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Blood cell formation in crustaceans

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A B S T R A C T

In crustacean animals the hemocytes are key players in immunity and of crucial importance for the health of the animals. Hemocytes are mainly produced in the hematopoietic tissue and from there released into the circulation where they finally mature. In this review we summarize the latest findings about crustacean hemocyte formation. The role of the extracellular matrix and crosslinking enzyme transglutaminase is discussed. Moreover, important growth factors, transcriptional regulation and recent findings about inducers of hematopoiesis are covered. Finally, we discuss the use of different markers for classification of crustacean hemocytes.

1. Introduction

Crustaceans belong to one of the most species-rich animal groups on earth, and the variations in appearance and way of life are very large. The majority of crustacean's lives in aquatic environments and are in constant contact with microorganisms of various kinds. This means that they regularly come into contact with substances foreign to the body in significantly larger quantities compared to terrestrial animals. This is clearly reflected and shown in their immune system, which is constantly activated in many ways, e.g. by having a continuous production of various anti-microbial peptides [1–3]. Such peptides are in terrestrial animals, like many insects, as a rule instead induced during an infection, while in aquatic crustaceans they are often produced continuously. The organs that produce these peptides in crustaceans are mainly in the hepatopancreas, which has an open contact with the outside world via the gastrointestinal tract, and in the blood cells, that circulate throughout the interior of the body and defend the animal against foreign substances and organisms that may enter the body cavity via damage to the external protection often consisting of a cuticle. The blood cells, the hemocytes, are thus of crucial importance for crustaceans to survive in the dangerous environment in which they live, and it is therefore understandable that blood cells in particular have been studied extensively in these animals for more than a century. Many crustaceans live a long life, some of which can live more than 30 years, and their hemocytes are continuously consumed. It is therefore crucial that these hemocytes are replaced by new ones, and this happens continuously throughout the life of the animals. Hemocyte formation, hematopoiesis, has been studied more intensively in the last 20 years, but still only by a few research groups. Due to their great economic and ecological importance, it is mainly crustaceans of the decapod group that studies of blood cells, the immune system and blood cell formation have focused on, and consequently these topics have been the subject of numerous review articles in recent decades (see e.g. [4–7]). In the following review, the latest research findings in crustacean hematopoiesis will mainly be treated, and the focus will be on the freshwater crayfish with some comparisons with other crustacean animals.

1.1. Structure of the hematopoietic tissue

The hematopoietic tissue (HPT) in crustaceans was identified as early as in the 19th century by Allen et al. [8] but some researchers early considered the tissue to be an ecdysone-producing gland. However, this was disproved, among other things by Böhm and Gersch already in 1983 [9]. The HPT tissue has been described morphologically in detail by several research groups during the second half of the 20th century (for a detailed overview see [4]). Its structure and localization in crayfish and shrimp are very similar, although the penaeid shrimp also have a so-called lymphoid organ where accumulation of, and protection against foreign particles take place and a certain amount of cell division occurs [10]. In the freshwater shrimp *Macrobrachium rosenbergii,* a corresponding role could be shown for the sinuses around the core frontale [11], near the part of the hematopoietic tissue with a high proportion of rapidly dividing cells, called APC, which has been previously identified in both P. leniusculus [12] and Procambarus clarkii [13]. The APC, is described as the part of the hematopoietic tissue located at the core frontale, and the detailed anatomy of the tissue has been described in considerable detail by Chaves da Silva et al., 2013 [13]. It is precisely in this part that rapid cell division occurs, and probably due to the high metabolic activity that this requires, APCs exhibit high levels of reactive oxygen species (ROS) [12,13]. It is also in the APC that a large increase

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in cell division frequency is found after an injection of laminarin or LPS in connection with a short pulse of increased production of ROS [12]. We later showed that this pulse of ROS is important for stimulating the release of newly formed hemocytes from the hematopoietic tissue [14], which also was previously shown in Drosophila [15]. Recently, this importance of ROS for hemocyte release was also confirmed in *E. sinensis* [16]. In Fig. 1 we have made a simple picture to demonstrate the localization of the HPT and APC in *P. leniusculus*.

1.2. The extracellular matrix

The newly formed hemocyte precursors in the hematopoietic tissue are packed in small lobules surrounded by an extracellular matrix (ECM). The least differentiated cells, i.e. stem cells in both *P. leniusculus* [17] and *M. rosenbergii* [10] are localized to the interior of each lobule, while more differentiated cells are more loosely associated in the distal part of the lobule opening into the central sinus, in which mature hemocytes are also found. An important observation is that the ECM is increasingly loosely organized primarily in areas with more differentiated cells. We recently demonstrated that the ECM in the hematopoietic tissue of the crayfish *P. leniusculus* consists, among other components such as collagen also large amounts of the clotting protein [18]. The clotting protein is the protein that also circulates in the plasma and is an important substrate for the cross-linking enzyme transglutaminase (TGase). This enzyme converts this clotting protein into polymers of the protein and in this way, wounds are rapidly and efficiently sealed to prevent blood loss and penetrating pathogenic microorganisms. Thus the clotting protein and transglutaminase are the most important components of the coagulation reaction in crayfish as well as in several other crustaceans [19].

Transglutaminase (TGase) is one of the proteins that is expressed in large amounts in the hematopoietic cells [20–22], and we have shown that this enzyme has an important role in the regulation of hematopoiesis, mainly in terms of the release of cells out to the peripheral circulation from the hematopoietic lobules. Recently, we were able to show the presence of two different TGases in *P. leniusculus*, of which the first identified variant, TGase1 [23] is highly expressed in HPT cells, APC and semi-granular hemocytes, while the other, TGase2, is specific for granular hemocytes and cells of the brain, nerves and green gland [24].

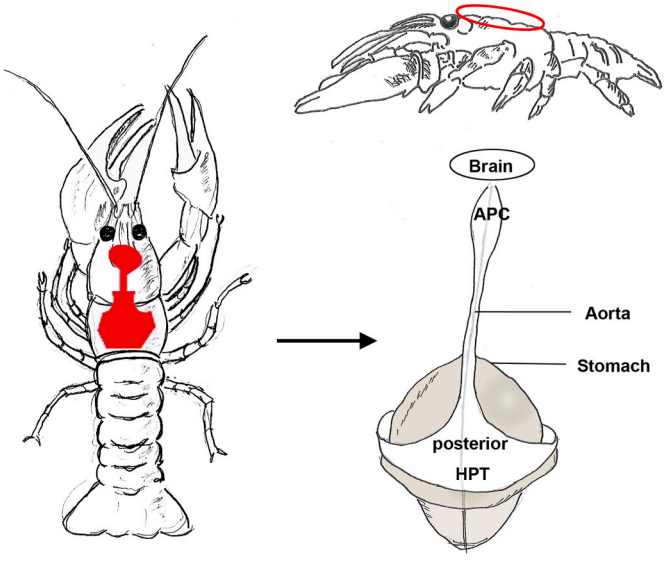

Fig. 1. The hematopoietic tissue (HPT) (marked in red) of freshwater crayfish is covering the stomach and consists of a thin layer of densely packed lobules with precursor cells in various stages of differentiation. The tissue surrounds the great ophthalmic artery and extends forward and merges into it. the so-called APC part around the core frontale.

TGase1 which is mainly responsible for the clotting reaction was also found to be active at low temperature in crayfish, which is an important characteristic in order to be able to protect the animal and allow clotting after wounding when these animals are living in cold water [25].This is the case for most crayfish in at least Northern and Middle Europe where water temperatures can go down to less than 4 C° during the winter season in lakes and rivers.

The ECM constitutes a physical barrier for newly formed hemocytes to be released into the circulation. The ECM is stabilized by TGase1 so that it forms a rigid matrix around the hematopoietic stem cells. If the activity of TGase1 was blocked or inhibited, it was possible to reduce the ECM strength, and this reduction in enzyme activity of TGase resulted in an increased release of blood cells [14,20]. We have recently shown that the TGase1 enzyme is colocalized with both collagen and the clotting protein in the ECM of the hematopoietic tissue [14,18], and thereby, contributes to strengthening the bonds between these extracellular matrix proteins. Likewise, in Drosophila, the importance of ECM proteins for differentiation has been demonstrated. In a loss of function (LOF) mutant, where the extracellular matrix protein Trol (terribly reduced optic lobes) was not present gave as a result an increased differentiation of hemocytes, clearly showing that intact ECM proteins prevent cell differentiation to occur [26]. Thus, our recent results give further evidence that control of the ECM is an important factor for the regulation of differentiation in the hematopoietic tissue and that the release of newly formed cells into the circulation is pending on a loosely organized ECM structure (Fig. 2). This control occurs at least in part via a regulation of TGase1 enzyme activity as we have shown in crayfish. Also, interesting to note is that the promoter region for TGase1 contains GATA-binding sites (GenBank: EU195879.1) and thus it is highly likely that GATA factors are involved in the regulation of TGase1 expression, as well as to be involved in regulating hematopoiesis in general. As mentioned above, we could show that a short pulse of a high ROS production is important to stimulate differentiation and release of new cells from HPT and the reason for this effect on hemocyte synthesis and their release into circulation was that this ROS pulse was found to inhibit the TGase activity in HPT (Fig. 2) [14]. Thus, by inhibiting TGase activity *in vivo* by treating crayfish with the reversible TGase inhibitor cystamine, we have further confirmed the importance of TGase1 enzyme in the regulation of hemocyte differentiation and release [27]. Cystamine was able to effectively inhibit TGase 1 activity, causing an increase in newly produced hemocytes in the circulation. A very interesting observation in connection with these *in vivo* experiments was that this cystamine-induced inhibition of the enzyme also produced a very clear and reversible effect on the crayfish's behavior, so that their movements and aggressiveness were radically reduced [27]. In this case, cystamine can also have effects other than blocking TGase activity, but the clear effect on the crayfish's behavior is noticeable and can hopefully lead to further studies since the mechanism by which this occurs or what substrate is affected is still largely unknown.

Inhibition of TGase activity also occurs naturally *in vivo*, via the hematopoietic growth factor astakine1 (Ast1), and we have also found a link between TGase activity and PVF/PVR signaling in hematopoiesis (see details below) [28,29]. Taken together, our experiments show that the physical strength of the ECM is an important regulatory factor for hemocyte homeostasis in crayfish and most likely also in other crustaceans (Fig. 2).

2. Induction of hematopoiesis and its regulation

Knowledge about blood cell synthesis, the so-called hematopoiesis, in invertebrates has mainly been generated through studies in *Drosophila melanogaster*. The formation of different hemocyte types in the hematopoietic organ of the Drosophila larva, the lymph gland, has been studied for many decades and has become an excellent model for the differentiation of blood cells. These studies can and have also provided a lot of knowledge about the corresponding processes in other

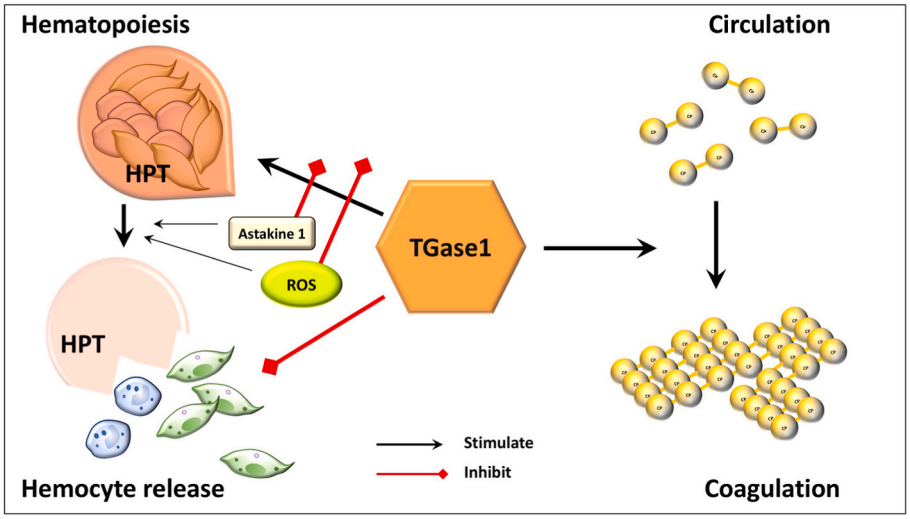

Fig. 2. Transglutaminase (TGase) is a multifunctional enzyme which has a biochemical function in forming cross-links between lysin and glutamine residues in proteins. In crustaceans, there are two TGases, and TGAse1 is involved in the clotting reaction in the animals by forming oligomers of a 200 kDa monomeric protein, the so called clotting protein (CP). In this way wounds are sealed in the animal. Another important function of TGase1 is to stabilize the matrix proteins in the HPT, again by crosslinking, among others the clotting protein and collagen, so that the HPT stem cells are prevented from migrating out of the tissue and are stimulated to divide. By inhibiting TGase1 activity by astakine 1 [28], or by a short pulse of ROS [14] this crosslinking will not take place and through action of peptidases the ECM will be loosened up and differentiation of the HPT cells will take place and these cells are then released into the circulation.

invertebrates as well as for humans [30,31]. The regulation of hematopoiesis via different signaling pathways and the importance of different transcription factors in Drosophila is well conserved also in vertebrates. The knowledge generated in the fly can therefore also be used to understand blood cell formation in crustaceans and other invertebrates.

The structure and localization of the hematopoietic tissue in the Drosophila larva and in crustaceans also resemble each other. The tissues are located in the dorsal part of the thorax and is closely associated with the dorsal median artery. In Drosophila, there are similar hematopoietic so-called hubs around this blood vessel in the adult fly as well [32]. The regulation of hematopoiesis via transcription factors, is highly conserved between Drosophila and vertebrates, and similarly, a corresponding regulation has also been demonstrated in crayfish. Thus, both GATA factors (zinc-finger DNA-binding proteins binding to a canonical GATA DNA sequence motif) and Runx transcription factors (runt-related transcription factors) have been shown to be important for the development of hemocytes in crustaceans [33,34]. We earlier showed that Runx was necessary for the differentiation of prophenoloxidase-expressing hemocytes in the crayfish *P. leniusculus* already back in 2003 [34], and recently, it was shown that both GATA and several Runx factors were important for hemocyte formation in the Chinese mitten crab *Eriocheir sinensis* similar to that previously found and described in the crayfish, *P. leniusculus* [35]. Thus a GATA factor was also recently found in *E. sinensis* and its expression was very high in the hematopoietic tissue, but also in the hepatopancreas and gonads, which suggests that it has a more general function in the differentiation of new cells in these tissues in this crustacean [33]. Other tested tissues such as gills, muscles and heart showed very low expression of this GATA factor and it is therefore likely that these tissues do not contain a significant number of stem cells [33]. GATA factors are regulated by other so-called "Friend-of-GATA" transcription factors, and in *E. sinensis* one such particular, EsUsh (similar to Drosophila U-shaped) has been identified and it was shown to regulate the formation of hyaline hemocytes so that these increase while the granular cell types decrease in proportion [36]. This is a similar function as for Ush in Drosophila, where Ush stimulates the formation of plasmatocytes and reduces the formation of proPO-containing crystal cells [37]. However, there are also clear differences between transcriptional regulation in fruit flies and crayfish. One example concerns the factor glial cell missing (gcm), which in the fly is required for plasmatocyte formation, while this transcription factor is missing or is expressed at a very low level in the hematopoietic tissue of the crayfish. *P. leniusculus* [38]. Instead we have recently shown that this factor is specifically expressed in the brain and there only in a few cells with specific morphology suggesting that this factor may be involved in brain development or brain repair [38].

However, it is important to point out that each species or group of animals has unique characteristics that have evolved in their specific habitat. Many crustaceans live in water, and many, such as lobsters and other decapods live for decades, and they are exposed to completely different potential pathogens compared to the fruit fly. It is also important to gain an increased understanding of stem cell development in genetically more complex animals other than simple model organisms in order to understand regenerative processes in long-lived animals, and also to gain a more detailed understanding of the immune system and how this specifically works against various pathogens in economic and ecologically important species. However, there are great technical difficulties associated with studying hematopoiesis in crustaceans, and several other invertebrates, compared to Drosophila. The fruit fly offers genetically homogeneous lines, and these can easily be genetically manipulated to produce mutants, which enable studies of the function of different molecules in a more direct way. So far, this has not been possible in decapod crustaceans. Many of these animals have large genomes, such as *Fenneropenaeus chinensis* with 1.88 Gb [39] and *Litopenaeus vannamei* with 1,66 Gb [40]. This fact makes the production of genetically homogenous populations difficult. Instead, hematopoiesis can be studied by exposing the animals to various types of stimulating or inhibiting interventions, which in themselves can and most likely will produce many side effects.

Examples of treatments that stimulate an increase in the number of hemocytes in the circulation are draining the animals of a larger amount of hemolymph, or injecting a sterile saline solution. Both of these methods produce a very rapid increase in the number of blood cells within 1–6 h, and recent studies have shown that this increase is mainly due to hemocytes being released from various depot tissues in the crayfish body [41,42]. Only a minor proportion new cells from the hematopoietic tissue is released during this early hours after such stimuli [41]. A study on *Cherax quadrcarinatus* [41], showed that bleeding the crayfish rapidly caused a release of hemocytes that had been stored in the gills. That gill filaments are important organs for hemocyte storage are well known and a study by Suleiman clearly showed that mRNA expression of the antimicrobial peptide Carcinin in gills is only due to infiltrated hemocytes [43]. Injections of different solutions are often used in studies of the immune system and its function in crustaceans, and we recently showed that an injection of only a sterile saline solution has large effects on differential blood cell counts, and thus of course also will affect drastically the mRNA expression in the total population of hemocytes [42]. The fact that a relatively large volume of fluid is injected into the circulation increases the pressure in the body cavity and causes cells stored in various tissues to be washed out into the

circulation. If, however, a pathogen, or a pathogen-associated molecule (PAMP) such as LPS or a β1,3-glucan is injected at the same time (dissolved in saline), other responses to these molecules will overlap with the response given by a saline injection alone,but more importantly the response to saline injection is disguised by the PAMPs. Thus, saline injection alone mainly results in that large quantities of mature blood cells that have infiltrated various tissues are being pushed out into the circulation, but this saline injection does not significantly affect the cell division frequency in the hematopoietic tissue.This is in contrast to an injection of a β1,3-glucan which increases cell division dramatically [34] For example the corresponding increase was demonstrated after an injection of *Aeromonas hydrophila* or LPS in *E. sinensis*, in that the mRNA expression of cyclin-dependent cyclase 2 (CDK2) doubled already 3 h after the injection [44]. However, the increased hematopoiesis after such inductions manifests itself later in the circulation, which has been demonstrated in studies where cells in S phase have been labeled into the hematopoietic tissue (HPT) [45], and the anterior proliferation center (APC) [46]. Taken together, these studies show that gene and protein expression studies in crayfish and other crustaceans to study the immune defense against specific pathogens are complicated, and require careful controls and time series both with and without injections of control saline or an effector [42]. Therefore, it is always essential to add an uninjected control so that the effect of vehicle injections can be analyzed properly.

3. Growth factors in crustacea hematopoiesis

While several growth factors and signaling pathways regulating hematopoiesis in Drosophila have been described, such as signaling through platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF)-pathways [47], Epidermal growth factor receptor (EGFR) [48], Nimrod-type receptor B5 [49], Unpaired (Upd) [50] and several others (for a recent review see [51]), similar studies are rare in crustaceans.

However, we have identified a number of different PDGF/VEGF-like factors (PVF) and corresponding receptors (for example the PDGF/VEGF-receptor like factor PVR1, GenBank accession No. KY444650) in hemocytes and in hematopoietic cells of *P. leniusculus* [22,29]. The receptor PVR1 is only expressed in a small percentage of both the hematopoietic cells and hemocytes. If the crayfish hematopoietic tissue was treated *in vitro* with the inhibitor Sunitinib malate, we were able to show an increased extracellular TGase1 activity, but in addition to this increase in enzyme activity there was also an increased migration of HPT cells [29]. Sunitinib malate is a relatively broad inhibitor of receptor tyrosine kinase such as PDGFR/VEGFR family receptors, and we could demonstrate that the signaling via these or similar receptors is involved in hematopoiesis in *P. leniusculus* [29]. However, it is necessary to perform more detailed studies to elucidate more specifically how PVF/PVR signaling regulates hemocyte synthesis and differentiation in crustaceans.

Considerably more experimental data is available for the growth factor, or cytokine, that we have identified in crayfish and shrimp and which we named Astakine [52]. Astakines (Ast) are small cysteine-rich proteins containing a prokineticin domain (pfam 06607), and after our initial identification in crayfish and shrimp, similar proteins have been found in a large number of crustaceans and insects,but notably and interestingly not in dipteran, lepidopteran or coleopteran insects. These latter classes of insects are considered to be of later evolutionary stand than other insects [53]. A more detailed review of astakine and its effect is described in a previous review [4], and here only the most recent experimental data will be reported.

That Ast has a role in the regulation of hematopoiesis has recently been shown in several crustaceans, such as e.g. *Scylla paramamosain* where Ast knockdown resulted in a significant reduction in the number of circulating hemocytes [54], and in *E. sinensis* where, in a similar way, Ast knock-down gave a delayed new formation of hemocytes [16]. Interestingly, even in the pacific oyster *Crassostrea gigas*, an Ast-like factor has been identified that is important for new formation of hemocytes [55].

In the crayfish *P. leniusculus*, two forms of astakine are present and both are also expressed in hematopoietic cells and hemocytes (Ast1 and Ast2 [56]), and it is mainly the function of Ast1 that we have studied in more detail. Ast1 affects cell division in the hematopoietic tissue as well as the release of newly formed cells from this tissue into the circulation. This release of cells occur when Ast1 is inhibiting the activity of TGase1 (Fig. 2) [28]. Thus, we were able to show that Ast1 purified from *P. leniusculus* plasma acted as a non-competitive inhibitor to both *P. leniusculus* TGase enzyme and its activity in an HPT cell lysate, and a purified commercial TGase from guinea pig. The clotting reaction which involves the TGase enzyme and the clotting protein in *P. leniusculus* was also inhibited by Ast1 in an *in vitro* experiment [28]. Purifying native Ast1 from crayfish plasma involves certain difficulties, as the protein under certain circumstances forms large molecular complexes with other molecules. We found that Ast1 inside the cells always occurs as a low-molecular monomer, while if the Ast1 is secreted into the plasma, it forms high-molecular complexes if the Ca^{2+} concentration is low and/or if the protein concentration is high [57]. This polymerization is most likely to prevent the peptide Ast from being degraded in circulation. Also this fact may be one of the key reasons why so far no further reports on the purification of Ast1 from crustaceans have been published, apart from our initial report in 2005 from crayfish plasma [52].

Apart from the astakines, and PVF/PVR, we have recently also shown that components that are formed after activation of prophenoloxidase (see review [58]), affect hematopoiesis. We produced a recombinant peptide with sequence corresponding to the peptide cleaved off from proPO upon activation by the proPO-activating serine protease [59]. This recombinant peptide was then injected into *P. leniusculus* hemolymph and we could then establish that this peptide affects hemocyte synthesis so that an increase in BrdU-labeled cells in the circulation and the total number of hemocytes increased [60]. One mechanism by which the peptide acts on the hematopoietic tissue appears to be via an increased ROS production in the APC [60].

The hematopoietic cells can be dissociated and maintained in *in vitro* cell cultures, and in our experiments, by adding partially purified plasma containing native Ast1, we could keep HPT cells from *P. leniusculus* in culture for several weeks [52]. These cultured cells can, especially if they are allowed to grow in a three-dimensional matrix of collagen and clotting protein, partially differentiate and express genes that are otherwise only expressed in circulating hemocytes in the hemolymph [12,18]. In Table 1, we summarize the present knowledge about genes and factors that participate in crustacean hematopoiesis.

There are also examples of other methods for culturing isolated hematopoietic cells using crude undefined muscle extracts. This was described for *Nephrops norvegicus* [61], and recently it was shown that nearly all isolated HPT cells from the crayfish *Cherax quadricarinatus* could differentiate into granular hemocytes after culture *in vitro* for over a month with the addition of fetal calf serum and a very crude muscle extract from the crayfish. However, no attempts were made to isolate or characterize the component(-s) responsible for this effect during a very long time in *in vitro* cultures [62]. Conversely, after intensive trials in *P. leniusculus*, we have unfortunately not succeeded in repeating these published results after cultivation in the presence of crude crayfish muscle extract as was described by Li et al. [62]. Thus, this shows that it is necessary to identify the components in this muscle extract to be able to properly repeat the published results from the crayfish *C. quadricarinatus* in other crayfish species.

4. Molecular markers for specific cell types in crustacean hematopoiesis

Crayfish hemocytes have traditionally been divided into three morphologically different types; hyaline, semigranular (SGC) and

Table 1
Factors involved in hematopoiesis in crustaceans.

Factor	Function	Species	References
Clotting protein	ECM component	*P. leniusculus*	[18]
Collagen	ECM component	*P. leniusculus*	[18]
TGase 1	Crosslinking ECM protein, blocking hemocyte release	*P. leniusculus*	[27–29]
Runx	Transcription factor, important for induction of proPO expressing cells	*P. leniusculus* *E. sinensis*	[34,35]
GATA-factor	Transcription factor, needed for hematopoiesis	*E. sinensis*	[33,35]
EsUSH	Friend-of-GATA factor, important for development of hyaline cells	*E. sinensis*	[36]
PVR1	A PDGF/VEGF-receptor like factor. Indirect, unknown mechanism, effect on hematopoiesis and hemocyte migration	*P. leniusculus*	[29]
Astakines	Prokineticin domain proteins, act as inducing growth factors of hematopoiesis. Inhibits TGase!	*P. leniusculus* *S. paramamosain* *E. sinensis*	[16,52, 54]
proPO-cleavage peptide	Induce hemocyte release, unknown mechanism	*P. leniusculus*	[60]
Crustacean hematopoietic factor (CHF)	Protect HPT cells from apoptosis	*P. leniusculus*	[84]

granular hemocytes (GC) [63,64]. These three cell types can be separated using centrifugation in a Percoll gradient, a technique developed by Söderhäll and Smith as early as 1983 [65,66]. Through this technique, we could already in the 1980s identify different functions in these three different cell types in a number of crustaceans (see review [64]). We found that the hyaline cells are phagocytic [67], while the granular cells contain the proPO-activating system, and also have cytotoxic properties [68]. The semi-granular hemocytes were found to have some phagocytic activity or rather some of the cells in this SG cell fraction could display some phagocytosis. Also it was detected that some of the SG cells contained the proPO system and that the SG cells could participate in encapsulation reactions [69]. Since then, we and other researchers have used this separation method to search for specific molecular markers for these hemocyte groups in crustaceans, and the method with slight modification have also been used for insects and various other invertebrate animal groups.

In a recent study, we separated the circulating hemocytes from *P. leniusculus* into an SGC and a GC fraction, and additionally isolated hematopoietic cells, which were divided into APC and HPT cells. We isolated proteins from these different cell populations and analyzed these protein extracts by LC-MS/MS analysis to obtain information about the total proteome in each cell group, separately [21]. We could then show that very few proteins were specific for the APC cells compared to the remaining HPT cells (without APC cells). However, we noted that differentiated cells were present in the remaining HPT, since proteins related to differentiated cells, such as several proteinase inhibitors were upregulated in these cells compared to the APC. As a consequence it then appears as if APC mainly contains less differentiated stem cells, and more importantly in this part of the tissue (APC) a higher cell division frequency occurs [21].

In our proteomic analysis [21] we found much greater differences between the circulating hemocytes and cells from the hematopoietic tissues, indicating that the final differentiation of hemocytes occurs after the cells are released from the HPT, which is in close agreement with our previous results [34]. We also found several markers in the GC fraction, some of which had already been identified in our previous study from 2008 when we analyzed proteins from SGC and GC using 2-D gel electrophoresis and MS analysis of the different proteins [70]. The antimicrobial peptide crustin 3 was uniquely expressed in GC, while other crustins were shown to clearly have higher expression in GC compared to SGC [21]. Other specific GC marker proteins were SOD, Mannose binding lectin (MBL), proPO, peroxinectin and several serine proteases. We also found that a large number of proteins were uniquely expressed in SGCs, of which several PDGF/VEGF-like proteins as well as their corresponding receptors are characteristic examples. Also, the previously identified "SGC-specific" Kazal protease inhibitor (KPI [70]), was now confirmed to be present in high amount in the SGC. In addition to this KPI, hemolectin (Hml), and TGase1 were also detected as specific biomarker proteins in this SGC population of cells [21].

A recent study in *C. quadricarinatus* has shown similar results and has identified peroxinectin [71], Mannose binding protein (MBP) (a MBL homologue [72]), ppA [73] and a serine protease inhibitor as specific markers for GCs, as well as a vitelline membrane outer layer 1 homologue (VMO-1b), a C-type lectin, Crustacean hematopoietic factor (CHF) [74], and a peptidase as specific markers for SGC [75]. Six of these markers, i.e. peroxinectin, MBP and ppA for GCs, and VMO1, a C-type lectin and a peptidase for SGCs were all confirmed for their presence in specific cell types by immunofluorescence, qRT-PCR and western blot [75]. In addition,this study in *C. quadricarinatus* identified SOD as specific for GCs, as well as Hml and a KPI as highly expressed in SGCs [75]. Thus, this is in agreement with our previous published studies in *P. leniusculus* [21,70].

These studies on Percoll gradient separated cells, and the classification of cells by morphological characters into three categories and then detection of different markers in these different morphological classes have generated knowledge that has helped us to understand the functions of different hemocyte types in the immune system. However, each of these three classes are highly likely to contain cells with some differences in morphology and then also different amounts of marker proteins and marker mRNAs. As a consequence, when examining mRNA expression in separated hemocytes and hematopoietic cells at the individual cell level by *in situ* hybridization, it is often found that certain transcripts are limited to a very low number of cells. This means that each morphological cell type is likely to contain cells with different expression profile [29]. In addition, when microscopic studies of primary cell cultures are carried out, it is clear that cells in the same cell fraction can acquire very different properties in terms of spreading, attachment, migration, etc. We have found that this applies especially to cells in the semi-granular fraction and as a conclusion of this we therefore consider that this fraction often contains several different cell or sub cell types, which is also indicated by the overlapping functions of cells in this SGC fraction such as for example rate of phagocytosis, amount of encapsulation and degree of melanization found in this fraction [64]. A recent study in *C. quadricarinatus* by Li et al. indicated that the SGC fraction contains cells at different stages of development, all on their way to differentiate into GCs [45]. This conclusion was made after transplantation of labeled hemocytes or labeled HPT cells into recipient crayfish, and in which after a long time of ca 10–12 weeks the labeling could only be found in GC [45].

That the SGC fraction contains cells that in the circulation mature and develop more cytoplasmic vesicles and adopt a GC morphology is absolutely likely. The SGC fraction is clearly diverse which is easily observed after gradient separation in Percoll, and also the HPT cell fraction contains a high number of cells which was suggested to be precursor cells in the GC lineage [17]. Nevertheless, we do not believe that this applies to all cells in the SGC fraction, and we see certain and several shortcomings in this study by Li et al. [45] such as e.g. that no consideration has been given to whether how many of the labeled cells that die or divide, nor is the total exact number of cells reported at the various time points but only a percentage of labeled cells. As a consequence, this could mean that only very few cells survived this very long time. This also means that it is impossible to judge whether any specific labeled cell develop into a granular cell type. At the start of the experiment, a very low amount of the HPT/SG (ca 5–8%) of the total cells

were labeled, At the end of the experiment it ends up with only ≈ 1% labeled GC cells [45],which is extremely low and no information of the exact number of labeled cells is provided All in all, since the exact number of cells in the SGC class which are converted into presumable granular cells are not detailed and all published data point to that the numbers are extremely low, this study needs more details such as actual numbers instead of percentage to be able to properly evaluate the published results [45].

However, it is undisputed that the SGC fraction and the cells easily dissociate from the hematopoietic tissue contain cells undergoing differentiation into a more granular morphology. This was already suggested by us and published in Chaga et al., 1995, where five different cell types in the HPT from the crayfish *P. leniusculus* were characterized morphologically [17]. We speculated that cell type 2, 3 and 4 which made up about 85% of the easily dissociated cells and most of these cells was considered to be different stages of granular hemocyte development [17]. On the other hand, although this may be partially true, our recent results indicate that the nature of hematopoietic cells and hemocytes is more diversified than previously understood. We have earlier seen that each class of hemocytes such as hyaline cells, SGCs and GCs apparently contains different subclasses since the morphology is slightly different as well as some of their functions, but with the tools that have existed until now it has not been possible to distinguish true sub cell types and therefore classification of cells has been based mainly on gross morphology and to a lesser extent molecular characterization. However, with new single-cell methods for analyzing mRNA and/or protein expression, it has become possible to find different cell types of both HPT cells and hemocytes [22,76]. With these new methods combined with previous information it has been possible, in recent years to identify and characterize several previously unknown types of cells in the hematopoietic system of Drosophila [77–80]. However, it must be emphasized that such studies are considerably more difficult to carry out in crustaceans,where fully annotated genomes are missing in the majority of species, and furthermore, we rarely or at least at present moment have access to genetically homogeneous individuals in defined synchronous developmental stages. Despite these drawbacks, a scRNA-seq study of hemocytes from *Marsupenaeus japonicus* using a transcriptome has shown the presence of six different hemocyte types in the circulation [76]. By performing a lineage-tree reconstruction and pseudo-temporal order analysis, Koiwai et al. [76] were able to show that it is likely that the hemocytes differentiate from a common stem cell that divides into two different branches [76] in a way similar to what we have proposed to occur in crayfish in our earlier studies [17,64]. In a recent paper where we have used transcriptome data, we have also performed scRNA-seq analysis in the crayfish *P. leniusculus*, of circulating hemocytes and dissociated APC + HPT cells [22]. Although there are major drawbacks to performing an scRNA analysis without access to a fully annotated genome as was the case also in shrimp [76], we were able to clearly demonstrate the presence of several different cell types both in the hematopoietic tissue as well as among the circulating hemocytes [22]. It was obvious, as was known before, that cell division does not take place to a significant extent in the circulating hemocytes, and instead it takes place in the HPT and APC, where proliferation has been demonstrated to occur avidly. We were also able to identify some cell types in the hematopoietic tissue that were not previously known. An example is a group of cells with high expression of genes involved in iron homeostasis as for example Pacifastin heavy chain [81], ferritin [82]and a previously uncharacterized transferrin. This may be an interesting finding, since the cells in this cluster may be involved in controlling and regulating the mitochondria turnover in HPT stem cells when they differentiate into more mature hemocytes containing a lower number of mitochondria, and presumably mitophagy has occurred. This finding may also be relevant for other stem cells and therefore needs more detailed and extensive functional studies. Another small group of cells in HPT expressed the transcript encoding the melanin-inhibiting protein MIP [83] and since proPO is not expressed in the HPT stem cells the MIP in HPT may be there to inhibit melanization in this tissue if any mature hemocytes expressing the proPO protein for some reason is activated in the tissue and thereby a MIP can prevent the deleterious effects of active PO in the HPT tissue. One group of cells showed higher expression of transcripts for mitochondrial proteins, and we judge that these may well be more stem cell-like as was also the case in the APC cells in our previous global proteome study [21].

We were also able to identify Hml and TGase1 as differentiation markers in HPT cells and these were likewise found in a small cell population in circulating hemocytes [22]. This is in line with the results in the shrimp *M. japonicus* [76]. Some of the cell clusters identified in this scRNAseq analysis were dominated by cells expressing previously identified SGC markers. Among these hemocyte clusters, we were able to identify some transcripts whose expression was limited to a small percentage of the cell populations. Some of these were cenB (an endoglucanase), PVF1 (GenBank:ASU10867.1), Duox (a dual oxidae homologue) and FAS2 (a Fasciclin-2-like transcript). These transcripts are found to be expressed in different cells with SGC morphology, meaning that not all SGCs express these but that there are several cells with different characteristics in this fraction.

It is important to remember, however, that each analysis in nearly all studies takes place at one time or at best a few times and thus provides a snapshot of gene expression, and to accurately identify the function of each cell, you need to know expression patterns over time. Even cells in the GC fraction show some diversity, and one example is the expression of a glycine-rich peptide that is only expressed in a minor proportion of GCs, indicating the presence of granular hemocytes with specific functions [22,42].

Therefore, the most important conclusion from our study is that studies should be made over longer times and not only at one time point and further that morphology combined with marker proteins and RNA should be used to separate hemocytes into classes of cells. But it is also important to expand our knowledge of specific subsets of hemocytes in these gross hemocyte classes to fully understand the complexity of crustacean and invertebrate immune processes.

5. Conclusions and future perspectives

The HPT is the organ in which hemocytes are produced in crustaceans and the main three morphological classes of hemocytes are still valid to use for studies of function and involvement in immunity with the notion that there are also sub-classes of these main classes some of which may have very important functions in hematopoiesis and immunity.

Future research on immunity should focus more on the immune reactions which are important and maybe also unique for crustaceans and invertebrates in general and perhaps not too much try to tag human or vertebrate immune processes into crustaceans. This because in most cases the complexity of an immune response in a crustacean is quite large and therefore requires detailed studies to be fully deciphered in its complexity before it can be compared and contrasted with vertebrate immune reactions.

CRediT authorship contribution statement

Irene Söderhäll: Conceptualization, Writing – original draft, Writing – review & editing. **Kenneth Söderhäll:** Conceptualization, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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