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# A Homologous form of Human Interleukin 16 is Implicated in Microglia Recruitment Following Nervous System Injury in Leech *Hirudo medicinalis*

**FRANÇOISE CROQ,[1] JACOPO VIZIOLI,[1] MARINA TUZOVA,[2] MURIEL TAHTOUH,[1] PIERRE-ERIC SAUTIERE,[1] CHRISTELLE VAN CAMP,[1] MICHEL SALZET,[1] WILLIAM W. CRUIKSHANK,[2] JOEL PESTEL,[1] AND CHRISTOPHE LEFEBVRE[1]***

[1]*Université Lille Nord de France, Université Lille 1, Laboratoire de Neuroimmunologie des Annélides, Centre National de la Recherche Scientifique, FRE 3249, IFR 147, F59655 Villeneuve d'Ascq, France*

[2]*Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts*

*KEY WORDS*

nerve cord repair; microglial cells; chemotaxis; invertebrate cytokine

*ABSTRACT*

In contrast to mammals, the medicinal leech *Hirudo medicinalis* can completely repair its central nervous system (CNS) after injury. This invertebrate model offers unique opportunities to study the molecular and cellular basis of the CNS repair processes. When the leech CNS is injured, microglial cells migrate and accumulate at the site of lesion, a phenomenon known to be essential for the usual sprouting of injured axons. In the present study, we demonstrate that a new molecule, designated *Hm*IL-16, having functional homologies with human interleukin-16 (IL-16), has chemotactic activity on leech microglial cells as observed using a gradient of human IL-16. Preincubation of microglial cells either with an anti-human IL-16 antibody or with anti-*Hm*IL-16 antibody significantly reduced microglia migration induced by leech-conditioned medium. Functional homology was demonstrated further by the ability of *Hm*IL-16 to promote human CD4+ T cell migration which was inhibited by antibody against human IL-16, an IL-16 antagonist peptide or soluble CD4. Immunohistochemistry of leech CNS indicates that *Hm*IL-16 protein present in the neurons is rapidly transported and stored along the axonal processes to promote the recruitment of microglial cells to the injured axons. To our knowledge, this is the first identification of a functional interleukin-16 homologue in invertebrate CNS. The ability of *Hm*IL-16 to recruit microglial cells to sites of CNS injury suggests a role for *Hm*IL-16 in the crosstalk between neurons and microglia in the leech CNS repair. 

## INTRODUCTION

In contrast to the mammalian CNS, which has a limited capacity for repair after damage, the medicinal leech can completely restore normal functions of its CNS after injury. In mammals, microglial cells are regulators of tissue homeostasis and are involved in pathological processes orchestrating tissue remodeling. In vertebrates, microglia are currently considered to also function as sensors in the brain (Kreutzberg, 1996). Under neuropathological conditions, microglia are activated and migrate to and within the lesions. Activated microglia are characterized by a morphological transition from a quiescent stellate form to a macrophage-like phenotype. Microglial cells were first designated "microglia" in the leech CNS (del Rio-Hortega, 1932). Indeed, leech microglia behave similarly to vertebrate microglia by migrating in response to tissue damage, by changing their morphology (Elliott and Muller, 1981; Masuda-Nakagawa et al., 1990) and by their potential phagocytic properties (Perry and Gordon, 1988). When the leech nerve cord is injured, microglia migrate toward the lesion and accumulate at the damage site (Chen et al., 2000; Kumar et al., 2001; Masuda-Nakagawa et al., 1990; von Bernhardi and Muller, 1995). This cell migration was shown to be an essential step for the axon sprouting required for an efficient regeneration (Ngu et al., 2007). Thus, to characterize soluble factors involved in microglia recruitment, we investigated the chemoattractant effect of culture medium generated by injured leech CNS.

Using this approach, we identified a chemoattractant factor that had significant homology with the mature form of human IL-16. This factor was designated *Hm*IL-16 (GenBank Accession EU629212). Human interleukin-16 is a proinflammatory chemoattractant cytokine originally termed lymphocyte chemoattractant factor (LCF; Center and Cruikshank, 1982; Cruikshank and Center, 1982). IL-16 is produced by numerous cell types including lymphocytes and microglia (Center et al., 2000). The mature (secreted) bioactive form of IL-16 corresponds to a C-terminal peptide which is generated following caspase-3 cleavage (Zhang et al., 2001). Mature human IL-16 is a ligand for CD4 and has been demonstrated to induce a migratory response in a variety of CD4+ cell

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*Correspondence to: Christophe Lefebvre, Université Lille 1, Cité Scientifique, SN3, 59655, Villeneuve d'Ascq cedex, France. E-mail: christophe.lefebvre@univ-lille1.fr

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types, such as T and B lymphocytes, monocyte/macrophages, dendritic cells, as well as to induce migration in mammalian CD4+ cells. In human brain, IL-16 is constitutively expressed by a microglia subpopulation and may attract CD4+ lymphocytes across the blood-brain barrier (BBB) under pathological conditions (Schluesener et al., 1996). A paracrine role of IL-16 was also demonstrated in inflammation following cerebral ischemia (Schwab et al., 2001a). IL-16 may be implicated in some neurodegenerative diseases such as multiple sclerosis (MS) lesions where enhancement of IL-16 production suggests a role in regulation of inflammation in axonal damages (Mittelbronn et al., 2001; Schwab et al., 2001a,b; Skundric et al., 2006).

In the present study, we demonstrate the chemotactic activity of *Hm*IL-16 contained in CNS-conditioned medium for leech microglia and further, provide evidence that the neuronal cells are the cell source. Interestingly, we showed that leech IL-16 (*Hm*IL-16) is functionally homologous to human IL-16 and is able to induce CD4+ T cells to migrate *in vitro*. Some data suggest that *Hm*IL-16 could bind to human CD4. To our knowledge, this is the first report of such a cytokine with an associated biological function in protostomes. Consequently, the present study highlights a new cytokine in invertebrates that is highly evolutionarily and functionally conserved in CNS and suggest a role for *Hm*IL-16 in the crosstalk between neurons and microglia in the leech CNS repair.

## MATERIALS AND METHODS

### Central Nervous System Preparation

*Hirudo medicinalis* adult leeches were obtained from Ricarimpex (Eysines, France). After anesthesia in 10% ethanol at 4°C for 15 min, animal CNS (nerve cords) were dissected out into a sterile Ringer solution (115 mM NaCl, 1.8 mM $CaCl_2$, 4 mM KCl, 10 mM Tris maleate, pH 7.4) under a laminar flow hood. After isolation, nerve cords were placed in three successive baths of antibiotics (100 UI $mL^{-1}$ penicillin, 100 μg $mL^{-1}$ streptomycin, and 100 μg $mL^{-1}$ gentamycin) for 15 min and further incubated in Leibovitz L-15 medium (Gibco, Invitrogen, USA) complemented with 2 mM L-glutamin, 0.6% glucose and 10 mM Hepes (complete medium).

### Study of Microglial Cell Morphology After Injury: Immunocytochemistry of CNS with Gliarin

The leech CNS consists of head and tail ganglia and 21 segmental ganglia. Each segmental ganglion contains about 400 neurons and is linked to its neighbors by thousands of axons that form the connectives. The entire central nervous system of the leech lies in a blood-filled sinus but no blood vessels penetrate the ganglia or connectives. Of interest, all experimental procedures were performed from sinus-free CNS, which excludes any possible involvement of blood cells. In the adult CNS, microglial cells are only resident circulating cells evenly distributed in leech ganglia and in the bundle of axons (ganglia) that connect to them. Of note, the CNS does contain neither astrocytes nor oligodendrocytes. To study the microglia morphology in normal conditions and after nerve crush directly in the connectives, an antibody against a glial-specific intermediate filament protein, gliarin, was used (a kind gift from Dr. Johansen, Iowa State University, USA) to whole mount preparations (Xu et al., 1999). Gliarin is an intermediate filament protein specifically expressed in glial cells of the leech nervous system. Nerve cords were dissected and fixed as described above. The connective capsules were opened with fine forceps for better antibody penetration. Fixed samples were washed in PBS, permeabilized by a 24-h incubation in PBS containing 1% Triton X100 at room temperature and preincubated in blocking solution [BS; 1% Triton, 3% Normal Donkey Serum (NDS) and 1% Ovalbumin in PBS] for 8 h at room temperature. Samples were then incubated with mouse monoclonal anti-gliarin antibody (1:2,500) diluted in the AB solution (PBS containing 1% BSA, 0.05% Triton, 1% NDS and 1% ovalbumin) overnight at 4°C. After three washes with PBS, goat anti-mouse IgG antibody (Invitrogen, USA) conjugated to Alexa Fluor 488 was added (1:5,000 in the AB solution). Negative controls consisted of samples incubated with the preimmune serum. Samples were observed with a Zeiss LSM 510 Laser Scanning Confocal Microscope.

### Transmission and Scanning Electron Microscopy (TEM and SEM)

Nerve cords were fixed in glutaraldehyde 2.5% in 0.1M phosphate buffer, pH 7.4 for one night. For TEM, the sample nerve cords were postfixed in 1% osmium tetroxide in the same phosphate buffer. Nerve cords were dehydrated through an ascending series of ethanol exposure. For SEM, ganglia were then dried using hexamethyldisilazane using the protocol described by Bray et al. (1993) and metalized by gold sputtering using Balzers union SCD 040 before observation using a Jeol CX 100 electron microscope coupled to ASID-4D (Bray et al., 1993). For TEM, ganglia were embedded in Epon in the conventional manner (polymerization at 60°C for 48 h). Ultrathin sections with gold to silver interference colors (80–90 nm) were cut from the Epon blocks, placed on 200-mesh copper grids, routinely counterstained with uranyl acetate and lead citrate, and observed using a Jeol CX 100 electron microscope.

### Microglial Cell Preparation

Nerve cords were placed in 35-mm Petri dishes with 200 μL of complete L-15 medium. Each ganglion was carefully decapsulated by removing the collagen layer surrounding the nerve cord with microscissors. Nerve cells, neurons (10–70 μm) and microglial cells (5 μm),

were mechanically resuspended by gentle scraping and filtered through a 7-μm nylon mesh. The microglial cell population was then specifically collected and centrifuged at 1,000*g* for 10 min at room temperature (RT). The cell pellet was resuspended in complete medium (100 μL per nerve cord) for migration assays.

### Leech CNS-Conditioned Medium (CM) Preparation

Eight nerve cords which have been crushed or cut were cultured during a 24-h period as described above. The medium (500 μL) was then centrifuged 20 min at 1,000*g* to eliminate cells and tissues. The cell-free supernatant was then used as conditioned medium (CM) for western blot and chemotaxis experiments.

### Molecular Characterization

*Hirudo medicinalis* nervous system EST databases were granted by Hirudinea Consortium (http://www.cns.fr/externe/English/Projets/Projet_PE/PE.html). Sequence analyses used the BLAST programs (Altschul et al., 1997; Karlin and Altschul, 1990).

### Fluorescent *In Situ* Hybridization

Nerve cords were fixed for an hour at 4°C in buffered 4% paraformaldehyde, pH 7.4 directly after dissection or 24 h after incubation in complete L15 medium. The 5′ biotin-labeled specific antisense probe and sense probe (negative control) were generated from a linearized cDNA plasmid by *in vitro* transcription using Biotin RNA-labeling-Kit according to the manufacturer's instructions (Roche, Switzerland). The hybridization protocol was conducted as previously described (Nardelli-Haefliger and Shankland, 1992). Nerve cords were incubated in secondary anti-sheep antibody conjugated to Alexa Fluor 488 (Invitrogen, USA) diluted 1:5,000 in water, rinsed with PBS, and finally mounted with glycergel (Sigma Life Science, USA). Samples were kept at 4°C in the dark until observed with an inverted microscope (Leica DMIRE2).

### Western Blotting

Ten nerve cords were dissected and both RNA and proteins were extracted using the TRIzol Reagent (Invitrogen, USA, manufacturer's protocol). SDS-PAGE was conducted with a 12% acrylamide with Tris 150 g/HCL 0.6N running gel and a 4% acrylamide with Tris 150 g/HCL 0.8N stacking gel (Tastet et al., 2003). Migration was performed using a cathode buffer (0.6% Tris base, 2.5% Taurine, and 0.1% SDS) and an anode buffer (0.6% Tris base, 2.8% Glycine, and 0.1% SDS). Twenty microliters of the nervous system protein extract were added to the Laemmli buffer (Tris 120 g/HCl 0.8N, glycerol 50%, SDS 10% and DTT 7.7%). Gels ran at 70 V for 15 min and at 120 V for 45 min. Nerve cord extracts were loaded on SDS-PAGE gel prepared as previously described. Separated proteins were transferred to Hybond$^{TM}$-C Extra membrane (GE Healthcare Life Sciences, Amersham, USA) by electroblotting. The nitrocellulose membrane was preincubated in blocking solution, washed three times in PBS containing 0.05% Tween 20 and further incubated overnight at 4°C with the specific rabbit polyclonal anti-*Hm*IL-16 antibody (1:1,000). The anti-*Hm*IL-16 rabbit polyclonal antibody (Ab) was raised against a synthetic peptide corresponding to Ile$^{48}$ - Ile$^{63}$ amino acid sequence (Agro-Bio, France). After three washes in PBS containing 0.05% Tween20, a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (dilution 1:2,000) (Jackson Immunoresearch, USA) was added for 1 h at room temperature. After the final wash, bands were revealed using an ECL Kit (GE Healthcare Life Sciences, Amersham, USA) on Kodak® X-Omat LS film (Sigma-Aldrich, USA).

### Immunohistochemistry

Nerve cords were dissected and fixed as described above. Fixed samples were washed in PBS, permeabilized by a 24-h incubation in PBS containing 1% Triton X100 at room temperature and then incubated in blocking solution [BS; 1% Triton, 3% Normal Donkey Serum (NDS) and 1% Ovalbumin in PBS] for 8 h at room temperature. Samples were then incubated with anti-*Hm*IL-16 rabbit antibody (1:2,500) diluted in the AB solution (PBS containing 1% BSA, 0.05% Triton, 1% NDS and 1% ovalbumin) overnight at 4°C. After three washes with PBS, donkey anti-rabbit IgG antibody (Invitrogen, USA) conjugated to Alexa Fluor 488 was added (1:5,000 in the AB solution). Secondary antibody incubation, washing sample mounting and confocal microscopy were performed. Negative controls consisted of samples incubated with the preimmune serum. In some experiments, the specificity of the anti-*Hm*IL-16 antibody was controlled by the addition of the synthetic peptide at 1.6 μg mL$^{-1}$ to samples incubated with anti-*Hm*IL-16 rabbit antibody diluted in the AB solution. Samples were then observed with a Zeiss LSM 510 Laser Scanning Confocal Microscope.

### Chemotaxis Assay on Leech Microglial Cells

Because of the nonadherence nature of the cells, chemotaxis assays were conducted using the double-P assay, as described by Köhidai, with minor modifications (Köhidai, 1995). Thirty-five millimeters Petri dishes were filled with 1 mL of a 0.5% agar and 1% gelatin solution. After drying, two 6-mm diameter wells were generated, each one presenting a parallel individual channel. One well was filled with 50 μL of L-15 containing purified microglial cells (10,000 cells) and the other well with different concentrations of the human IL-16 (0, 0.2, 1, and 50 μg mL$^{-1}$) or cell-free conditioned medium (1, 1:2, 1:4, 0). Another channel was generated perpendicu-

larly to others using a coverslip. One hour later, the number of cells present in the chemoattractant containing well was determined. To assess specificity for *Hm*IL-16 and to correlate bioactivity with that of human IL-16, neutralizing experiments were conducted by preincubating microglial cells for 30 min at room temperature with either mouse monoclonal anti-human recombinant IL-16 (1:1,000) or with specific rabbit anti-*Hm*IL-16 antibody (1:2,500) before using conditioned medium as chemoattractant. Rabbit preimmune serum (dilution 1:2,500) was used as a negative control. The number of migrating cells was counted on a hemocytometer (three different counts for each condition) with a Zeiss axioscope microscope. Experiments were performed in triplicate. In the negative control condition, 10,000 microglial cells were loaded and only 2% (~200 cells) were finally counted in the other side (L-15 medium-containing well), representing baseline, or control migration. To compare this L-15 control with stimulated migration, the results are expressed as 100% of control migration in all chemotaxis assays. Comparisons between means were made using Student's *t*-test. Statistical differences were considered to be significant if $P$ was $<0.01$.

### Human Lymphocyte Isolation

Following informed consent, human primary peripheral blood mononuclear cells were isolated from healthy volunteers. The blood was collected in accordance with the guidelines established by the Boston University School of Medicine Institutional Review Board. Briefly, blood was collected in the presence of 100 U of heparin-sodium (American Pharmaceutical Partners) per mL of blood. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque centrifugation and suspended in complete M199 medium (Mediatech), consisting of M199 supplemented with 0.4% bovine serum albumin (USB Corp.), 240 U of penicillin and 240 μg of streptomycin (Gibco), and 20 mM HEPES (Gibco). T cells were negatively enriched using nylon wool (Polysciences) adherence, resulting in nylon wool nonadherent T cells. Purity, as assessed by fluorescence-activated cell sorting (FACS) analysis, consistently demonstrated >93% $CD3^+$ cells, of which on average 64% were $CD4^+$ and 30% were $CD8^+$. The cells were routinely incubated overnight in complete M199 medium before use in the chemotaxis assay or subset generation. Purification of T cells into $CD4^+$ and $CD8^+$ subsets was accomplished using a magnetic bead negative isolation technique (Dynal/Invitrogen). Resulting purity was assessed using FACS analysis and was routinely demonstrated at >95% for both $CD4^+$ and $CD8^+$ subsets. Following subset generation the cells were again incubated overnight prior to the chemotaxis assay.

### Chemotaxis Assay on Human T Cells

Cell migration was measured using a 48-well Boyden Chamber (Neuro Probe). For the migration assay, various concentrations of recombinant human (Fitzgerald, Concord, MA) or leech IL-16 were added to the bottom chamber at the designated concentrations. Fifty-five microliters of a suspension of $10^7$ cells per mL was added to the top chamber and allowed to migrate through 8-μm-pore-size cellulose nitrate filters for 1 h. Filters were removed, fixed with ethanol, and stained with hematoxylin (Sigma). Migration was calculated as the number of cells that had migrated beyond 50 μm into the filter. For the neutralization studies, the IL-16 preparations were incubated with either monoclonal anti-human recombinant IL-16 (0, 0.5, 1, and 5 μg $mL^{-1}$) or polyclonal anti-*Hm*IL-16 (0, 1:1,000, 1:500, and 1:250 dilutions) for 30 min prior to adding the cells. To assess specificity for IL-16, either anti-IL-16 antibody (1 μg), an IL-16 antagonist peptide (5 μg) or soluble CD4 (5 μg) were added to the cells before addition of the leech conditioned medium. The peptide sequence was RRKSLQSKETTAAGDS corresponding to amino acids Arg $^{106}$ to Ser $^{121}$ of human IL-16 and has been reported to block IL-16 bioactivity compared with a negative control which corresponds to a scrambled peptide containing the same residues in a randomly chosen sequence (Nicoll et al., 1999). An unrelated IgG2a was used as a supplementary negative control for neutralization. The cell counts from experimental conditions were compared with the migration from unstimulated (media alone, control) cell migration, which is expressed as 100%. In the typical migration assay for these studies, 10 to 15 cells per high-powered field are counted under control conditions. Ten high-powered fields are counted for each control and experimental condition, and the data are then expressed as a percentage of control cell migration.

### Effect of Anti- *Hm*IL-16 on the Accumulation of Microglia at a CNS Lesion

Segmental ganglia 2, 3, 4, and 5 were carefully dissected from the animal and pinned in separate plastic 35-mm Petri dishes (Falcon 3005 Becton Dickinson, Franklin Lakes, NJ) coated with silicone rubber (Sylgard 184; Dow Corning Midland, USA) and placed in L-15 complete medium. Injections of either specific anti-*Hm*IL-16 rabbit polyclonal antibody (1:2,500), preimmune serum (1:2,500) or PBS were done in the connective between ganglia 3 and 4, which were tied up with a loop of fine nylon thread to confine the effect of the injected solutions to this region. For injections, patch pipettes were pulled from borosilicate glass capillaries (outer diameter 1.5 mm, Clark GC 150F-10) using a two-stage horizontal micropipette puller (model P-97, Sutter instrument, USA) (pipette resistance 3–5 MΩ). One of the connectives was immediately cut or crushed with fine forceps on both sides of the injection site and the tissues were fixed 4 h after injection as described above. Microglial cell nuclei were stained with Hoechst 33342 fluorescent dye (1:1,000; Invitrogen, USA) for 10 min to observe accumulation of cells.

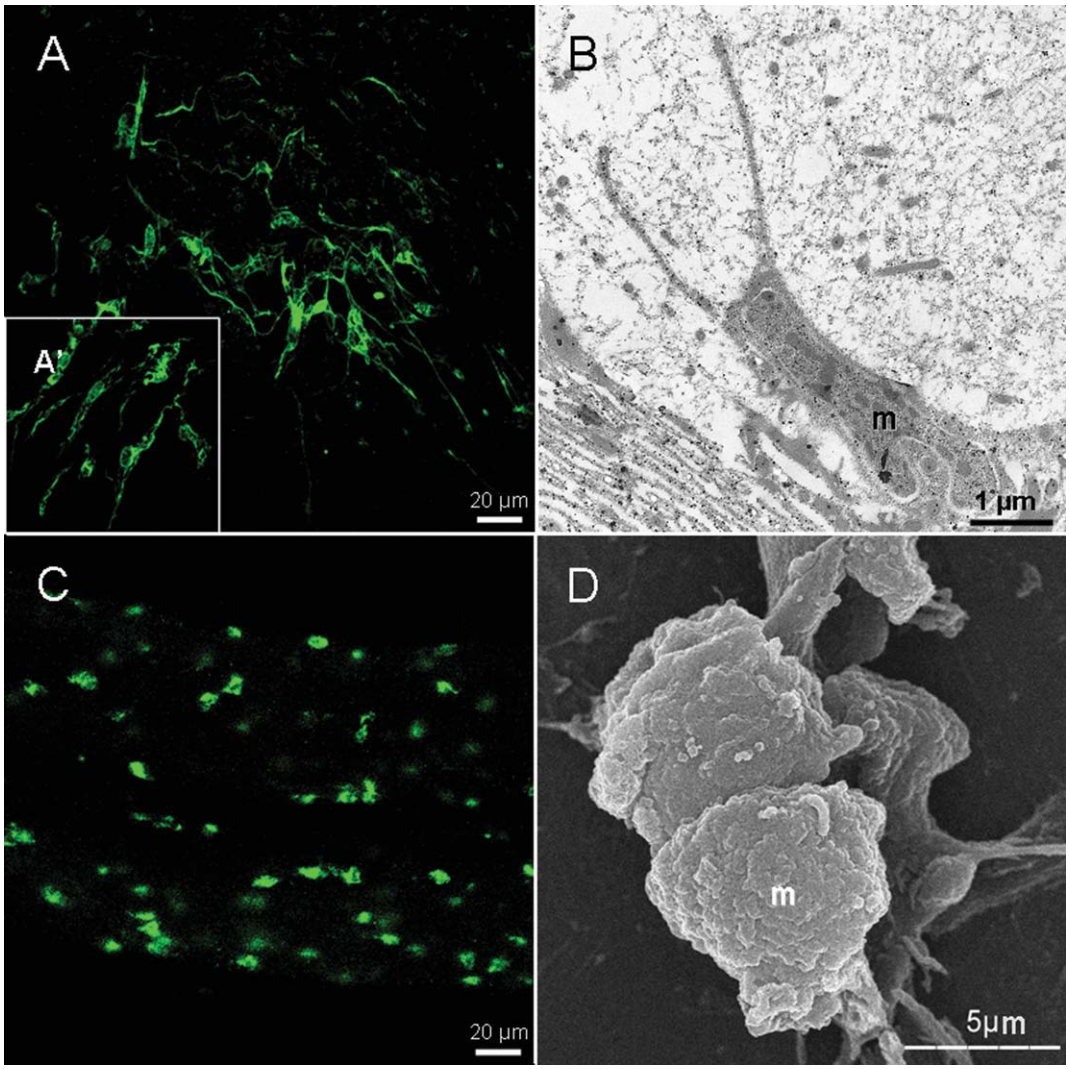


Fig. 1. Transformation of resting microglia (m) into activated cells after injury in a ganglion and in a connective. (**A**, **C**) Immunohistochemical staining using anti-gliarin antibody of quiescent stellate form of microglial cells in connectives (A) or in ganglia (**A**′) and activated microglial cells (C) with rounded shape after injury of a connective. (**B**) Transmission electron microscopy of an elongated microglial cell (m) with thin pseudopods in a ganglion. (**D**) Scanning electron microscopy of 24 h activated microglial cells (m) at the cut end of a connective. Typically, within hours after the lesion of a ganglion, some microglial cells have emerged from cut nerve roots and appeared as rounded cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

## RESULTS

### Changes in Microglial Cells Morphology After Injury

Immunohistochemical and ultrastructural techniques were used to study the transformation of resting microglia into activated cells after injury in a connective. For immunohistochemical studies to whole mount nerve cords, we used the monoclonal antibody anti-gliarin. Gliarin is an intermediate filament protein specifically expressed in glial cells of leech nervous system (Xu et al., 1999). By confocal microscopy, microglial cells stained with the anti-gliarin antibody appeared in the connective (Fig. 1A), as well as in ganglia (Fig. 1A′) as ramified spindle-shaped cells. TEM of a non-injured ganglion showed an elongated microglial cell with thin pseudopods (Fig. 1B) located in a packet of neurons between processes of a glial cell. The microglial nucleus appeared elongated and large compared with the entire cell. Twenty-four hours after injury, changes in morphology occurred in the connective, as demonstrated by staining with anti-gliarin antibody, indicating retracted, rounded, activated microglial cells that had been recruited to the lesion within the connective (Fig. 1C). As shown in Fig. 1D, several microglial cells with a rounded shape can be observed by SEM at the cut end of a connective. These morphological changes underline the key-observation indicating that, when the leech central nervous system (CNS) is injured, microglial cells migrate to the site of the lesion (Morgese et al., 1983).

### Molecular Characterization

Interestingly, leech CNS-conditioned medium induces a significant dose-dependent migratory response of

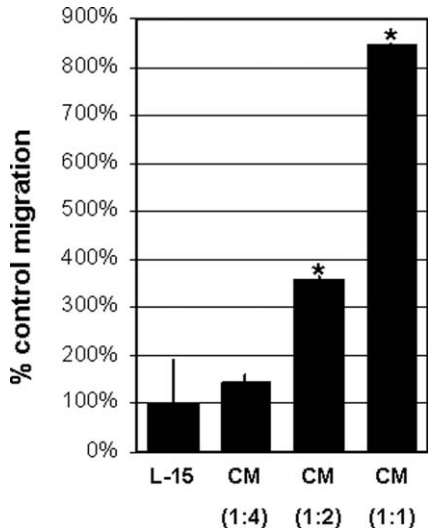


Fig. 2. Chemotactic effect of CNS-conditioned medium (CM) on leech microglia. CM obtained after the nerve cords incubation in L-15 medium for 24 h was used pure (1:1) or diluted (1:2, 1:4) and compared with the L-15 alone. Chemotaxis results were realized from three independent experiments and the cell counts from experimental conditions were compared with the migration from unstimulated (L-15, control) cell migration, which is expressed as 100%. Asterisks denote that CM-induced cell migration values were significantly different ($P < 0.01$) from the control sample.

microglial cells in defined chemotaxis assay (Fig. 2). The observed chemoattractant activity suggests that nerve cells could release some chemotactic factors, which trigger the microglia accumulation. To investigate which chemoattractant factors might be responsible for microglia migration, we first screened our leech CNS cDNA library for transcripts with homology to mRNA transcripts coding for known chemotactic factors. Using this approach a homologous transcript to vertebrate interleukin-16 (IL-16) was identified. The mRNA sequence extracted from the leech CNS databases is composed of 784 nucleotides coding for a 115 amino acid protein containing a PDZ domain at the C-terminus end (Fig. 3A). BLAST-P analyses demonstrated a significant homology of this partial amino acid sequence to the secreted form of vertebrate IL-16 (65% homologous with mouse and rainbow trout, 64% with pufferfish, 62% with human and 58% with chicken) as well as other putative invertebrate IL-16-related sequences (67% with purple urchin, 65% with mosquito, 64% with wasp, 60% with pea aphid and 58% with honey bee) (Fig. 3B). The GenBank accession numbers are detailed in Fig. 3B. To date, invertebrate sequences were annotated from automated computational analyses without any associated functions (Fig. 3B). Using the Multalin program (Corpet, 1988), sequence alignment with vertebrate IL-16 (mature form)

**A**

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  1 gaaggaattcaacgttcaatcccacgtaattccacgctaagaacatccgggatcgaaagt
    E  G  I  Q  R  S  I  P  R  N  S  T  L  R  T  S  G  I  E  S  20
 61 tcaaaggtcagagatgaagttgtgaactttgacacaaccacggtgaagttggagaagggc
    S  K  V  R  D  E  V  V  N  F  D  T  T  T  V  K  L  E  K  G  40
121 ttccttggggttggattctgcatcgagggtgggagggcctcaccctacgggacaaacca
    F  L  G  V  G  F  C  I  E  G  G  R  A  S  P  Y  G  D  K  P  60
181 atcctgataaaaagggttgtcagaggtgacgttcccttgagagcaggtgatgagttggtg
    I  L  I  K  R  V  V  R  G  D  V  P  L  R  A  G  D  E  L  V  80
241 tccgtgaatggaaaggaggtgtcatctatgacgtcagcccaggtgtggagtttactcaaa
    S  V  N  G  K  E  V  S  S  M  T  S  A  Q  V  W  S  L  L  K  100
301 tcacttccaggtggagtgatggtgctggagttgagaaggaaggaatgaagattgtaatga
    S  L  P  G  G  V  M  V  L  E  L  R  R  K  E                115
361 tgactgagaaggaaggaatgaagattgtaatgatgactgagaaggaaggagtgaagattg
421 taatgatgactgagaaggaaggaatgaagattgtaatgatgactgagaaggaaggagtga
481 agattgaaaagttgaaagcttctcttcatgtgactacttttaaatgagctgtctcacatc
541 ttcagtgcttgcatttctttcgattggaatttttgcgaccaattttcatcgagtttatt
601 ttgcaaataattgcgaaaatgactttgattttggggattatttcgatttgcctcgaaaat
661 tgaatttattaaaattgaatttatgcaatcttcaaaccttgcattttcatgacttttgac
721 actcattatgtcaagcggagaattgataaaaacaaataaaaagcacaaaattccaattta
781 aaaa
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**C**

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                1                                                  70
Leech   EGIQRSIPRNSTLRTSGIESKVRQEVVNFDTTTVKLEKGFLGVGFCIEGGRASPYGDKPILIKRVVRG
Mouse           SAASASAASDISVESK-EATVCTVTLEKTSAGLGFSLEGGKGSLHGDKPLTINRIFKG
Rat             SAASASVASGISVESV-EATVCTVTLEKTSAGLGFSLEGGKGSLHGDKPLTINRIFKG
Human           SAASASAASDVSVESTAEATVCTVTLEKMSAGLGFSLEGGKGSLHGDKPLTINRIFKG

                71                                                 132
Leech   AASEQSETVQPGDEILQLGSTAMQGLTRFEAWNIIKALPDGPVTIVIRKSLQSKETAAGDS
Mouse   ----DVPIRAGDKLVSVNGKEVSSMTSAQVWSLLKSLPGGVMVLEIRKE
Rat     --DRTGEMVQPGDEILQLAGTAVQGLTRFEAWNVIKALPDGPVTIVIRETSLQCKQTTASADS
Human   --TEQGDAVQPGDEILQLAGTAVQGLTRFEAWNVIKALPDGPVTVVIRENGLEGKQTTASADL
```

**B**

| Protein ID | Common name (*scientific name*) | Homology | Studies |
|---|---|---|---|
| ref\|XP_784502.2\| similar to interleukin-16 precursor | **Purple urchin** (*Strongylocentrotus purpuratus*) | 67% | automated computational analysis (genome or EST) |
| ref\|XP_317940.4\| AGAP011384-PA | **African malaria mosquito** (*Anopheles gambiae*) | 65% | automated computational analysis (genome or EST) |
| emb\|CAD70074.2\| interleukin-16 | **Rainbow trout** (*Oncorhynchus mykiss*) | 65% | automated computational analysis (genome or EST) |
| gb\|AAC04383.1\| interleukin-16 precursor | **House mouse** (*Mus musculus*) | 65% | functional analysis |
| ref\|XP_001606112.1\| similar to prIL-16 | **Jewel wasp** (*Nasonia vitripennis*) | 64% | automated computational analysis (genome or EST) |
| gb\|AAX36076.3\| interleukin 16 | **Pufferfish** (*Tetraodon nigroviridis*) | 64% | phylogenic analysis |
| gb\|AAB36371.2\| prIL-16 | **Human** (*Homo sapiens*) | 62% | functional analysis |
| XP_001951758 similar to IL-16, partial mRNA | **Pea aphid** (*Acyrthosiphon pisum*) | 60% | automated computational analysis (genome or EST) |
| ref\|XP_001121687.1\| similar to interleukin 16 precursor | **Honey bee** (*Apis mellifera*) | 58% | automated computational analysis (genome or EST) |
| gb\|AAO18640.1\| interleukin 16 | **Chicken** (*Gallus gallus*) | 58% | automated computational analysis (genome or EST) |

Fig. 3. Molecular characterization of *Hm*IL-16 molecule in leech nerve cord. (**A**) *Hm*IL-16 partial sequence contains the C-terminal end of the protein (115 residues). The region highlighted in grey represents the C-terminal PDZ domain. The GVGF motif and W residue, essential for the particular conformation of known IL-16, are indicated in frames (A, **C**). (**B**) BLAST-P analyses showing homologies with sequences corresponding to putative sequence or characterized IL-16. The state of studies is detailed in the table according to the literature. (C) Dark and light grey residues, respectively, denote the high and medium rate of sequence identity between different IL-16 active forms. $Arg^{107}$ residue in human IL-16 which is critical for chemoattractant activity is highlighted in black on the alignment.

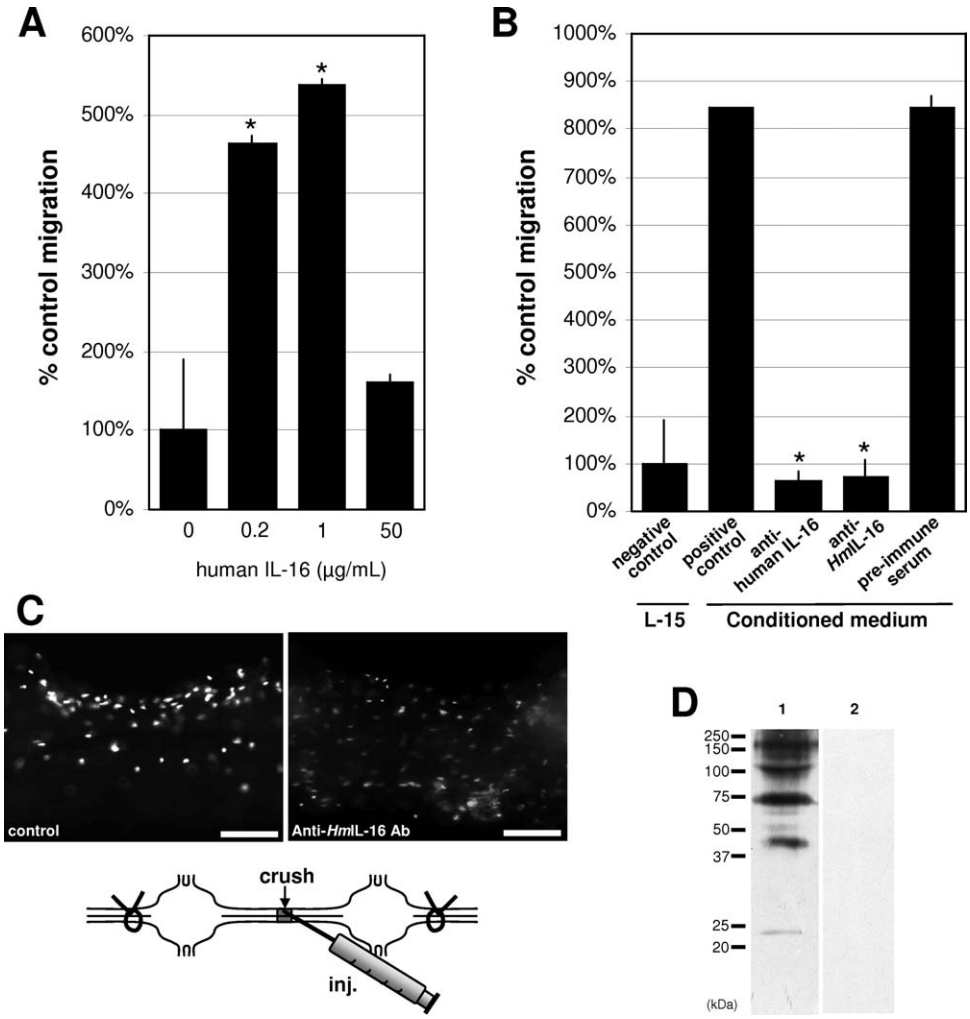


Fig. 4. Chemotactic effect of IL-16 on leech microglia. (**A**) Microglial cells were tested against human IL-16 gradient (0, 0.2, 1, and 50 µg $mL^{-1}$) to study the chemotactic property and determine the optimal concentration of human IL-16 in chemotaxis assays. (**B**) Conditioned medium from leech nerve cords was used as chemoattractant for leech microglia. A negative control was performed with L-15 medium. By using leech CNS conditioned medium, cells were pre-incubated with: L-15 medium alone (positive control); with mouse monoclonal anti-human IL-16 antibody; with rabbit polyclonal anti-*Hm*IL-16 antibodies and with its pre-immune serum as negative control. Chemotaxis results were realized from three independent experiments. Cell counts from experimental conditions were compared with the migration from unstimulated (L-15, control) cell migration, which is expressed as 100%. Asterisks denote that cell migration of the indicated sample was significantly different ($P < 0.01$) from the conditioned medium alone. (**C**) Visualization of Hœchst 33342-labeled microglia accumulation following experimental injury. As shown in the diagram, simultaneously to the lesion, connectives were injected with rabbit polyclonal anti-*Hm*IL-16 Ab or with pre-immune serum (control). The images show in the crush area the altered microglia accumulation with anti-*Hm*IL-16 perfusion compared with a normal migration with the control perfusion at the lesion site 4 h after experimental crush. Scale bars correspond to 100 µm. (**D**) Western blot analyses were performed from CNS protein extracts (Lanes 1 and 2) by using the rabbit polyclonal anti-*Hm*IL-16 Ab (Lane 1) and preimmune serum as negative control (Lane 2).

showed the conservation of essential residues in the PDZ (postsynaptic density/disc large/zona includens-1) domain contained within the C-terminus (Fig. 3C). Of interest, similar to fish IL-16, leech IL-16 contains a GVGF motif instead of the GLGF tetrapeptide identified in mammals (Wen et al., 2006). The tryptophan residue, which is represented in frame, is known to be essential to the specific conformation of active IL-16, which allows the interaction with the tetrapeptide. Presence of the tryptophan in leech IL-16 therefore represents conformational homology with vertebrate IL-16. In addition, *Hm*IL-16 also contains a RRK sequence in the C-terminal end ($Arg^{112}$-$Lys^{114}$) consistent with human IL-16. This amino acid sequence has been shown to be critical for human IL-16 bioactivity (Nicoll et al., 1999) and suggests the potential for functional cross-reactivity.

## Human IL-16 Exerts a Chemotactic Effect on Microglia

Since *Hm*IL-16 presents homologies with human IL-16, the chemotactic activity of human IL-16 on leech microglial cells was assessed. Three dilutions (0.2, 1, 50 µg $mL^{-1}$) were used in the chemotaxis experiments (Fig. 4A). Microglial cell migration was stimulated in a dose-

dependant manner, which peaked at 1 μg $mL^{-1}$ and demonstrated high dose inhibition (a characteristic of chemoattractant cytokines).

### Anti-IL-16 Antibodies Neutralize Microglial Cell Migration

To determine the specificity of IL-16-mediated microglia migration and conditioned medium-induced chemotaxis of microglial cells, anti-IL-16 antibodies were used to inhibit induced cell migration. The preincubation of microglial cells either with mouse monoclonal anti-human IL-16 Ab, directed to the RRK sequence, or with specific rabbit polyclonal anti-*Hm*IL-16 Ab showed a significant decrease in the chemotactic effect of the conditioned medium on microglia migration (Fig. 4B). Control experiments performed with rabbit preimmune serum at the same concentration had no effect on induced migration (Fig. 4B). Finally, when microglial cells were pretreated with specific rabbit anti-*Hm*IL-16 Ab, the chemotactic response to the optimal concentration of human IL-16 (1 μg $mL^{-1}$) was also significantly reduced. Taken all together, these data indicate that leech CNS-conditioned media contains IL-16 bioactivity that is structurally and functionally homologous to human IL-16.

### Anti-*Hm*IL-16 Inhibits the Accumulation of Microglia at a CNS Lesion

The *in vitro* experiments suggest that IL-16 may play a role in the recruitment of microglial cells in the leech CNS. To confirm this concept, recruitment of microglial cells were assessed following a crushed nerve cord model. It has been revealed that leech microglia normally lie scattered among axons as an apparently homogeneous population of cells within the nerve cord. Microglia accumulate at the site of lesion within 24 h of nerve injury (Morgese et al., 1983; Ngu et al., 2007). As previously described, these cells can be identified unambiguously without specific markers, because the only other nucleated cells among the thousands of axons that extend between segmental ganglia are two large glial cells, up to 5 mm in length, that sheathe the axons and are located midway between ganglia. Thus, microglia can be tracked in the living cord with the aid of nonspecific fluorescent nuclear dyes (McGlade-McCulloh et al., 1989). In the present experiment, nerve cords were crushed and then recruitment of microglial cells is determined using the flurorescent nuclear dye Hoechst 33342. Our results indicate that cords fixed 4 h after crushing showed an accumulation of microglia at lesions (data not shown). To determine the contribution of IL-16 to the overall recruitment of microglia, crushed connectives were injected with the specific anti-*Hm*IL-16 antibody prior to assessment 4 h later: the migratory process of microglial cells at the injured site was abolished (Fig. 4C). In control experiments, the perfusion of preimmune serum did not affect microglia accumulation (Fig. 4C).

### IL-16 is Detected in Leech CNS by Western Blot

To determine if the biological activity found in the chemotaxis assays could correspond to the presence of a homologous of IL-16, conditioned medium was analyzed by Western blot assays using the specific anti-*Hm*IL-16 antibody. The results revealed in CNS protein extract a pattern of products ranging from 23 to 140 kDa (Fig. 4D, Lane 1). A similar pattern was observed from CNS-conditioned medium protein extract (data not shown). Preimmune serum was used as negative control (Fig. 4D, Lane 2). The pattern of detected products is in accordance with other previous studies which demonstrated in human the presence of numerous processed and auto-aggregated forms of IL-16 (Chupp et al., 1998).

### Effect of Conditioned Medium on Human CD4+T Cell Chemotaxis

To study the functional analogy of *Hm*IL-16 with its human homolog, the chemotactic property of the leech molecule was assessed on human cells. Because human IL-16 was described as a chemotactic factor for CD4+ T cells through a CD4-dependent pathway and because a CD4-like molecule is not characterized in human microglia yet, the effect of leech conditioned medium (1:10) containing *Hm*IL-16 was evaluated using human T cell migration in comparison with recombinant human IL-16 ($10^{-8}$M). Human T lymphocytes were observed to migrate toward conditioned medium and cell migration percentages were comparable to those obtained toward human IL-16 (Fig. 5A). Incubation of T cells with different concentrations of either monoclonal anti-human IL-16 or polyclonal anti-*Hm*IL-16 antibodies induced a significant dose-dependent decrease in cell migration (Fig. 5A). Interestingly, mixed T cells were observed to migrate specifically toward leech conditioned medium containing *Hm*IL-16 in a dose-dependent manner with a maximal migration at a dilution 1:10 (Fig. 5B). Higher or lower concentration produced a diminished CD4+ T cell migration, consistent with typical chemoattractant responses. Similar results were obtained using CD4+ T cells alone. Specificity of the activity of *Hm*IL-16 on CD4+ T cell migration was demonstrated using specific neutralizing experiments with anti-IL-16 antibodies (Fig. 5C). Indeed the preincubation of CD4+ T cell with either anti-human IL-16 antibody (1 μg) or the IL-16 antagonist peptide (5 μg) strongly reduced cell migration. The addition of soluble CD4 (5 μg) to the cells prior to the chemotaxis assay was able to significantly reduce the human CD4+ T cells migration induced by leech conditioned medium. Controls realized either with an anti-IgG2a antibody or a scrambled peptide showed no effect on CD4+T cell migration induced by conditioned medium (Fig. 5C). Although a CD8+ T cell migration was observed at the highest concentration of conditioned medium (1:1), this response was not dependent on *Hm*IL-16 as incubation with the monoclonal anti-human IL-16 did not affect the induced migration.

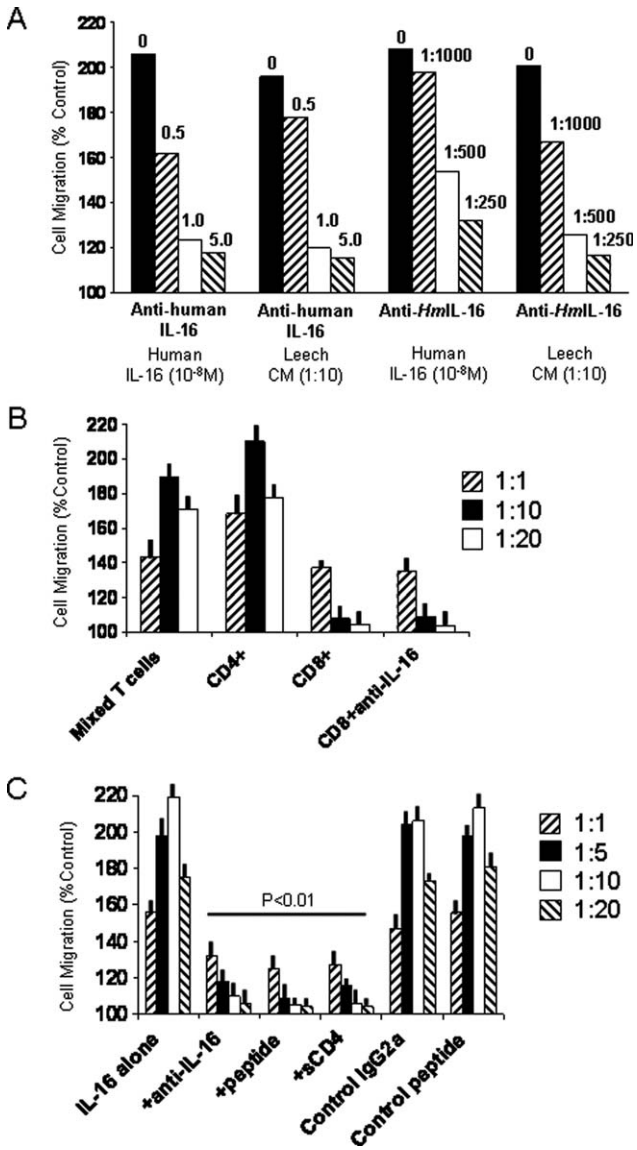


Fig. 5. Chemotactic effect of *Hm*IL-16 on human T cells. (**A**) Anti-sera inhibitory effect on human T cell migration induced by either human recombinant IL-16 ($10^{-8}$M) or leech CNS conditioned medium (CM, 1:10) containing *Hm*IL-16. The antibodies were used at 0.5, 1, and 5 μg $mL^{-1}$ for monoclonal anti-human IL-16 and at 1:1,000, 1:500, and 1:250 for polyclonal anti-*Hm*IL-16 antibody. (**B**) CD4 specificity of *Hm*IL-16 for migratory effect on T cells. Either CD4+ T cells or CD8+ T cells were tested against a gradient of leech conditioned medium (1:1, 1:10, and 1:20) containing *Hm*IL-16 compared with mixed T cells. For CD8+ T cells, a preincubation with human anti-IL-16 antibody was performed as control before the addition of conditioned medium attesting a slight *Hm*IL-16-independent chemotaxis. (**C**) Inhibitory effect on *Hm*IL-16-mediated chemotaxis on human CD4+ T cells. CD4+ T cells were tested against leech conditioned medium (1:1, 1:5, 1:10, 1:20) containing *Hm*IL-16. Preincubation of CD4+ T cells with an anti-humanIL-16 mAb (1 μg), an IL-16 antagonist peptide (RRKSLQSKETTAAGNS) (5 μg) and soluble CD4 (5 μg) were performed previously to the addition of *Hm*IL-16. CD4+ T cells were preincubated with either IgG2A or a scrambled peptide using the same amino acids (5 μg) as controls for respectively mAb and antagonist peptide.

### Distribution of IL-16 in the Nervous System Following Injury

Cells expressing *Hm*IL-16 protein and mRNA were investigated by immunohistochemical staining and by *in situ* hybridization on nerve cord at different times after injury (Fig. 6). Just after the crush, IL-16 was detected, using an anti-*Hm*IL-16 antibody, in neuron bodies (Fig. 6A). A similar sample, treated with rabbit pre-immune serum as a negative control did not show any signal (Fig. 6A′). Interestingly, 24 h after injury, a stronger *Hm*IL-16 signal appeared in neurons (Fig. 6B). The saturated control samples using the synthetic peptide did not show any signal (Fig. 6B′) further confirming specificity of the anti-*Hm*IL-16 antibody. *Hm*IL-16 appeared distributed within the cytoplasm in a scattered pattern (Fig. 6C). Immediately following the lesion, staining was observed at the cut end of a connective before accumulation of any microglial cell (Fig. 6D). Immunolocalization of the *Hm*IL-16 at the cut end of axons suggests production and a rapid release of the cytokine by the neurons. To get further information on the origin of *Hm*IL-16 observed at the lesion site, ligature experiments were performed. It appeared that by contrast with the external ligatures, the external and internal ligatures of ganglia 3 and 4, which isolate the cell body of neurons from nerve fibers, blocked the accumulation of *Hm*IL-16 at the lesion site (Fig. 6E). Twenty-four hours following the injury, no *Hm*IL-16 positive microglial cells were detected at the lesion site (see frame, Fig. 6F′) whereas Hoechst staining of the same sample confirmed the accumulation of microglial cells (Fig. 6F). As described above, this nuclear dye is specifically used to follow the only circulating cells in the connective which are microglial cells (Chen et al., 2000; McGlade-McCulloh et al., 1989). Of interest, although these cells are negative for the protein, *in situ* hybridization analyses performed on crushed connective (Fig. 7A) demonstrated that migrating and accumulating Hoechst positive microglial cells (Fig. 7B) produce *Hm*IL-16 mRNA 24 h following lesion (Fig. 7C). The uncrushed connective on the other side of the same nerve cord was used as an internal reference. Interestingly, neither the accumulation of microglial cells (Fig. 7B) nor mRNA was detected in microglia of the uncrushed connective (Fig. 7C). Despite the accumulation of microglial cells at the crushed site, no specific signal was detected with sense probe (negative control) (Fig. 7D).

To determine whether *Hm*IL-16 protein was detectable later in microglial cells of the connectives, nerve cords were cultured for 72 h after injury and stained with anti-*Hm*IL-16 antibody. Three days after injury, *Hm*IL-16 immunoreactivity was detected in microglial cells accumulated at the cut end of the lateral roots of the ganglia (Fig. 7E). A similar sample, treated with rabbit preimmune serum as a negative control did not show any signal (data not shown).

## DISCUSSION

Vertebrate microglial activation is generally characterized as a gradual transition from a quiescent stellate form to a macrophage-like morphology. When the brain is injured or affected by brain diseases (e.g., degenerative, infectious, or autoimmune diseases), the resident ramified microglia morphologically transform into cells

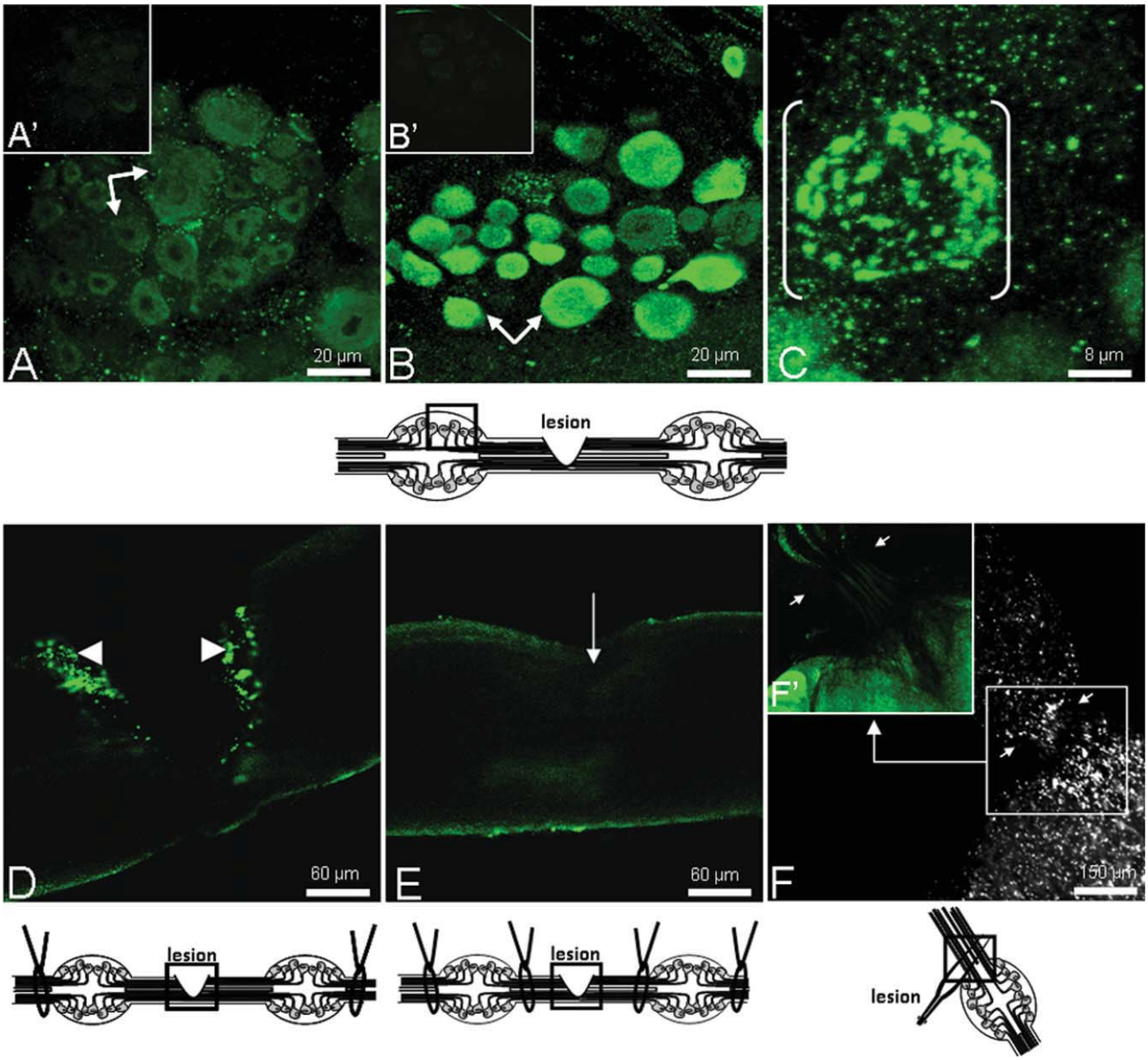


Fig. 6. Immunostaining of leech CNS using rabbit polyclonal anti-*Hm*IL-16 antibody. Ganglia were analyzed immediately (**A**, **A′**) or 24 h (**B**, **B′**, **C**) following injury. (A) The anti-*Hm*IL-16 immunostaining was detected at $t = 0$ inside a ganglion in some neurons of different size (see arrows as examples). (B) Twenty-four hours following injury, the immunostaining was stronger in neuron bodies (see arrows as examples) and sometimes concentrated in peripheral vesicles (C, one neuron in brackets). Connectives were analyzed immediately (**D**, **E**) or 24 h (**F**) following injury. Positive immunostaining at the injury site is observed close to the axonal cut ends when ganglia were ligatured outside (D, arrow heads). No signal was detected when additional internal ligature was performed (E, arrow). (F) Twenty-four hours following injury (arrows), once microglial cells were migrated to the crush site (F, Hœchst 33342 labeled cells) they were still negatives for *Hm*IL-16 immunostaining (**F′**). Negative controls were performed using rabbit pre-immune serum (A′) and anti-*Hm*IL-16 preincubated with the synthetic peptide used for polyclonal antibodies production (B′). The diagrams representing two consecutive ganglia of leech CNS indicate in frames the position of presented images. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with retracted processes and enlarged cell bodies, and increase in cell number at the affected site. Microglial cells with this particular form are generally referred as "activated microglia" or "reactive microglia." Of interest, the present study showed for the first time from whole injured leech CNS the same morphological changes of microglia by using a specific leech microglial marker.

In this report, a chemotactic molecule, designated as *Hm*IL-16, was demonstrated to be involved in the microglial cell migration following leech nerve injury. This cell accumulation was shown to be a primary event required for sprouting of severed axons in the leech CNS (Ngu et al., 2007). In contrast to vertebrates, invertebrates such as leeches can repair their nervous system following injury and therefore offer unique opportunities to study the molecular and cellular steps of this vital process (Blackshaw et al., 2004; von Bernhardi and Muller, 1995; Wang et al., 2005).

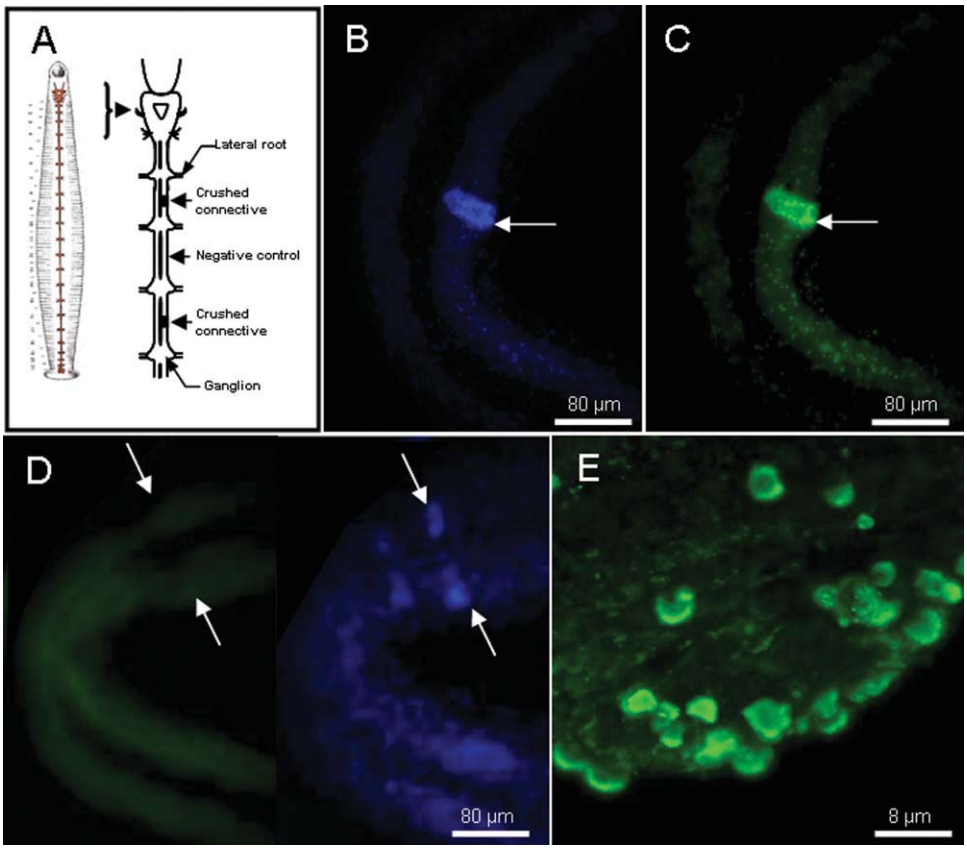


Fig. 7. Induction of *Hm*IL-16 in microglial cells following recruitment at the injury site. (**A**) Diagram of experimental procedure for CNS dissection and crushes on one of the two connectives. Tissues were analyzed 24 h (**B**–**D**) and 72 h (**E**) following injury. (B) Fluorescence microscopy image showing Hœchst 33342-labeled microglial cells accumulated at the lesion site (arrow). (C) *HmIL-16* mRNAs were located by fluorescence *in situ* hybridization on connectives joining two ganglia. Transcripts were specifically detected (antisense *HmIL-16* riboprobes) in recruited (arrows) and circulating microglial cells. (D) In the control sample (sense riboprobe), no signal was detected in microglial cells (arrows) accumulated at the lesion site of both connectives (left) and evidenced by Hœchst 33342 labeling (right). (E) *Hm*IL-16 protein was located by using fluorescent immunostaining (rabbit polyclonal anti-*Hm*IL-16 antibody) of microglial cells recruited at a damaged connective end, 72 h after injury. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

By using leech CNS databases, a molecule having significant homologies with vertebrate interleukin-16 was identified. In its C-terminus end, the molecule contains a PDZ domain, specialized for binding of C-termini in partner proteins potentially involved in cellular signaling (Jelen et al., 2003). Bioactive IL-16 is the first known extracellular protein with a PDZ domain-like fold whose structural alterations avoid consensus peptide binding properties. Indeed, the GLGF cleft of IL-16 is much smaller than those of PDZ-domains and is blocked by a tryptophan side chain at its center (Muhlhahn et al., 1998). Interestingly, the leech molecule contains a GVGF motif similar to IL-16 in rainbow trout or pufferfish (Wen et al., 2006). Moreover, *Hm*IL-16 sequence showed significant similarities with putative IL-16 sequences from protostomes and nonchordate deuterostomes. However, although sequencing of several invertebrate genomes (i.e., insects and echinoderms) recently identified the presence of such molecules, their role was not elucidated by functional analyses. In that regard, we have now demonstrated that *Hm*IL-16 can induce chemotactic activity in leech microglia, as well as human T cells, and is able to cross-react with antibodies directed towards human IL-16. Indeed, the cross-reactivity between human and leech IL-16 activity likely resides in their sequence homology at the bioactive sites and in the PDZ domains. Reports suggest that IL-16+ human cells exert chemotaxis on microglia precursors during neurogenesis (Schwab et al., 2001a,b) but such activity of IL-16 for microglial cells has not been reported. The specificity of IL-16-mediated microglia migration was clearly demonstrated by using anti-IL-16 antibodies and by showing that the conditioned medium activity was dependent on a bioactive IL-16 form. The use of a peptide corresponding to the human IL-16 C-terminal end ($Arg^{106}$ to $Ser^{121}$, RRKSLQSKETTAAGDS), known to specifically inhibit human IL-16 activity (Keane et al., 1998), will confirm this concept. Within the 16 C-terminal residues, the peptide RRK shown to be critical for vertebrate IL-16 function is present in the leech sequence. Point mutations in IL-16 protein revealed that $Arg^{107}$ is critical for CD4+ T cell chemotaxis (Nicoll et al., 1999). $Arg^{107}$ is conserved in the mouse IL-16 sequence as in six primate species (Bannert et al., 1998). In *Hm*IL-16, this critical arginine is also conserved (Fig. 3C). Using human CD4+ T cells as target

cells, the leech-conditioned medium exhibited similar activities as human IL-16. The fact that *Hm*IL-16 has no effect on CD8+ cell migration further indicates a similar relationship with CD4 as has been described for human IL-16. While a CD4-homologous protein has not been identified in leech, our data indicating conservation of function would suggest its presence. Experiments carried out with soluble CD4 indicate that *Hm*IL-16 might act via a CD4-like ligand expressed by leech microglial cells as reported for human microglia (Schluesener et al., 1996). A specific anti-human CD4 antibody can reduce the microglia migration induced with either human IL-16 or conditioned medium (data not shown). Additionally, the human anti-CD4 antibody allowed (i) detection of two products (55 and 75 kDa, consistent with human CD4) in the CNS protein extracts, and (ii) was capable of labeling 4.5% of microglial cells by FACS analyses (data not shown) indicating subset specificity. Thus, these experiments with human or leech IL-16 on either human T cells or leech microglia showed functional cross-reactivity and suggest a cross-species conservation of IL-16 receptor structures. The study of mammalian microglial recruitment will be undertaken consequently to these results. But, this information would be suitable following the molecular characterization of a CD4 related molecule in leech microglia which is under way. If so, leech data will be originally useful to highlight, through a further publication, the presence of CD4-like receptor in mammalian nerve cells, distinctly to the infiltrating blood cells.

We have also determined that leech microglial cells are capable of generating IL-16. Based on our analysis of the IL-16, it appears that the process for generation is similar to that described for human IL-16. Western blot analysis of leech CNS using the anti-*Hm*IL-16 antibody revealed a pattern of bands (from 23 to 140 kDa) indicative of multiple forms with a putative pro-IL-16 and auto-aggregated active form(s). In mammals, IL-16 is initially synthesized as a large precursor protein (pro-IL-16), identified in two related isoforms. The pro-IL-16 isoform 1 generated by hematopoietic cells (Pro-IL16) is a 68 kDa molecule (Bellini et al., 1993; Laberge et al., 1997; Lim et al., 1996; Rumsaeng et al., 1997; Sciaky et al., 2000; Sharma et al., 2000) but is also generated by microglia (Guo et al., 2004; Liebrich et al., 2007; Schluesener et al., 1996; Schwab et al., 2001b; Zhang et al., 2009; Zhao et al., 2004). The pro-IL-16 isoform 2 is a 141 kDa neuronal protein (NIL-Pro-IL-16) detected in neurons localized in the cerebellum and hippocampus. Both pro-IL-16 proteins are cleaved by caspase 3 at an aspartate site located before the last PDZ domain to produce an N-terminal sequence (Zhang et al., 1998) and a C-terminal peptide which can subsequently function as mature IL-16 by forming multimers (Zhang et al., 2001). Indeed, the homo-tetramerization of human IL-16 was reported as necessary for biological activity (Center et al., 1996). C-terminal regions of mammalian IL-16 and NIL-16 are completely homologous (Kurschner and Yuzaki, 1999). As the specific *Hm*IL-16 antibody binds to the C-terminal portion of *Hm*IL-16, the two isoforms could not be distinguished. Additional studies are needed to characterize the processing from proIL-16 to the bioactive IL-16 form(s) in the leech-conditioned medium. The *in vitro* biological activity of *Hm*IL-16 was confirmed by *ex-vivo* experiments using leech nerve cords. The injection of specific anti-*Hm*IL-16 antibodies in the living nerve cord following injury reduced the microglia migration at the injury site. Since such accumulation is essential following nerve cord lesion, *Hm*IL-16 might play a crucial role in leech CNS repair (Duan et al., 2005; Ngu et al., 2007). In mammals, treatments that neutralize IL-16, which is a proinflammatory cytokine, with anti-IL-16 antibodies successfully reversed paralysis and ameliorated relapsing of experimental autoimmune encephalomyelitis. In treated mice, diminished infiltration by CD4+ T cells, less demyelination and increased sparing of axons was observed (Skundric et al., 2005a,b). In leech, the microglia recruitment at lesions suggesting an inflammatory response is possible with the participation of *Hm*IL-16. Nevertheless, the regulation of inflammation leading to the nerve repair is still unclear.

Otherwise, the neuronal production of *Hm*IL-16 immediately after injury suggested a constitutive expression of its gene and the existence of a neuronal form as already suggested by the results of western blots. The IL-16 immunoreactivity is mainly localized at the periphery of the neuron cytoplasm and sometimes appeared vesicular suggesting an active transport outside of neurons and/or toward axons. Interestingly, when ganglia are ligatured to isolate cell body of neurons from nerve fibers leading to the cut connective, accumulation of *Hm*IL-16 at the lesion site is not detected. Thus, these observations strongly suggest that IL-16 protein present in the neurons is rapidly transported and stored along the axonal processes to promote the recruitment of microglial cells close to injured axons. Although numerous microglial cells were recruited following the lesion, no *Hm*IL-16 protein was detected in connective's microglia either immediately or 24 h after injury. Nevertheless, *Hm*IL-16 transcripts were observed 24 h after injury in activated microglial cells where *Hm*IL-16 protein was detected from 72-h postinjury. We hypothesize that microglial cells are first activated by neuronal IL-16 released from damaged neurons and then migrating microglial cells can release their own IL-16 to maintain cell accumulation at the lesion. In human brain, IL-16 is constitutively expressed by a subset of microglial cells (Mittelbronn et al., 2001). Numerous studies have reported the induction of IL-16 in pathological neurodegenerative situations (Glass et al., 2006; Guo et al., 2004; Liebrich et al., 2007; Mueller et al., 2006; Schwab et al., 2001a,b; Zhao et al., 2004 no. 306). The increased rate of IL-16 was correlated with lymphocyte infiltration but that recruitment was not directly dependent on neuronal and/or microglial IL-16 (Skundric et al., 2005a). Microglia in mammals are considered as sensors for micro-inflammatory processes (Hanisch and Kettenmann, 2007; Hanisch, 2002). Of interest, other cytokines from microglia/macrophage are also required for the

accumulation of T cells which mediate damages in the nervous system (Schwab et al., 2001a).

In summary, we have identified a protein in leech CNS (*Hm*IL-16) that has high functional homology to mammalian IL-16. Our studies also suggest that, similar to mammalian IL-16, *Hm*IL-16 can associate with CD4 further confirming a strong evolutionary association between IL-16 and CD4. Its implication in the microglia migration was clearly demonstrated as *Hm*IL-16 was identified as a key molecule involved in the crosstalk between neurons and microglia during the leech CNS repair. Further experiments will be undertaken to characterize subsets of leech responsive microglia and their sequential mobilization along the time course of nerve repair. Thus, our first purpose is to clarify the leech CNS processes thanks to its simplicity. Indeed, the respective implication of blood cells and microglia in mammalian nerve repair is difficult to specify because *in vivo* analyses in mammals cannot exclude the presence of blood cells in brain. The present report does not consider this "mammalian" question prior to better understand microglia implication in leech nerve repair. Finally, molecular mechanisms involved in only leech microglial activation might be potent tools to evaluate the implication of microglia in mammalian nerve repair.

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