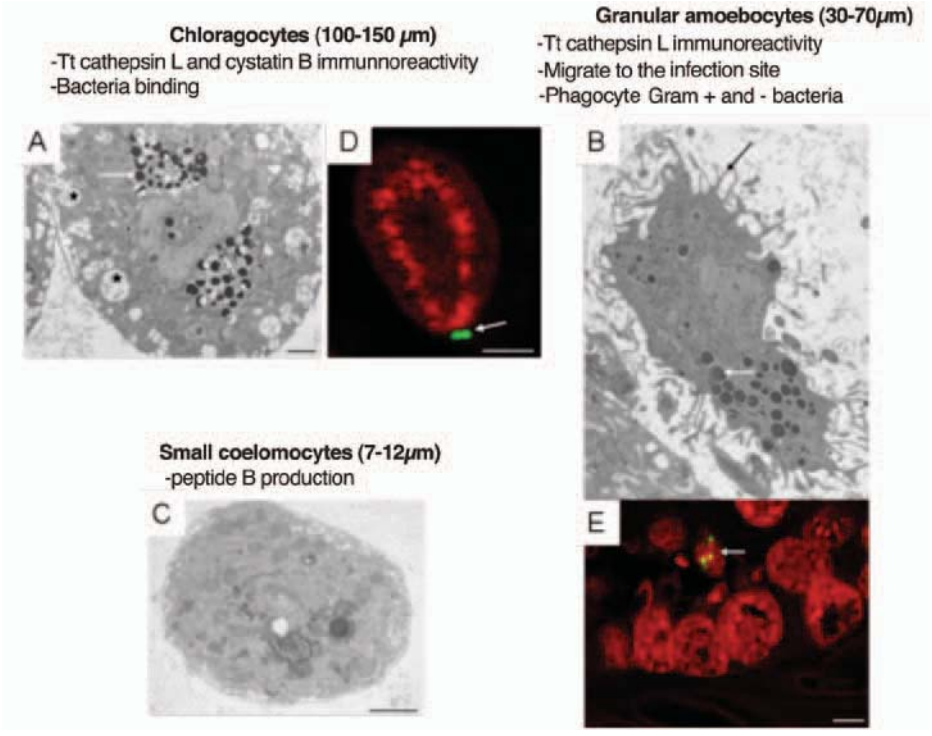

Figure 14. Coelomocytes of *T. tessulatum*. A) Large coelomocytes present large electron-dense granules (white arrow) and electron-lucent vesicles (black stars). B) Intermediate size coelomocytes show long cytoplasmic pseudopods (black arrow) and large electron-dense granules (white arrow). C) Small circulating cells are rich in endoplasmic reticulum and small granules. D-E) Animals were injected with FITC-labelled bacteria (killed *M.luteus* or *E. coli*). After 24 hours of incubation, bacteria are still observed near chloragocytes (D, white arrow) but phagocytosed bacteria are observed in granular amoebocytes only (E, white arrow).

were investigated by essentially focusing on the phagocytic activity and the migrating property of these circulating cells. As resumed in the Figure 14, the granular amoebocytes are able to migrate to the injection site of microorganisms and to phagocyte without any apparent distinction both killed Gram positive and Gram negative bacteria. The molecular mechanism of recruitment may imply enkephalin peptides derived from the PEA maturation process, such as ME or MERF. By contrast, leech chloragocytes are not able to phagocyte bacteria. However, while no phagocytosis was detected, confocal microscopy analysis evidenced chloragocytes-bacteria interaction suggesting the presence of recognition molecule expressed at the surface of these cells (Fig. 14D). Leech chloragocytes may also be implicated in encapsulation reactions as described in oligochaeta annelids. The third type represented by the small coelomocytes presents the morphology of invertebrate hyaline cells i.e., a cytoplasm deprived of granules. However the leech cells have been considered not to be hyaline cells due to their incapacity to phagocytose killed bacteria. Interestingly, our group has very recently observed that the use of live bacteria was a prerogative for inducing the phagocytosis process in a population of leech blood cells. Thus, the small coelomocytes should be incubated with live bacteria to determinate whether they could be assimilated to hyaline cells or not.

This heterogeneous population of coelomocytes reminds the population described in other annelids. Numerous studies performed in oligochætes as *Lumbricus sp*. and *Eisenia sp*[59] used optical and electron microscopy. Although monoclonal antibodies were carried out against various invertebrate taxa, the majority of the studies related to insect hæmocytes. De Eguileor *et al* identified three coelomic cell populations, i.e., macrophage-like, NK-like and granular cells, using human monoclonal antibodies in the hirudinea *Glossiphonia complanata*.[58] Engelmann *et al* produced monoclonal antibodies against coelomic cells in *Eisenia fetida* earthworm.[60] While anti-EFCC1 antibody (*Eisenia fetida* coelomocyte differentiation cluster) is able to recognize antigenic motifs on various tissues, three other antibodies named anti-EFCC2, anti-EFCC3 and anti-EFCC4 allowed to respectively discriminate chloragocytes, hyaline amoebocytes and granular amoebocytes.[60] Because oligochætes and leeches are closed relatives, it should be interesting to test these anti-EFCC antibodies for discriminating our coelomic cells and possibly identify the presence of phagocytic hyaline cells in *T. tessulatum*.

Unlike other invertebrates, one of the particularities of annelids is to possess a closed circulatory system separated from the coelomic cavity. Our group is investigating the immune function of the circulating blood cells of the medicinal leech. Most of the reported data being focused on coelomic cells, the obtained results will constitute the first description of the function of the blood cells in an annelid. Moreover since the leech central nervous system permanently baths into the blood, these data may open up new avenues for discovering the impact of the immune response on the neural repair of the medicinal leech.

CONCLUSION AND PERSPECTIVES

The observations we have reviewed in this chapter attest to the broad range of inquiry, from structure to function, and to the breadth of the techniques currently employed to study the defence, repair and maintenance of the leech nervous and immune systems. Clearly, the increasing application of biochemical and molecular genetic tools is beginning to yield insights into the nature of the molecular mechanisms responsible for these phenomena.

But, while progress is being made, it is also clear that there is a strong need to accelerate the implementation and application of genomic, transcriptomic and proteomic tools to the leech. The leech model has many important advantages, perhaps the most important being the ability to bridge from the immune response to neural repair, in the context of having, or the possibility of having, detailed knowledge about all the neurons in the CNS and thus an unparalleled level of completeness. Most other invertebrate systems, as well as all vertebrate systems, under current study, can only afford partial, because many or most of their neurons are inaccessible and only population properties are attainable. However, other systems, including *C. elegans*, *Drosophila*, zebrafish, and some rodents, greatly benefit from the possibility of doing standard genetics, their shorter reproductive cycles (particularly in comparison to Hirudo, not as much with respect to small leeches like Helobdella), and the availability of the complete sequence of their genomes. Nonetheless, the ability to conduct systems level functional studies, and the possibility of relating the physiological programs to genetic programs that encode the underlying circuitry and its properties, the capacity of the system to defend itself, regenerate and repair, and the availability of reverse genetic tools to study gene expression and regulation, all strongly justify continuing and enhancing our efforts to understand neuroimmune responses in the leech.

ACKNOWLEDGMENTS

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