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**INVERTEBRATE HEMATOPOIESIS: AN ANTERIOR PROLIFERATION CENTER AS A
LINK BETWEEN THE HEMATOPOIETIC TISSUE AND THE BRAIN**

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Abstract

During evolution, the innate and adaptive immune systems developed to protect organisms from nonself substances. The innate immune system is phylogenetically more ancient and is present in most multicellular organisms, whereas adaptive responses are restricted to vertebrates. Arthropods, lack the blood cells of the lymphoid lineage, and oxygen-carrying erythrocytes, making them suitable model animals to study the regulation of the blood cells of the innate immune system. Many crustaceans have a long life span and need to continuously synthesize blood cells, in contrast to many insects. The hematopoietic tissue (HPT) of *Pacifastacus leniusculus* provides a simple model to study hematopoiesis because the tissue can be isolated and the proliferation of stem cells and their differentiation can be studied both *in vivo* and *in vitro*.

Here we demonstrate new findings of a physical link between the HPT and the brain. Actively proliferating cells were localized to an anterior proliferation center (APC) in the anterior part of the tissue near the area linking the HPT to the brain, whereas more differentiated cells were detected in the posterior part. The central areas of HPT expand in response to lipopolysaccharide-induced blood loss. Cells isolated from the APC divide rapidly and form cell clusters *in vitro*; conversely, the cells from the remaining HPT form monolayers, and they can be induced to differentiate *in vitro*. Our findings offer an opportunity to learn more about invertebrate hematopoiesis and its connection to the central nervous system and thereby to obtain new information about the evolution of different blood and nerve cell lineages.

Keywords: Hematopoietic stem cells, Arthropods, Reactive oxygen species,

Introduction

Ninety five percent of all animal species are invertebrates, which include a diverse range of organisms, from unicellular protozoans to more complex echinoderms and protochordates. . Invertebrates lack an adaptive immune system, and they rely on very efficient innate immune systems, in which blood cells play a key role in immobilizing or destroying invading microorganisms. Within this large number of species, several strategies for recognition and defense have been described. From an evolutionary standpoint, some constituents of the innate immune system have been substantially conserved through the lineages leading to the chordates, whereas other factors may be unique to invertebrates. Because of this great diversity, the potential for finding new molecules that may explain the evolution of different blood cell lineages is considerable.

Invertebrate blood cells (hemocytes) are essentially comparable to cells of the myeloid lineage in vertebrates because they participate in innate immunity and blood clotting [1]. *Drosophila melanogaster* and the crayfish *Pacifastacus leniusculus* are well established as model organisms in the field of invertebrate innate immunity [2-4], and these species have provided major contributions to hematopoiesis research in invertebrate animals. Since they lack oxygen-carrying red blood cells (erythrocytes) and blood cells of the lymphoid lineage, which participate in adaptive immune defense, these animals offer a simple model system to study the development of the cells of the innate immune system. In *Drosophila*, mature hemocytes are formed in the early embryonic head mesoderm and during the larval stage in a specialized organ called the lymph gland (LG) [3]. The progenitor cells in the LG differentiate during the larval stages but are not released into the hemolymph until the pupal stage, when most hemocytes are involved in phagocytosis of degraded larval tissues [5]. Thus, no LG exists in the adult flies. By contrast, crayfish hematopoietic development is an ongoing process throughout the entire life cycle and takes place in the hematopoietic tissue (HPT) covering the dorsal part of the stomach [1]. It is well established that several transcription factors and signal transduction pathways are involved in hematopoiesis and that they are highly conserved between *Drosophila* and mammals [6]. We have also confirmed the importance of GATA transcription factors in crayfish HPT [7]. Further, the Runx protein homologues in crustaceans and *Drosophila* are of special interest because they are closely associated with the differentiation of cells that express the prophenoloxidase

(proPO) gene [4, 7]. ProPO is the key enzyme in the proPO-system, an important innate immune system in invertebrates [2]. In the freshwater crayfish, three morphologically different classes of hemocytes, hyaline cells (HCs), semigranular cells (SGCs) and granular cells (GCs), are observed within the hemolymph, and we have demonstrated that Runx expression is a prerequisite for the final differentiation of SGCs and GCs, both of which express proPO [7].

The transcriptional regulation of hematopoietic cell development in invertebrates and vertebrates shares many common features. Hematopoiesis is regulated by humoral factors in vertebrates, such as numerous cytokines, but the knowledge about cytokine regulation of hematopoiesis is limited in invertebrates [1]. The evolution of these factors appears to be far more complex, and few hematopoietic cytokines are found in invertebrate genomes [1]. A technique to culture the crayfish hematopoietic progenitor cells *in vitro* has enabled us to isolate and characterize a new group of cytokines named astakines (Ast1 and Ast2) from crayfish plasma [8, 9]. Astakines contain a conserved domain that is similar to vertebrate prokineticins (PROKs), which are known to influence processes related to development of the nervous system, immunity and hematopoiesis [10, 11]. Ast1 is an important regulator at the crossroads between proliferation/differentiation and apoptosis [8, 12]. Ast1 acts by regulating the expression of a protein that is responsible for preventing apoptosis, crustacean hematopoietic factor (CHF), which has similarity to the N-terminal region of the human cysteine-rich transmembrane BMP regulator 1 (chordin-like), also known as CRIM1 [12]. A schematic illustration showing the different hematopoietic lineages in crayfish is shown in Figure 1. When searching GenBank, we found at least 20 astakines in different crustaceans, scorpions, spiders, ticks, and hemipteran, hymenopteran and blattodean insects. Surprisingly, these small peptides appear not to be present in dipterans, such as *Drosophila* or *Anopheles* [9].

Because nothing was known about the location of different cell types and precursor cells in the HPT, we decided to analyze this tissue in more detail. In this study, we have characterized different areas of the HPT and found that the tissue has more connections in the animal than was previously thought. We revealed for the first time that the crayfish HPT has a connection to the brain, and we have identified a distinct area of highly proliferative cells close to this connection that constitutes a stem

cell center (APC). We also demonstrated that APC cells can be cultured in vitro and that they differ from other HPT cells in culture.

Material and Methods

Animals

Pacifastacus leniusculus (crayfish) from Lake Hjälmaren in Sweden was maintained in aquaria at 10 °C with aerated running fresh water. Intermolt crayfish were used for the experiments.

Tissue preparation

The whole stomach covered with the HPT was dissected from crayfish and immediately fixed in 4% paraformaldehyde in PBS at 4 °C overnight. The HPT was then carefully removed from the stomach, dehydrated and embedded into paraffin blocks. The embedded tissue was horizontally sectioned with the thickness of 7-11 µm and placed on MENZEL super frost glass slides. For whole mount staining, the tissue was collected from crayfish at 0, 2 or 4 h after LPS or laminarin injection. After fixation and washing with PBS, the tissue was placed on super frost glass slides, air dried and subjected to hematoxylin and eosin staining.

Transmission electron microscopy

The HPT was fixed for 1.5 h in ice-cold 1.25 % glutaraldehyde in 0.1 M sucrose, 0.1 M sodium cacodylate buffer pH 7.2. The samples were subjected to 15-min-rinsing in cold veronal buffer and 2-hour-postfixed in 2 % osmium tetroxide, followed by dehydration in ethanol and acetone. Finally the samples were embedded in TAAB 812 resin and thin sections were stained with uranyl acetate, lead citrate and analyzed with a Philips CM 10 transmission electron microscope.

BrdU incorporation and detection

Crayfish were injected with 10µl/g fresh weight of 50 mM BrdU in crayfish saline (0.2 M NaCl, 5.4 mM KCl, 10 mM CaCl₂·2H₂O, 2.6 mM MgCl₂·6H₂O, 2 mM NaHCO₃, pH 6.8). At 1 and 3 hour post-injection the whole stomach covered with the HPT and its connected brain was isolated and immediately fixed as described above. Then the HPT and the brain were carefully removed from the stomach and used for sectioning or whole mount BrdU detection as described previously [7, Supplementary material].

Detection of mitotic cells in HPT

Mitotic cells in the HPT were detected using a rabbit-anti-Histone H3 (phospho S10) antibody (Abcam). For whole mount staining, the tissues were treated with 2M HCl for 30 min at room temperature before overnight incubation with rabbit-anti-Histone H3 antibody (1:500) at 4 °C. Then the tissues were incubated in the dark with FITC-conjugated anti-rabbit Ig (1:500) (Sigma) for 1 h at room temperature and observed under a fluorescence stereomicroscope. For detection of mitotic cells in HPT sections, deparaffinized and rehydrated sections were treated with 10 mM sodium citrate buffer, pH6 at 95 °C for 10 min and incubated overnight with rabbit-anti-Histone H3 antibody (1:1000) at 4 °C. The sections were then incubated in the dark with Cy3-conjugated anti-rabbit Ig (1:500) (Sigma) for 1 h at room temperature. The sections were later mounted in Vectashield mounting medium containing DAPI (Vector) and observed under a fluorescence microscope.

Detection of phenoloxidase (PO) activity in the HPT

Live crayfish were fixed by injection of 12.5 % glutaraldehyde in PBS. After 15 minutes, the HPT was dissected and washed five times for 10 minutes in 55 mM sodium phosphate buffer pH 6.8 (PB) containing 0.3 % Triton X-100, followed by three washes with PB, for 5 minutes each. For PO activity detection, the tissues were incubated in 5 mM L-dopa, 5 % sucrose in PB, with or without 10 mM phenylthiourea (PTU) as a control.

***In situ* hybridization**

Sense and anti-sense specific RNA probes were generated following the manufacturer's instruction of the DIG RNA Labeling Kit (SP6/T7) (Roche). A DNA template of CHF was amplified (see Table S1 for primers used) and the PCR products were then purified and used as templates for DIG-labeled RNA synthesis. The labeling efficiency was determined using DIG Quantification Teststrips (Roche) following the manufacturer's instruction. After deparaffinization and rehydration, the tissue sections were treated with 20 µg/ml of proteinase K (Sigma) for 15 min, and immediately washed with PBS and post fixed in 4% paraformaldehyde and washed again. The sections were acetylated with 0.25% acetic anhydride in 0.1M triethanolamine pH 8, and prehybridized in 4x SSC buffer containing 40% deionized formamide at 37 °C for at least 30 min. Each section was incubated 16 h at 52 °C with 100 µl hybridization solution (40% deionized formamide, 4x SSC, 1x Denharts solution, 0.1 mg/ml yeast

tRNA, 0.1 mg/ml salmon sperm DNA, 1mM DTT, and 7.4% dextran sulfate) containing 60 ng cRNA probe. Post-hybridization washes were performed at 52 °C using 5x SSC and 0.2x SSC, respectively. The sections were then treated with 20 µg/ml RNase A and washed with 0.2x SSC before blocking at room temperature (RT) for 1 h in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) containing 1% Blocking Reagent (Roche) and incubated at RT for 1.5 h with 1% Blocking Reagent containing 1:500 of sheep anti-digoxigenin-AP IgG (Roche). A dark purple color was developed for about 12 h in the dark in detection buffer (100 mM Tris-HCl, 100mM NaCl, pH 9.5) containing NBT/BCIP substrate (Roche), and then methyl green nuclear staining was performed. Finally, the sections were dehydrated and mounted in Euparal mounting medium (WALDECK).

Preparation of collagen I as matrix for the HPT cells

A collagen I solution was prepared by diluting 10.6 mg/ml collagen I stock solution (BD™) to 0.106 mg/ml with PBS and then 150µl of the diluted solution was added into 96 well plates, and allowed to form a gel at 37°C for 80 min. The collagen I-matrix-gel plate was sealed and stored at RT and used within 1 week.

***In vitro* cell culture**

The HPTs were collected and washed several times with PBS. The most anterior part of the HPT that connected to the brain was dissected separately and was named stem cell center (APC) in the following text. The rest of the tissue was named HPT_{rest}. Both parts were treated with collagenase (0.1 % of Type I and Type IV, Sigma) as previously described [7]. After suspension in modified L15-medium [8], the cells (1×10^5 cells/well, 150 µl) were seeded directly into 96-well plates for 2D cell culture, or were carefully dropped onto the surface of the collagen-I- matrix gel for 3D culture. The cell culture was maintained at 16°C and one-third of the medium was replaced every second day.

RNAi *in vitro*

RNAi was performed by mixing 5-10 µg dsRNA (prepared according to Supplementary Material and Methods) with culture medium and directly added to the HPT cell cultures. After one week, total RNA was extracted and quantitative RT-PCR was performed to monitor the transcript of crayfish hemocyte maker genes (primers listed in Table S1).

Reactive oxygen species (ROS) detection in HPT

The whole fresh HPT including the brain was dissected from crayfish and washed three times with PBS. Freshly prepared 5 mg/ml of 2',7'-dichlorofluorescein diacetate (DCF-DA) (Sigma) in DMSO (Sigma) was diluted with PBS (1:1000) and the tissue was incubated in this solution for 10 min in darkness. The tissues were then washed 4 times with PBS and transferred to 30% glycerol in PBS, and immediately observed under fluorescent stereomicroscope.

Results

There is a physical link between the HPT and the crayfish brain

The HPT of *P. leniusculus* is a thin sheet-like tissue with a lobule-like structure, and it is composed of hemocyte precursor cells in different developmental stages. Previously, this tissue was thought to be restricted to the dorsal part of the stomach. However, because previous reports have indicated that neuronal stem cells in the crayfish brain are derived from an external source [13, 14] and because several genes that we have isolated from crayfish are limited in expression to the HPT and cells in nerve and brain tissues [9, 12], we asked whether there is a physical link between the HPT and the brain. By careful dissection of this thin and fragile tissue, we can now demonstrate that the HPT extends forward and connects to the brain (Figure 2 A-C), which thereby supports that there is a morphological and possibly a functional link between these organs.

The HPT has an APC close to the brain

In the *Drosophila* lymph gland, the stem cells cluster together next to the dorsal vessel (medullary zone; MZ), whereas differentiating cells that develop mainly in the late larval stages, are located in the surrounding area, called the cortical zone (CZ) [15, 16]. To identify similar areas in the crayfish HPT, we first studied the structure of the HPT by light microscopy and hematoxylin and eosin staining. The tissue consists of numerous tightly packed cells, and some areas located in the middle of each side of the ophthalmic artery (OA) have more loosely associated lobules with large amounts of space between them (Figure 2C, arrows). A difference in nuclear staining intensity after hematoxylin and eosin treatment was found in the HPT cells (Figure 2D-G). Some cells show dark nuclear staining with

mainly condensed chromatin (heterochromatin) indicating more differentiated cells (black arrow in Figure 2G). Other cells show light nuclear staining indicating mainly loose chromatin (euchromatin) as in immature cells and stem cells (white arrow in Figure 2F, Figure S1A-B). Different amount of those cells were found at different locations of the HPT. The cells with dark nuclear staining were found with high percentage in the dorsal HPT (75%) and were present at significantly lower amount in the APC (43 %). In contrast, the cells with light nuclear staining were high in number in APC (57 %) and were found with significantly lower numbers in the dorsal part of HPT (Figure 2H). Moreover, the area with loosely associated lobules, which we named the growth zone (arrows in Figure 3A), was filled with cells following an injection of either LPS or laminarin, both of which are infection-mimicking treatments known to induce the recruitment of new hemocytes from the HPT (Figure 3C-D). To determine whether this growth zone close to the vessel was a center for cell division, we examined the proliferation of the HPT by using BrdU incorporation. However, proliferating cells were found mainly in the most anterior part of the HPT, especially around the area connected to the brain and in the edges around the HPT (Figure 4A), whereas significantly fewer BrdU incorporating cells were detected in the center or in the growth zone. When the incorporation time was extended to 3 h, the area with a strong BrdU signal expanded over the anterior part of the HPT from the edges and moving inwards (Figure 4B). However, the area surrounding the OA and the growth zone still contained few BrdU-positive cells (Figure 4D-I), but the percentage of BrdU positive cells in this area is lower than in the anterior part of the tissue (Figure 4C). In addition to investigating cell proliferation by BrdU incorporation, mitotic cell division in the tissue was also detected (Figure 5-6). In agreement with BrdU incorporation, more mitotic cells were found in the APC, which is the most anterior part of the HPT (Figure 5 A and C, Figure 6A) when compared to the posterior HPT (Figure 5 B and D, **Figure 6B**). To investigate whether the cells in the growth zone were induced to proliferate only during emergency or during an infection, mitotic cells in the HPT were examined at 30 min and 4 h after LPS injection. The amount of these cells were significantly increased at 30 min after LPS injection and especially in the anterior part of the HPT, and the level of mitosis decreased to the normal level again at 4-hour post LPS injection (Figure 6).

In summary, our data indicate that the HPT in crayfish is organized differently from the *Drosophila* lymph gland and that cells with a high proliferation rate are located in the anterior part of HPT, especially in the area close to the brain. We suggest that this region is the major location of the multipotent hematopoietic stem cells of the *P. leniusculus* HPT, and thus, we named it the APC.

ROS signaling is high in the anterior part of the HPT

Because increased levels of reactive oxygen species (ROS) have been found to induce the differentiation of hematopoietic stem cells in *Drosophila* and in the myeloid lineage of mammals [16, 17], we decided to test ROS activity during HPT differentiation. High ROS production was only found in a small area in the most anterior part of the HPT between the APC and the brain in normal crayfish and crayfish injected with CFS (Figure 7A, C). When laminarin was injected, the hemocyte number in the peripheral blood circulation was dramatically reduced to approximately 5% within 30 min (Figure 7B) and at 60 min post injection, the hemocyte number started to increase, and differentiation was initiated. At 30 min post injection with laminarin, a stronger signal of ROS was detected that also extended throughout the APC (Figure 7D) relative to that observed 30 min after CFS injection (Figure 7C). However, at 1 h after the injection, no obvious difference in the ROS signal was observed between CFS- and laminarin-injected crayfish (data not shown). The increase in ROS activity after laminarin injection corresponds in time with a rapid loss in circulating hemocyte number that is followed by a rapid recruitment of new hemocytes from the HPT (Figure 7B), which suggests that an increase in metabolic activity is associated with differentiation. In a previous study we demonstrated that hematopoiesis in crayfish is regulated in a circadian rhythmic manner [18]. Interestingly, the ROS activity in close proximity to the APC was very low or nearly absent, at 30 min after the light comes on in the morning (Figure 7E-F). This time coincides with the time of the day when the hemocyte number in the circulation is at its highest, which could indicate that the hematopoietic activity goes down when there is a lesser need for new hemocytes.

Low expression of the anti-apoptotic factor, CHF, is found in proliferative areas

Apoptosis is an ongoing process in the crayfish HPT [7], and CHF is important to limit apoptosis in the HPT when new hemocytes have to be produced and secreted into the circulation [12]. Previously, this gene was found to be exclusively expressed in HPT and mature SGCs [12]. In order to establish whether SGCs and GCs develop in the same or in separate lobules, we investigated where CHF-expressing cells are located in the HPT. We performed *in situ* hybridization and the results revealed CHF mRNA expression in all parts of the HPT excluding the edges areas (Figure 8A-B), where cell proliferation was high. Cells expressing CHF were spread over the remaining tissue; however, not all cells in each HPT lobule had high CHF expression (Figure 8D), which may reflect that CHF is restricted to SGC and HPT cells and that cells of the GC lineage do not express this transcript [12]. In some lobules no CHF mRNA was detected, indicating that these lobules are only producing GCs (Figure 8D black arrow). When we further analyzed the localization of apoptotic cells by TUNEL assay the highest rate of apoptosis was detected in the lobules near the edges of the tissue, whereas apoptosis (green areas Figure S2A) was very low or even absent in the central part near the artery. Positive cells were carefully examined for typical morphology to confirm apoptosis (Figure S2A insert). To stimulate new hemocyte synthesis, we injected LPS or laminarin into the hemolymph and observed that CHF expression was increased in all parts of the HPT (Figure S2B). New hemocytes are needed to compensate for the loss following laminarin injection (Figure 7B) and a similar decrease in apoptosis was observed in corresponding areas of the HPT after an LPS injection (data not shown).

Melanization of cells owing to PO activity does not occur inside the HPT lobules

Phenoloxidase (PO) is a marker for hemocytes that are close to full maturation and for mature hemocytes; nondifferentiated cells do not have PO activity. Mature SGCs as well as GCs and the late GC precursors (type 4 cells, Figure 1) express proPO mRNA. Therefore, we decided to localize the HPT cells, which are almost mature, by staining for PO activity *in situ* (by incubating the tissues in L-dopa). The result indicated that most PO-active cells in the HPT were freely circulating between the lobules and few, if any, PO-active cells were contained inside the lobules (Figure 9 A-C) and hardly any PO-active cells were found in the anterior part of the HPT. Preincubation with PTU, a strong PO

inhibitor, resulted in the absence of PO-active cells (data not shown), demonstrating that the observed staining is due to PO activity. A high number of PO-active hemocytes were also observed in the AO (Figure 9A, arrow), whereas PO-active cells were never detected in the anterior part of the HPT (Figure 9A and D). Instead, these more mature cells are mainly found in the central and posterior parts (Figure 9A and E).

Our new findings show proliferating cells at the edges of the HPT and an APC which is adjacent to the brain. More developed cells are found in the central parts of the tissue near a growth zone and around the large artery.

HPT cells from the APC appear to be different from cells from other parts of the HPT in culture

We have previously reported a method to culture HPT cells *in vitro* and used this method to isolate the astakine cytokines. Differentiation of the stem cells can be judged by the induction of the marker transcripts KPI for SGCs, and SOD for GCs [1]. However, in our previous culture system, APC cells were not included, and cells belonging primarily to types 2 and 3 (Figure 1) were cultured (Figure 10A).

In our new study, we were able to isolate single cells from the APC and compared their appearance in culture with that of cells of the remaining part, now named HPT_{rest}. Cells from HPT_{rest} could grow as monolayers (Figure 10B), and they can be induced to differentiate into GC and SGC precursor cells (type 4 and type 5) in accordance with our previous results [9]. However, cells isolated from the APC had a different appearance in culture. These cells divided rapidly during the first days, and after 3–5 days, small densely packed cell clusters appeared (Figure 10C-E). Several cells formed bowl-shaped cell clusters after more than 1 week in culture. However, after reaching a certain size at approximately 12–14 days, the cell clusters did not increase, and growth stopped. We did not find any signs of differentiation of these cells even when Ast1 or 2 was added to the culture. Conversely, as we have already demonstrated, HPT_{rest} cells can be induced to differentiate *in vitro* [9]. The survival and differentiation into SGC of the HPT_{rest} when cultured in a collagen I matrix, could be improved dramatically, as indicated by the increased transcription of PCNA (proliferating cell marker), KPI, and SOD (Figure 10D). Notably, the transcription of KPI increased more than seven fold. This may imply

that the collagen I matrix is favorable for the differentiation of HPT cells along the SGC lineage and also that the differentiation of SGCs is a faster process compared to that of GCs. The HPT cells cultured in the collagen I matrix exhibited higher CHF expression, which implies lower apoptosis. Downregulation of TGase further stimulated differentiation along the SGC lineage (Figure 10E), in accordance with our previous results showing that Ast1 stimulates HPT cells to differentiate and induces a decrease in the extracellular TGase activity of these cells [19].

Discussion

Hemocyte production in crayfish occurs in the HPT, which is located in the dorsal part of the stomach surrounding the ophthalmic artery [1]. The HPT contains densely packed hematopoietic lobules, and the cells in the APC and the dorsal HPT have different morphology. More than 50% of the cells in the APC contain nuclei with loose euchromatin whereas most of the cells in the dorsal HPT contain condensed heterochromatin. The structure of the chromatin has been reported to be less condensed in undifferentiated embryonic stem cells in mammals, and the chromatin rearranges to form highly condensed heterochromatin during cell differentiation [20]. Thus we speculate that the cells in the APC constitute the multipotent stem cells of the HPT. Further, we were able to culture cells from the APC *in vitro*, and these cells developed into densely packed cell clusters after 3–7 days of culture; conversely, the cells from the posterior HPT (HPT_{rest}) as were previously demonstrated to form monolayers, divide sporadically and differentiate *in vitro* after induction [8].

When LPS or laminarin is injected, cells accumulate in the central areas of the tissue, resulting in the release of new hemocytes. This injection also induced the proliferation of the HPT cells especially in the anterior part of the tissue. This observation indicates that there is a dynamic change in the HPT in response to LPS-induced blood loss, similar to that observed in response to an infection by bacteria (which contain LPS) or fungi (which contain laminarin). This is in agreement with our previous reports demonstrating that the injection of LPS or laminarin into crayfish results in elevated synthesis of hemocytes and subsequent release of hemocytes from the HPT [7, 8]. A similar response has also been observed during physiological stress in mice. Bleeding or LPS injection into mice induced the release of hematopoietic progenitors from the BM into the blood circulation through the activation of

osteoclasts, which degraded the BM endosteal compartment. Osteoclasts are bone cells derived from HSCs, and they require signals from osteoblasts to proliferate, differentiate and function [21, 22]. Here, we demonstrate that the anterior part of the HPT extends forward and links the HPT with the brain. This anatomical connection possibly facilitates physiological communication between the HPT and brain. For example, it has been reported that hemocyte number and the plasma level of the hematopoietic cytokine, Ast1, oscillates in a typical circadian rhythmic manner in crayfish [18]. Moreover, a study in adult *P. clarkii* crayfish suggested that HPT cells possibly serve as a primary source of neuronal precursor cells [13]. In mammals, a brain-bone-blood link has been proposed to require signals delivered from the central nervous system directly to the hematopoietic stem and progenitor cells or indirectly to the niche that controls HSC proliferation, mobilization and differentiation [22]. In humans, the percentage of BM cells in S-phase and colony-forming units (CFUs) exhibited circadian variations [23]. In mice, circadian-regulated HSC release from the BM is correlated with the secretion of adrenergic hormones from the suprachiasmatic nucleus [24]. In this study, we discovered an APC in the anterior part of the HPT in close connection to the brain. This location is very different from that in the fly, in which an APC is localized in the MZ close to the dorsal vessel [15]. No connection between the *Drosophila* lymph gland and the brain has been described, which may indicate different functions for arthropod hematopoietic tissues. However, the peripheral nervous systems (PNS) has been reported to be a place where mature circulating larval hemocytes of *Drosophila* resides and the PNS has been shown to be important for hemocyte survival [25]. The primary lobes of the *Drosophila* lymph gland develops a larger CZ during the final larval stages, and since crayfish are much more long living animals with adult hematopoiesis, the major parts of the HPT may correspond to this CZ of the *Drosophila* lymph gland, whereas a putative MZ may be minor and difficult to detect without molecular markers. However, our results showing changes in ROS activity linked to proliferation indicates that the SSC may play a similar role in crayfish as the MZ in *Drosophila*.

Aerobic metabolism normally causes the production of ROS, which exposes cells for the risk of oxidative stress. The maintenance of an optimal O₂ level is therefore important for proper cell

function, especially in stem cells. In mammals, low O₂ levels or hypoxic conditions have been reported to play a role in establishing a stem cell niche and controlling stem cell behavior [26]. However, different stem cells require different optimal O₂ environments. For example, mesenchymal stem cells occupy a 2–8% O₂ niche, whereas ranges of 1–8% and 1–6% O₂ are required for neural stem cells and HSCs, respectively [27]. Moreover, in the mouse BM, two different niches that are related to different O₂ levels are present and required for two subpopulations of HSCs. The low-O₂/hypoxic osteoblastic niche is possibly necessary for maintaining long-term HSC quiescence and protecting the HSCs from the harmful effects of ROS accumulation, whereas the high-O₂/vascular niche in the central BM is a residence for short-term HSCs and primes the HSCs to proliferate [28]. In mice, ROS were demonstrated to play a role in hematopoietic reconstitution [17], the HSC transition to myeloid progenitors, self-renewal capacity and HSC quiescence maintenance [29, 30]. In *Drosophila*, high ROS levels were found in the hematopoietic progenitor cells in the MZ. Stimulating an elevation of ROS resulted in the differentiation of the progenitor cells to all hemocyte types in the *Drosophila* lymph gland (LG) via the activation of the JNK and FOXO signaling pathways and the downregulation of Polycomb [16]. In addition, the increase of ROS was detected in the posterior signaling center (PSC) of *Drosophila* LG after a wasp infestation. This elevation regulates the *Drosophila* immune response since it serves as a signal that primes the progenitor cells in LG to differentiate into lamellocytes [31]. Here, high ROS levels were detected in a specific area between the brain and the APC of the HPT, an area that we propose to be crayfish HSCs, and the level of ROS formation was increased in response to a laminarin injection. The high ROS levels are possibly the result of a high metabolic rate of the cells in this area, which could serve as a unique microenvironment or a niche to maintain crayfish HSC behavior. However, it is difficult to conclude whether the increase in ROS production is essential for differentiation or if it only reflects a high metabolic rate during the differentiation process.

Melanization is an important innate immune reaction in invertebrates [2, 32] that is mediated by mature hemocytes expressing proPO. PO-positive cells were mainly found in mature hemocytes in the ophthalmic artery and in a small population of HPT cells in the posterior part of the HPT. These results confirm our previous observations that a low percentage of HPT cells express proPO transcript,

whereas mature hemocytes have high proPO expression [7]. Another report in *Drosophila* revealed that differentiation into PO-active crystal cells is a postmitotic event [4]. From this information, we propose that the APC is where multipotent HSCs are located and that more differentiated cells reside in the central and posterior parts of the tissue. These differentiated cells are possibly already committed to undergoing final maturation upon release from the HPT.

Conclusion

The HPT of *P. leniusculus* provides a simple model system to study hematopoiesis because the tissue can be isolated and the proliferation of stem cells and their differentiation into mature hemocytes can be studied both *in vivo* and *in vitro*. Moreover, our discovery of a stem cell center in close connection to the brain offers an opportunity to explore the regulation of invertebrate hematopoiesis and its connection to the central nervous system.

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Author Disclosure Statement

The author hereby certify that they have no non-financial or commercial associations that might create a conflict of interest in connection with submitted manuscripts.

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Figure legends

Figure 1. Schematic overview of the hematopoietic lineages in crayfish

Hemocytes are produced in HPT located on the dorsal side of the stomach. Five different cell types are identified in the HPT, where cell type I is a stem cell and type II is a SGC/GC precursor. Cells in the HPT follow two main cell lineages: one is from type I-II (the stem cells) via type V cells to SGCs and the other is from type I-II to types III and IV cells to GCs. Two cytokines are identified in this process: Ast1 and Ast2. Ast1 stimulates the proliferation of HPT cells, induces differentiation of the SGC lineage and stimulates the release of hemocytes into the circulation by affecting extracellular TGase activity [1,19]. Ast1 induces the expression of CHF, an antiapoptotic factor, and KPI, an SGC marker. By contrast, Ast2 increases the number of mature GCs by an unknown pathway. SOD is a GC lineage marker. proPO and the Runt transcription factor are expressed in mature hemocytes of both the SGC and the GC lineages.

Figure 2. Location and structure of crayfish HPT and brain

The HPT is located in the dorsal part of the stomach, with the anterior part of the tissue extending forward and linking the tissue to the brain (A-C). Hematoxylin and eosin staining of the longitudinal section of the tissues show organization of the APC, dorsal HPT, stomach and the brain (D-E). HPT cells in APC and the dorsal HPT show different nuclear staining (F-G). The APC contains higher percentage of the cells with light nuclear staining (immature cells) whereas the dorsal HPT contains mainly the cells with dark nuclear staining (maturing prohemocytes) (H). Scale bars in F and G are 20 μ m. AO = ophthalmic artery, asm = anterior stomach muscles, b = brain, c = connective tissue, e = esophagus, psm = posterior stomach muscles, s = stomach.

Figure 3. Accumulation of the cells in the growth zone after LPS injection

Some areas of the HPT consist of tightly packed cells, whereas some areas have loosely connected cell lobules (A). The area with low cell density, indicated by the light red color of hematoxylin and eosin staining (arrow in Figure 2A), contains a higher density of cell lobules after crayfish were injected

with LPS for 2 h (C) and 4 h (D) relative to that at 5 min (B). The experiment was repeated twice with 4 animals in each experimental group. Scale bars in are 100 μm .

Figure 4. BrdU incorporation in the HPT

At 1 h (A) and 3 h (B) after BrdU injection, HPTs were collected and used for whole-mount BrdU staining (green). BrdU detection was performed using mouse anti-BrdU IgG, and mouse IgG was used as the negative control (the inset in A shows the green stain in the area of the brain that stains nonspecifically). BrdU staining of HPT sections was also performed 3 h after BrdU injection (D-I), and percentage of BrdU positive cells in HPT section was calculated (C). Nuclear staining for HPT was performed using DAPI (blue). Images of HPT sections from different regions of the tissue are shown in D-I according to the letters indicated in B. Scale bars are 1 mm for A and B and 20 μm for D-I.

Figure 5. Mitotic cells in APC and posterior/dorsal HPT

Mitotic cells (red) were detected in APC (A-C) and also in posterior HPT (B-D). Nuclear staining for HPT was performed using DAPI (blue). Scale bars in A-B are 300 μm . Scale bars in C-D are 20 μm . b = brain, c = connective tissue, s = stomach.

Figure 6. Increase of mitotic cells in HPT after LPS injection

The mitotic cells in HPT were detected using anti-phospho-histone H3 antibody (shown in green). Under normal condition the mitotic cells are mainly present in the anterior part (A) when compared to the posterior part (B). At 30 min after LPS injection significantly higher amount of mitotic cells are present in the tissue (C-D), but no obvious difference was observed at 4 h after injection (E-F) when compared to A-B. The green spots of mitotic cells were counted and calculated as mitotic cells/ cm^2 of the tissue. Scale bars are 200 μm .

Figure 7. ROS activity in the HPT

ROS (green) was detected in fresh HPT. In normal crayfish (A) and crayfish injected with CFS (C), a strong ROS signal was found in a small area of the most anterior part of the HPT between the APC and the brain. Circulating hemocytes dramatically decreased in number approximately 30 min after laminarin injection (B). Conversely, ROS levels were increased at this time point, and ROS signals were also found in the APC (D).

Low levels of ROS were detected in the morning at 30 min after onset of light (E), whereas high levels were found concomitant with lower hemocyte counts at night (2 h after lights comes off) (F).

A, C-D are merged pictures of the tissues taken under visible and UV light. Scale bars are 1 mm. APC = stem cell center. THC = total hemocyte count.

Figure 8. Expression of CHF in different parts of the HPT

Expression of CHF in the different parts of HPT detected by in situ hybridization (ISH) indicates that CHF is evenly distributed throughout the tissues excluding the edges (A-B). The arrow in A denotes the low expression at the edge of HPT, and a control with a sense RNA probe is shown in (C). Higher magnification of CHF ISH in the HPT lobules (D), note a mature GC with no CHF expression (thin black arrow). In some lobules, most of the cells are CHF-positive (white arrow), whereas in some lobules, no expression is observed (black arrow). Scale bars are 40 μm for D-F and 20 μm for G.

Figure 9. Phenoloxidase activity in the HPT

PO-positive cells are found in the posterior part of the HPT, and PO-positive hemocytes can also be observed within the ophthalmic artery (arrow) (A). Most PO-positive cells are found outside of the lobules in the interlobular spaces (B-C). Details of the anterior edge of the HPT show no PO-positive cells (D), whereas several melanized cells can be detected in the posterior part (E).

Figure 10. Culture of HPT cells isolated from different parts of the tissue

Type 2 cells are the major dividing cells in HPT_{rest} as shown by TEM (A), and the cells isolated from this part form monolayers in *in vitro* culture (B). HPT cells isolated from the stem cell center (APC)

form cell clusters during *in vitro* culture (C-E). HPT_{rest} cells cultured in a collagen I matrix had a higher proliferation rate as monitored by PCNA transcription and differentiated along the SGC lineage as monitored by the transcription of the KPI transcript. Moreover, the transcription of CHF was also significantly upregulated (F). RNAi of TGase in 3D HPT cell culture showing that downregulation of TGase preferentially promoted the differentiation of HPT cells along the SGC lineage as indicated by increased KPI expression (G).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Supplementary Figure S1A

Supplementary Figure S1B

Supplementary Figure S2

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