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The spider hemolymph clot proteome reveals high concentrations of hemocyanin and von Willebrand factor-like proteins

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ABSTRACT

Arthropods include chelicerates, crustaceans, and insects that all have open circulation systems and thus require different properties of their coagulation system than vertebrates. Although the clotting reaction in the chelicerate horseshoe crab (Family: Limulidae) has been described in details, the overall protein composition of the resulting clot has not been analyzed for any of the chelicerates. The largest class among the chelicerates is the arachnids, which includes spiders, ticks, mites, and scorpions. Here, we use a mass spectrometry-based approach to characterize the spider hemolymph clot proteome from the Brazilian whiteknee tarantula, *Acanthoscurria geniculata*. We focused on the insoluble part of the clot and demonstrated high concentrations of proteins homologous to the hemostasis-related and multimerization-prone von Willebrand factor. These proteins, which include hemolectins and vitellogenin homologous, were previously identified as essential components of the hemolymph clot in crustaceans and insects. Their presence in the spider hemolymph clot suggests that the origin of these proteins' function in coagulation predates the split between chelicerates and mandibulata. The clot proteome reveals that the major proteinaceous component is the oxygen-transporting and phenoloxidase-displaying abundant hemolymph protein hemocyanin, suggesting that this protein also plays a role in clot biology. Furthermore, quantification of the peptidome after coagulation revealed the simultaneous activation of both the innate immune system and the coagulation system. In general, many of the identified clot-proteins are related to the innate immune system, and our results support the previously suggested crosstalk between immunity and coagulation in arthropods.

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1. Introduction

Two key differences between vertebrates and arthropods are the absence of a closed circulation system in arthropods and that the arthropods' immune defense system primarily is based on innate immunity. As a consequence of the open circulation system, the arthropods are i) more exposed to loss of hemolymph upon injury, ii) more prone to infections, compared to vertebrates [1], and iii) less vulnerable to develop thrombosis and its fatal consequences. In order to cope with the different challenges related to the open circulation system, the arthropods have developed a fast coagulation mechanism that not only plays a role in wound closure, but also is a central part of the immune system [2,3], since bacteria are entrapped and immobilized in the clot, and eventually killed [4,5]. Recently, functionally similar mechanisms have been identified as part of the human innate immune response where neutrophil extracellular traps (NETs), as well as the human clot, can immobilize and kill bacteria [6,7].

To use coagulation and entrapment of microorganisms as an effective part of the innate immune system, the arthropods have developed sensitive mechanisms. The horseshoe crabs (Limulidae) (from here 'horseshoe crab' refers to members of the Limulidae family), have extreme sensitivity to lipopolysaccharides (LPS) from Gram-negative bacteria or β-1,3-D-glucan from fungi, and the effect on coagulation have attracted considerable attention for decades [8,9]. Consequently, the coagulation in this chelicerate is the best described among the arthropods, and all clotting factors have been identified [10]. Upon binding of LPS and β-1,3-D-glucan to the serine protease zymogens Factor C and Factor G, respectively the proteolytic coagulation cascade is activated. Parallel to the human conversion of soluble fibrinogen to insoluble fibrin, the final step in horseshoe crab coagulation is the proteolytic conversion

Abbreviations: α_2M, alpha-2-macroglobulin; AMP, antimicrobial peptides; C8, cysteine 8; LC–MS/MS, nano-liquid chromatography-tandem mass spectrometry; NET, nucleophilic extracellular traps; TEP, thiol ester-containing protein; TIL, trypsin inhibitor-like domain; vWF, von Willebrand factor; VWA, von Willebrand factor A domain; VWD, von Willebrand factor D domain; VWE, von Willebrand factor E domain; XIC, extracted ion chromatography.

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of soluble coagulogen to insoluble coagulin. This clot is subsequently stabilized by transglutaminase activity, similar to the FXIIIa-mediated stabilization of the human clot [11]. In general, transglutaminases are widely conserved during evolution [12], and these protein–protein cross-linking enzymes are critical for the interplay between coagulation and the innate immune defense as these enzymes immobilize bacteria on the clot surface [7]. In contrast to humans, all components of the horseshoe crab's coagulation system are stored intracellularly until degranulation is initiated on contact with pathogens [4]. In spite of the comprehensive characterization of the horseshoe crab's coagulation system [13,14], the entire clot proteome has never been analyzed for the horseshoe crab or for any other chelicerate. This is probably, at least in part, due to the absence of a well-annotated horseshoe crab genome, which hinders comprehensive proteome analyses of the clot.

In contrast to the horseshoe crab, the clot has been intensively studied in *Drosophila* [15–17]. Hemolectin is the most abundant clot protein in *Drosophila* [17] and the importance of this protein is substantiated by hemolectin-knock down experiments causing bleeding defects [18]. Another hemolectin-like protein, called hemocytin, with agglutinating activity has been identified in the silkworm, *Bombyx mori* [19]. Alignment of hemolectin and hemocytin demonstrates that hemolectin contains an extended N-terminal region of approximately 1200 residues [20]. These two proteins are large (340 kDa and 430 kDa) and homologous to the vertebrate von Willebrand factor that is involved in hemostasis [19,20]. In addition to hemolectin, a protein called fondue is essential for coagulation in *Drosophila* [15,17]. In crustaceans, the clottable protein is present in the hemolymph, while the coagulation factors are stored intracellularly [21–23]. The clottable protein is part of the vitellogenin protein family, and similar to the clottable proteins in insects it displays sequence similarity to von Willebrand factor [22,23]. The coagulation reaction in crustaceans does not involve a proteolytic cascade; the release of transglutaminase from hemocytes into the hemolymph is sufficient to initiate protein polymerization and clot-production [22–24].

In general, proteolytic cascades are important in arthropod immunity, and in addition to the coagulation cascades, also activation of other parts of the immune system such as 'complement', activation of prophenol oxidase (proPO), and activation of the Toll receptor, involve proteolytic cascades [25]; these cascades are all triggered by microorganisms. Similar to clot formation, the activation of proPO results in encapsulation of intruding pathogens. This mechanism depends on the formation of tyrosine derivates with cross-linking activity and in turn the production of melanin. Consequently, infections initiate two cross-linking activities, namely transglutaminases and PO, and both activities are involved in covalent clot-stabilization [3]. Recently, it was suggested that the basic structure and the soft clot is formed by transglutaminases, while the melanization secures further cross-linking and hardening of the clot [12]. In the horseshoe crab, as well as in all analyzed arachnid (spiders, scorpions, ticks, and mites) genomes, the PO-enzymes apparently have gained an oxygen-transporting function during evolution [26,27]. Thus, in the chelicerate species the proteins that originate from the ancient PO-enzymes are called hemocyanin. However, the PO activity is not lost; instead it is exerted upon activation [27–30]. This functional conversion of hemocyanin can be mediated by treatment with detergents or lipids, by limited proteolysis, or by hemocyanin-binding proteins [27–30]. In the horseshoe crab, the clotting enzyme and clotting factor B mediate the functional conversion [27], emphasizing the interplay between coagulation and the proPO activation system.

In terms of species numbers and ecological importance, the most successful class of chelicerates is the arachnids. As described above, coagulation and the innate immune system have been studied quite intensively in the horseshoe crab, but the biochemical characterization of these systems in the arachnids is sketchy at the best. The coagulation factors, which have been functionally characterized in the horseshoe crab, are all serine proteases and include Factor C, Factor B, Proclotting enzyme, and Factor G. In contrast to the other arachnids, the analyzed spider genomes encompass Factor C with the same domain structure as in the horseshoe crabs [26]. Here the protease is involved in recognition of intruding pathogens and the activation of the enzyme triggers the coagulation cascade. The sequence similarity indicates a similar function in the spiders. Our recently published spider hemolymph proteome revealed that Factor C, and also proteins with similarity to the horseshoe crabs' Factor B, and the proclotting enzymes, are found in the spider hemolymph [31]. Overall, our previous spider genomics, transcriptomics, and proteomics [31] as well as comparative analyses [26] indicate that a coagulation cascade, similar to the horseshoe crabs', is present in spiders. However, the gene encoding coagulogen, which is the coagulating protein in the horseshoe crab, is apparently not present in the genome of any arachnids [26]. This prompted us to investigate the protein composition of a hemolymph clot from an arachnid species. For this analysis, the Brazilian whiteknee tarantula spider, *Acanthoscurria geniculata*, was chosen due its large body size and our recent comprehensive transcriptomics-based sequence database [31], which facilitates sensitive mass spectrometry analyses of the clot. The present study is the first global characterization of the protein composition of a chelicerate hemolymph clot. We focused on the insoluble part of the natural formed clot, defined as the clot formed in the presence of both hemolymph, as well as hemocytes. A proteomics-based approach was employed resulting in the identification of 293 proteins that are covalent incorporated in the clot. The main components are i) proteins encompassing von Willebrand factor-like domains (e.g. hemolectins), ii) hemocyanins, and iii) complement C3. Furthermore, the peptidome after clot formation was quantified and revealed the generation of activation peptides from both the coagulation and the innate immune system. The data underlines the crosstalk between the innate immunity-related proteolytic cascades, including coagulation cascade, PO activation, and complement activation. The presence of hemolectins and the absence of coagulogen indicate that the arachnid clot is functionally and structurally more similar to the clot generated in insects, than that of the closer relative, the horseshoe crabs.

2. Materials and methods

2.1. Hemolymph collection and clot formation

The hemolymph was collected from four male individuals of the Brazilian whiteknee tarantulas (*A. geniculata*) classified as Theraposidae, Mygalomorphae, Araneae, Arachnid, Chelicerata. The *A. geniculata* used in this study originated from a captive bred stock and were obtained from a commercial dealer (www.polyped.de). Upon purchase they were kept in individual terrariums. The daily light:dark cycle was 14:10 h, temperature was 27–29 °C, and air humidity around 80%. The *A. geniculata* were fed on cockroaches on a weekly basis and increased body mass during captivity.

In order to obtain hemolymph samples, the spiders were removed from their cages and sedated using CO_2. Afterwards hemolymph was sampled from the book lungs with a sterile syringe. Hemolymph (150–200 μl) was added LPS to a final concentration of 65 μg/ml. Coagulation was also obtained without the addition of LPS, probably due to bacterial contaminations from the spider exoskeleton. However, for consistency, LPS was added to all analyzed samples. Then the samples were incubated at room temperature to allow clot formation. Subsequently, the samples were centrifuged for 2 min at 1000 × *g*, the supernatants removed, and the remaining pellets containing the clot were snap frozen in liquid nitrogen and stored at −80 °C.

2.2. Clot purification and sample preparation for mass spectrometry analyses

The clots were washed with 1 ml of phosphate buffered saline (PBS) that was incubated with the clots for 3 min at 4 °C before centrifugation and gently removal of supernatant without touching the pellet. It was repeated three times, and then approximately half of each of the four

pellets was transferred to new tubes where SDS-gel electrophoresis sample buffer was added and the samples boiled for 5 min and then centrifuged. The supernatants were removed and SDS-sample buffer was added again to the insoluble pellets and then the samples were subjected to SDS-polyacrylamide gel electrophoresis. The insoluble clots were visible and retained in the stacking gels, and after the electrophoresis they were manually collected and the SDS was removed by washing the clots on 3 kDa molecular weight cut-off centrifugal filters, as described before for purification of human clots [11]. The clots were removed from the filters and the clot-proteins denatured and reduced by incubation at room temperature for 1 h in the following buffer: 15 mM dithiothreitol, 100 mM ammonium bicarbonate, and 8 M urea. Subsequently the reduced cysteines were blocked by alkylation with iodoacetamide added to a final concentration of 30 mM. The samples were incubated for an additional hour and then diluted 5 times in 100 mM ammnonium bicarbonate. Afterwards, 2.5 μg sequence-grade modified trypsin was added and the samples digested for app. 16 h at 37 °C. The resulting tryptic peptides were micro-purified on self-packed columns containing POROS R2 media (Applied Biosystems), eluted, and lyophilized.

2.3. Isolation and quantification of hemolymph peptidome

Hemolymph was collected from three spiders, divided in two where EDTA (5 mM final concentration) was added to one portion to prevent clotting. The clotted samples and the EDTA controls were centrifuged at 14,000 ×*g* in a 10 kDa cut-off spinfilter. The filters were washed sequentially with 1 M NaCl and 0.1% formic acid. The collected peptides were reduced and alkylated as described above, micro-purified and analyzed by mass spectrometry. Based on the Mascot Search result (see below) the amount of the identified peptides was calculated using MS1 Full-Scan filtering in Skyline [32,33]. Only peptides identified with more than 3 matching MS/MS spectra were included. After subtraction of background signal the total and summed ion intensity for clotted and control (EDTA) samples were calculated.

2.4. Mass spectrometry analyses

LC–MS/MS analyses were performed on a nanoflow HPLC system (EASY-nLC II, Thermo Scientific) connected to a mass spectrometer (TripleTOF 5600$^+$, AB Sciex) equipped with an electrospray ionization source (NanoSpray III, AB Sciex) and operated under Analyst TF 1.6 control. The samples were dissolved in 0.1% formic acid, injected, trapped and desalted isocratically on a precolumn. The peptides were eluted and separated on an analytical column (15 cm × 75 μm i.d.), which was pulled (P2000 laser puller, Sutter Instrument Co.) and packed with ReproSil-Pur C18-AQ 3 μm resin (Dr. Marisch GmbH) in-house. The peptides were eluted at a flow rate of 250 nl/min using a 50 min gradient from 5% to 35% phase B (0.1% formic acid and 90% acetonitrile). An information dependent acquisition method was employed allowing up to 25 MS/MS spectra per cycle of 1.6 s. Four technical replica were performed of each of the four biological samples resulting in 16 nano-liquid chromatography-tandem mass spectrometry (LC–MS/MS) analyses in total. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [34] via the PRIDE partner repository with the dataset identifier PXD002500.

2.5. Protein identification and quantification

The collected MS files were processed by Mascot Distiller 2.5.0 (Matrix Science) and subsequently these processed files were used to interrogate an improved version of the previously generated transcriptomics-based *A. geniculata*-sequence database [31,35]. The database contains 36.071 annotated protein sequences and is fully accessible via the PRIDE partner repository with the dataset identifier PXD002500. The following search parameters were selected, i) trypsin as enzyme and up to one missed cleavage allowed, ii) carbamidomethyl as fixed modification, iii) oxidation of methionine as variable modification, iv) peptide mass tolerance of 20 ppm, v) fragment mass tolerance of 0.2 Da, vi) instrument setting ESI-QUAD-TOF, and vii) significance threshold (p) of 0.01. For the peptidome analysis the search parameters were identical except an enzyme with no specificity was selected. The quantification of proteins in the clot is based on extracted ion chromatography (XIC) and for this purpose the average quantitation protocol was selected in Mascot Distiller using significant threshold at 0.01 and the number of peptides required for quantification was set to 3, matched rho was 0.7, XIC threshold was 0.1, and isolated precursor threshold was set at 0.5. The result obtained by the Mascot Distiller searches was subsequently parsed using MS Data Miner v. 1.3.0 [36]. Protein identifications were only accepted if they were based on at least two unique peptides with one of the peptides having a minimum score of 30, and if the protein was identified in at least 5 of the 16 LC–MS/MS analyses. Quantification, with the criteria described above, in three out of the four technical replicas was required in order to accept quantification of a protein in one of the biological replica. Proteins were only included on the final list of quantified proteins, if they were quantified in three or more the biological replica. The relative abundance of the quantified proteins was calculated as the average MS intensity for the three most intense peptides for each protein divided by the total sum of the average signal for all quantified proteins in the sample providing the relative amount (%) of each protein in each of the 16 individual analyses (Table S1, sheet 2). Subsequently, the average relative amount (%) of the four technical replica analyses was calculated (Table S1, sheet 2), and finally we calculated the average relative amount (%) based on the four biological replicas (Table 1 and Table S1, sheets 1 and 2).

Table 1
The most abundant proteins in the insoluble *Acanthoscurria geniculata* clot.
The protein composition of the insoluble part of the clot was revealed by LC–MS/MS and XIC-quantification. The table includes the 20 proteins, which constitute 0.5% or more of the clot (see Table S1 for the full list and further details). The data reveals that hemolectin-like protein-1 is the most abundant protein in the spider clot. In addition, it shows that hemocyanin is dominating and accounts for more than half of the total protein content.
The accession numbers refer to the transcriptome database, which can be downloaded from the PRIDE Archive (identifier: PXD002500).

Accession nr	Name, based on annotation	Mass (kDa)	Avg. (%)
L11662_T3/6_T_WB	Hemolectin-like protein 1	549.3	12.6
L780_T1/2_T_WB	Hemocyanin G chain	72.3	11.8
L1998_T1/1_T_WB	Hemocyanin F chain	72.5	10.4
L2916_T1/1_T_WB	Hemocyanin A chain	60.6	10.2
L9283_T1/1_T_V	Hemocyanin E chain	26.2	8.4
L810_T1/1_T_WB	Hemocyanin E chain	53.2	7.1
L1701_T1/2_T_WB	Hemocyanin D chain	72.4	6.3
L212_T9/9_T_WB	Vitellogenin-1	130.8	6.0
L2750_T1/1_T_WB	Hemocyanin A chain	18.9	3.8
L3573_T1/5_T_WB	Hemolectin-like protein 2	380.2	3.6
L4395_T2/4_T_WB	Hemocyanin C chain	73.2	3.5
L4600_T1/1_T_WB	Hemocyanin B chain	62.3	3.5
L18306_T1/2_T_WB	Histone H2B	14.4	2.3
L2980_T1/1_T_V	Hemocyanin B chain	8.2	2.0
L4278_T1/1_T_V	Histone H2B	13.8	1.5
L29510_T1/1_T_WB	Histone 1, H4c	14.2	1.2
L1442_T1/2_T_WB	Zonadhesin-like	370.3	0.6
L70_T2/4_T_WB	Complement component 3	67.8	0.5
L627_T5/8_T_WB	Complement component 3	127.4	0.5
L15008_T1/1_T_WB	Hydroxybutyrate dehydrogenase	38.7	0.5

3. Results

3.1. The spider hemolymph clot is a complex mixture of many different proteins stabilized by covalent cross-links

Hemolymph from Brazilian whiteknee tarantulas (*A. geniculata*) was allowed to coagulate in the presence of hemocytes to mimic the in vivo

situation, where both secreted proteins from hemocytes as well as extracellular hemolymph proteins are present. Although the resulting clots were washed extensively and subjected to boiling in SDS sample buffer followed by SDS-PAGE, as described in the method section, the clots were not dissolved. This suggests that the clot is covalently stabilized, similar to the human FXIII-stabilized clot [11]. To reveal the protein composition of the insoluble clot, the proteins were digested with trypsin and the resulting peptides analyzed by LC–MS/MS. We have previously used next-generation sequencing to generate a profound *A. geniculata* transcriptome database [31]. In the present study, we interrogated the sequence database with the obtained LC–MS/MS data. The sequence database, as well as the mass spectrometry data, is available via ProteomeXchange with identifier PXD002500 and the project name "The spider clot proteome" [34,37]. Protein identifications were only accepted when based on two unique peptides and identification in minimum 5 of 16 LC–MS/MS analyses (see Materials and methods section). Using these stringent criteria, 293 proteins were successfully identified in the spider clot, and 54 of these were quantifiable using a XIC-based protocol (Table S1).

3.2. Von Willebrand factor-like proteins are present in high concentration in the spider hemolymph clot

The most abundant protein in the insoluble spider clot is a hemolectin (Table 1). The hemolectins are known from the insects, and the first protein identified in this family, was hemocytin from the silkworm (*B. mori*), where it was suggested to play a role in hemostasis or in encapsulation of foreign substances [19]. The finding of a homologous protein in the clot suggests that the protein, at least in spiders, plays a role in both hemostasis as well as in pathogen encapsulation. This is probably also the case in *Drosophila*, where hemolectin is a major component of the clot [17]. As hemocytin is closely related to hemolectin, we named the two identified spider variants hemolectin-like protein 1 and 2 (Fig. 1). These proteins were both highly abundant in the clot (Table 1). Hence, our results indicate that the spider clot has functional and structural overlap with the hemolymph clot observed in insects.

The insects' hemolectins, as well as the zonadhesion proteins, encompass sequentially the following three domains: von Willebrand factor D domain (VWD), cysteine 8 (C8) domain, and trypsin inhibitor like domain (TIL). This arrangement is conserved and also known from gel-forming mucins [38]. These mucins have three N-terminally located VWD-C8-TIL motifs, known to facilitate oligomerization [38]. An InterProScan sequence search reveals that the most abundant protein in the spider clot (the hemolectin-like protein 1, sequence L11662_T3/6_T_WB) also contains this arrangement of VWD-C8-TIL domains (Fig. 1), suggesting that oligomerization of this protein is essential for the clot-formation in spiders. In addition to the hemolectins and zonadhesion proteins, vitellogenin also encompasses a VWD, and in total, these von Willebrand factor-related proteins constitute approximately 25% of the spider clot (Table S1).

3.3. Hemocyanin is the major protein in the insoluble spider hemolymph clot

Another major component of the clot is hemocyanin (Table 1). *A. geniculata* hemocyanin is a very large protein complex with an estimated molecular mass of approximately 1800 kDa [39]. The majority of arthropod and molluscan species use hemocyanin as an oxygen carrier in the hemolymph, and in addition hemocyanin displays PO activity in chelicerates upon activation [27–30]. The present study reveals that in addition to these two important functions of the protein, spider hemocyanin apparently also plays a role in coagulation, underscored by the finding that more than half of the protein content of the clot is hemocyanin.

Hemocyanin from the North American tarantula *Eurypelma californicum* is a 24-mer protein consisting of two 12-mer units, which again consists of 2 copies of subunits A, D, E, F, and G, and of one copy of subunits B and C [39]. The 7 subunits vary in molecular mass between 70 and 75 kDa. Our previously published *A. geniculata* transcriptome, as well as hemolymph proteome, contained all 7 hemocyanin subunits (A–G) [31], and the present study demonstrates that all subunits appear in the clot. All subunit sequences, identified in the clot, are annotated based on homology with the hemocyanin subunits identified in the

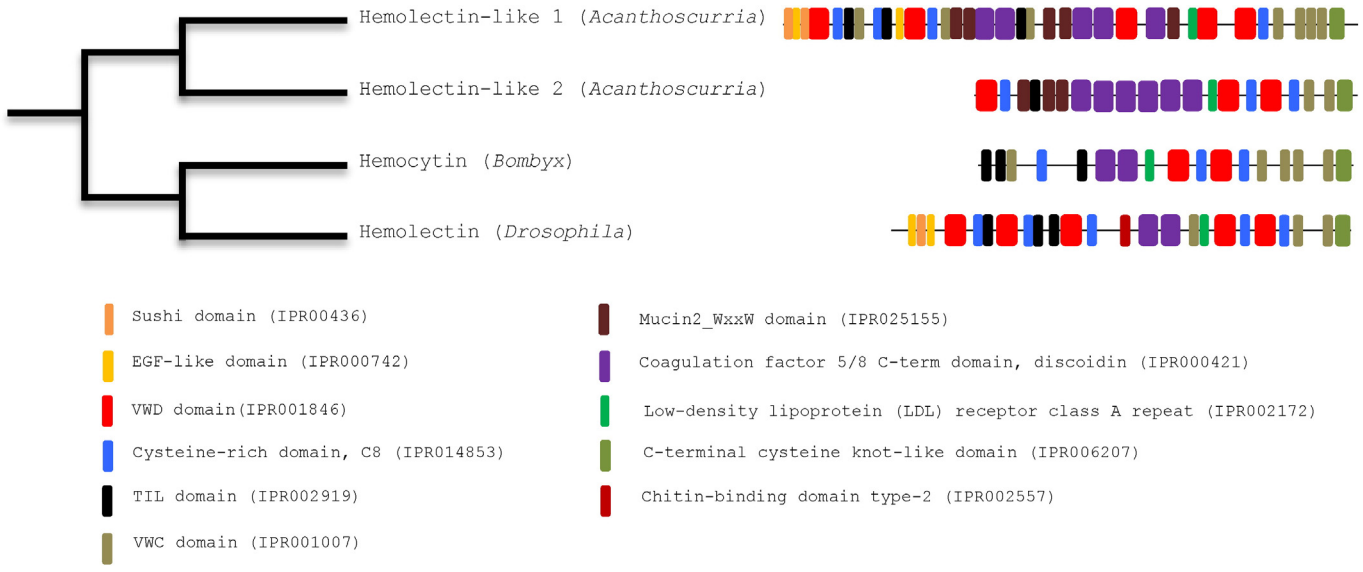

Fig. 1. Spider hemolectins display similarity with insect hemolectins. The most abundant protein in the insoluble spider clot hemolectin-like protein 1 (L11662_T3/6_T_WB) was aligned with the similar hemolectin-like protein 2 (L3573_T1/5_T_WB), and with two hemolectins that are part of the hemolymph clot in insects (*Drosophila* accession: Q9U5D0 and *Bombyx*, accession: P98092), and the resulting phylogenetic relationship is shown. Furthermore the Simple Modular Research Architecture Research Tool (SMART) from EMBL-Heidelberg was used to analyze the domain architecture of the four sequences. The most abundant domain in the spider hemolectins is the discoidin-domain that is known from human coagulation factors. The hemolectins are large (between 434 kDa and 530 kDa) and contain many domains. Many of these are involved in protein polymerization; a feature that most likely is exploited during clot formation and stabilization.

closely related *E. californicum*. As expected, alignment analyses of the hemocyanin sequences from these two mygalomorph species show that the sequences are very similar, and that the six putative copper-ion binding histidine residues are conserved in all hemocyanin sequences from both of the species. The finding of two variants of hemocyanin A, B, and E, most likely reflects that the *A. geniculata* sequence database is based on next-generation sequencing transcriptomics and automated sequence assembly [31]. Consequently, incomplete sequences could be present. Indeed, alignments with the corresponding *E. californicum* hemocyanin subunit sequences demonstrate that the two identified hemocyanin A, B and E sequences, represent respectively the N- and the C-terminal of the three subunits (data not shown). Taken together, our results suggest that i) the *A. geniculata* hemocyanin is almost identical to the *E. californicum* hemocyanin [39], ii) that all subunits are covalently associated with the clot, and iii) that the protein constitutes a substantial part of the spider clot.

3.4. The clot composition substantiates crosstalk between coagulation and the innate immune system

In humans, approximately 90% of the protein content of the insoluble clot is fibrin [11]. Parallel to this, we show that the major part of the insoluble spider clot is composed of hemocyanin and vWF-like proteins. The remaining approximately 10% is a mixture of many different proteins, but among the more abundant are histone- and complement -related proteins (Tables 1 and 2). Histones are the most abundant proteins in NETs [40]. Immune cells produce the NETs, and in human these structures are a part of the innate immune defense [6,41]. It is intriguing to speculate that the finding of histones covalently incorporated into the *A. geniculata* clot, could indicate that NETs are of ancient origin and part of the innate immune system in the arachnids.

We previously showed that complement protein C3 is a substrate for the human clot-stabilizing transglutaminase FXIIIa and that complement protein C3 is incorporated in the human clot [11,42]. The identification of complement protein C3 in *A. geniculata* demonstrates, for the first time, that spiders also exploit the complement system. Furthermore, the identification in the clot emphasizes the early origin of the crosstalk between complement and coagulation. This is also substantiated by the results obtained in horseshoe crab studies, where coagulation factor C acts as a C3 convertase [43]. The complement protein C3 and factor C in *A. geniculata* are both annotated based on horseshoe crab similarity, and both are identified in the clot. Thus, it is possible that factor C, also in the spiders, acts as a C3 convertase. To support this hypothesis we analyzed the peptidome in the hemolymph after clotting. We identified and quantified peptides produced during hemolymph clotting (Table 3). Peptides corresponding to the activation of Factor C were clearly produced [44]. These peptides (SGEVRTAIQ… and AAPVPASQ) represent the C-terminal of factor C heavy chain and thereby the activation of factor C. The peptide SVSGTIDR from Complement C3 match the region cleaved by C3 convertase in human Complement C3. The peptide represents a C-terminal extension to C3a anaphylatoxin [45]. Finally, peptides from the N-terminal of Histone H1 are produced. This indicates that the incorporation of histones in the clot is regulated by proteolysis, most likely by factor C.

In the present study, 5 complement protein C3 proteins were identified (Table S1). A thorough bioinformatic analysis of the sequences shows that this is not due to the presence of N-terminal and C-terminal partial sequences corresponding to the same full-length sequence, as was the case for the analyzed hemocyanin subunits. Instead, alignments of the sequences show that they indeed represent complement protein C3 variants with differences in the primary structure (Fig. 2A). This was also observed in other species e.g. human, fish, and in another spider [46–48]. All *A. geniculata* variants were annotated based on the complement protein C3 originally identified in the Japanese horseshoe crab, *Tachypleus tridentatus* [43], and alignment of the two most complete *A. geniculata* complement protein C3 sequences with this protein, as well as with the human homolog, shows that the domain structure is conserved across species (Fig. 2B). Interestingly, the isoglutamyl cysteine thiol ester is not conserved in one of the *A. geniculata* C3 sequences (L1971_T5/13_T_WB), although the domain structure is identical to the other C3 sequences, suggesting a C3 mechanism that is independent of covalent attachment to target acceptors. In conclusion, these C3-results suggest that i) spiders have an active complement system, that ii), parallel to humans, C3 is incorporated into the clot, and that iii), different C3 variants exist in spiders. Although complement protein C3 is present with the highest abundance, also complement component factor B/C2 is present in the clot (Table S1). The sequence encompasses complement control protein domains, a von Willebrand factor type A domain (VWA), and a trypsin-like serine protease domain, but the function of the protein in non-vertebrate species is only vaguely described [49,50]. Comparative analyses of the *A. geniculata* sequence (L16377_T1/1_T_WB) demonstrate higher similarity with lancelet, sea anemones, and acorn worms, than with the Bf/C2 homologous in the horseshoe crabs. It not only underlines that differences exist between the different chelicerate species, it also substantiates a potentially very ancient origin of complement.

Table 2
The coagulation-related immune response in spiders.
The table highlights the different groups of innate immunity-related proteins that are covalently immobilized in the hemolymph clot (proteins can be listed in more than one group). In addition to these, non-covalently associated defense proteins most likely also participate in the coagulation-related immune response.

Protein group	Proteins	Putative functions in the clot
Hemocyanin	Hemocyanin subunits A–G	PO-activity mediated protein-cross-linking, melanization (bacterial encapsulation) and antimicrobial activity
von Willebrand factor-related proteins	Hemolectins, vitellogenin-1, complement component factor B/C2, sushi, and zonadhesin-like proteins	Clot formation (protein polymerization) and antimicrobial activity
Thiol ester-containing proteins	Complement component C3 and α_2M	Immobilization of pathogens, antimicrobial activity, and protease inhibition
Histones	Histone 1, H3, H2A, and H2B	Formation of NETs (bacterial encapsulation)
Lectins	Hemolectins, Tachylectin 5A, Techylectin 5A, carcinolectin 5A, calreticulin, Galectin 4 and 9	Bacterial recognition, agglutination and immobilization
Coagulation factors	Proclotting enzyme and coagulation factor C and D	Bacterial recognition, hemocyanin conversion, antimicrobial activity

4. Discussion

4.1. The 'omics-based approach facilitates the hitherto deepest analysis of an arthropod clot proteome

In the present study, we allowed the clot to form in the presence of both hemolymph and hemocytes to simulate the situation in-vivo. This is in contrast to previous arthropod studies, where, i) the hemocytes were removed to focus on the hemolymph components of the clot [17,51], or ii) the hemolymph was removed prior to clot formation (in the horseshoe crab the content of hemocyte granulates is enough to facilitate clot formation) [5], or iii) the coagulation of purified components were studied [10,22]. These different approaches have revealed valuable information, but do not necessarily reflect the natural formed clot where proteins of hemolymph and cellular origin could synergize in clot formation and killing of microbes. In the present study, the access to fast LC–MS/MS equipment and a large spider sequence database allowed us to analyze the clot composition of a clot formed in the presence of both hemolymph and hemocytes in the spider *A. geniculata*. In total, we identify, using very stringent criteria, 293 proteins that are

Table 3
The peptidome of hemolyph after coagulation.
The peptidome of the hemolymph was quantified before and after coagulation. Quantification is based on eXtracted Ion chromatography (XIC) and the relative increase of the identified peptides after coagulation is listed. Only peptides with a more than 100 fold increase are shown. See Table S1 for the full list of identified peptides and quantitative information. The accession numbers refer to the transcriptome database, which can be downloaded from the PRIDE Archive (identifier: PXD002500).

Accession nr	Name	Peptide sequences	XIC		Relative increase
			After	Before	
L171_T1/1_T_S	Alpha-2-macroglobulin	TEAADYNGVRK	82,786	167	495.7
L17932_T1/1_T_WB	Histone H1	ATASPAVTTPK	56,204	N.D.	>500
L17932_T1/1_T_WB	Histone H1	ATASPAVTTPKK	212,912	741	287.3
L17932_T1/1_T_WB	Histone H1	SESTAAATASPAVTTPKKKSKSSS	87,065	648	134.4
L33319_T1/1_T_WB_fr6	L33319_T1/1_T_WB_fr6[a]	HGGAGLGGGGQGGGKGL	237,933	N.D.	>2300
L5295_T4/7_T_WB	Tolloid like	YDDNANDESNLR	60,940	N.D.	>600
L53773_T1/1_T_WB	L53773_T1/1_T_WB	SDEQEDENLLKHENFPR	197,063	N.D.	>1900
L627_T6/8_T_WB	Complement component 3	NHADMIGALG	844,727	N.D.	>8400
L627_T6/8_T_WB	Complement component 3	IANHADMIGALG	571,466	N.D.	>5700
L627_T7/8_T_WB	Complement component 3	SVSGTIDR	638,845	N.D.	>6300
L7785_T1/1_T_WB	Limulus clotting factor C	SGEVRTAIQA	3,646,791	N.D.	>36,000
L7785_T1/1_T_WB	Limulus clotting factor C	SGEVRTAIQ	245,219	N.D.	>2400
L7785_T1/1_T_WB	Limulus clotting factor C	AAPVPASQ	460,494	4465	103.1

N.D.: not determined.
[a] Similar to the antimicrobial protein Acanthoscurrin-1 and -2 from *Acanthoscurria gomesiana*.

covalently immobilized in the clot. Since the applied reference sequence database is based on transcriptomics, this number includes splice variants, and in addition overlapping and incomplete sequences could be present. However, in the lack of a complete *A. geniculata* protein sequence database that only contains manually curated and reviewed entries (like the human UniProt database), the deep-sequencing based transcriptome provides a very good alternative. Of the 293 proteins, 54 proteins were present in high enough concentrations to allow a semi-quantitative analysis based on XIC (Table S1).

One of the major findings in the present study is the large content of hemocyanin in the clot. The horseshoe crabs' innate immune system, and especially the coagulation cascade, has been studied in detail, and it is known that the clotting enzyme transforms the soluble coagulogen to the insoluble coagulin, but the overall clot composition has not been determined. However, an antibody-based approach revealed that hemocyanin is associated with the coagulin fibrils [52], and although this interaction, in contrast to the finding in spiders, was non-covalent, it indicates that hemocyanin also plays a role in the horseshoe crab clot.

4.2. Recognition and immobilization of microbes

To activate the innate immune system, the horseshoe crabs not only rely on the previously mentioned factor C and G, but also different lectins with specificity for carbohydrates exposed on the surface of pathogens [4]. We have previously identified different lectins in the hemolymph [31], and in the present study hemolectin-like proteins, techylectin 5A/tachylectin 5A, carcinolectin and galectin homologous were identified in the clot (Table 2). The *A. geniculata* techylectin 5A and carcinolectin were annotated based on the horseshoe crab sequences, and in the horseshoe crab, techylectin 5A displays an important role as a non-self-recognizing lectin that mediates bacterial

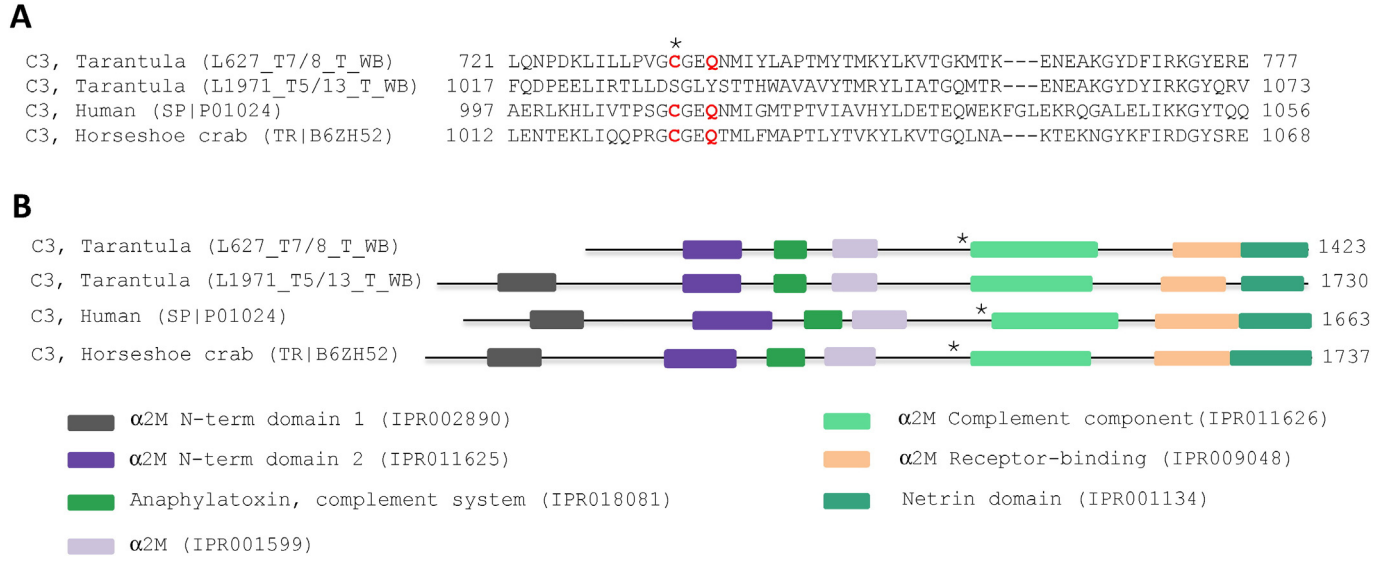

Fig. 2. Complement protein C3 is conserved across species. A) The two most complete *Acanthoscurria geniculata* complement protein C3 sequences (both identified in the clot) were aligned with the human and Japanese horseshoe crab, *Tachypleus tridentatus* homologous, and the region containing the thiol ester-containing region shown. The amino acid residue numbers are shown before and after the sequences. The alignment illustrates the sequence difference between the two *Acanthoscurria geniculata* variants underlined by finding that the amino acid residues (shown in bold red) involved in the formation of the isoglutamyl cysteine thiol ester cross-link is not conserved in the L1971_T5/13_T_WB sequence. B) The four listed C3 sequences were subjected to EMBL-EBI's InterPro tool for prediction of domains. The (*) marks the position of the cysteine residue involved in thiol ester cross-link formation and the color-coding is described below the alignment. The analysis shows that the domain structure in C3 is conserved between the chelicerates and human.

agglutinating activity [53,54]. Thus, it is likely that this protein, and probably also the more abundant hemolectins (See Fig. 1), have a similar function in the spiders.

Transglutaminases are conserved and involved in clot formation in all characterized animals. In humans, FXIIIa stabilizes the clot [11] and immobilize bacteria within the clot [7]. Transglutaminases were not identified in the spider clot, but we have previously shown that all analyzed arachnid genomes contain transglutaminase-encoding genes [26], and that transglutaminases are present at the mRNA and protein-level in the *A. geniculata* [31]. In horseshoe crabs and especially in crustaceans [22,55], transglutaminases play an important role in clot-stabilization and these enzymes could potentially also be central for protein-protein cross-linking, as well as protein-bacteria cross-linking during clot formation in spiders, although not covalently incorporated into the clot.

In addition to PO and transglutaminase activity, also the complement system facilitates covalent immobilization of intruding pathogens. Here, the cross-link is mediated by an intramolecular thiol ester in complement protein C3 (see Fig. 2). It marks the pathogen for removal by phagocytosis, and it is an important component of the innate immune defense, both among vertebrates and invertebrates [25]. Similarly to complement protein C3, the protease inhibitor α_2-macroglobulin (α_2M) also contains a thiol ester, and in general, sequence similarity exists between these two proteins. In insects, thiol ester-containing proteins (TEPs), which displays C3 function, but cluster with vertebrate α_2M in a phylogenetic tree analysis, are present [25]. The *A. geniculata* transcriptome and hemolymph proteome contain both C3 and α_2M homologous [31], and the present study reveals that the spider clot, similar to the human clot [11], contains both C3 and α_2M (Table 2). However, α_2M is present in amounts that are below the detection limit for the quantitative analyses (Table S1), indicating that, in relation to clot-biology, C3 is the most important TEP in spiders. An observation supported by the activation of C3 observed in the peptidome analysis.

4.3. Antimicrobial activity in the clot

Arthropods use coagulation as an active part of the innate immune system where microorganisms are entrapped and subsequently killed within the clot. In the present study, we have shown that complement and hemocyanin, which most likely display PO activity [28–30], are incorporated into the spider clot suggesting that these are involved in neutralization of the entrapped microbes (Table 2). Characterization of other arthropods innate immune system has shown that antimicrobial peptides (AMP) are an essential part of the immune system [4]. The AMPs are probably also part of the innate immune system in the spiders [26], substantiated by the finding of gomesin, an 18-residue cysteine-rich AMP [56], in the *A. geniculata* transcriptome. However, the AMPs are probably not covalently incorporated into the clot, because it would likely abolish the functional activity of these small peptides. This and the technical difficulties related to the identification of short sequences by transcriptomics and proteomics explain the lack of AMPs in the clot proteome. However, in the peptidome after clotting we observe a peptide homolog to the antimicrobial protein acanthoscurrin-1 and −2 from *Acanthoscurria gomesiana.* The peptidome therefore supports the hypothesis that spiders also use AMPs as part of the immune system.

Previous studies on another tarantula, *A. rondoniae*, revealed that the C-terminal part of hemocyanin subunit D can be released, and that this 10 residue peptide is an AMP (entitled “rondonin”) [57]. The *A. geniculata* subunit D's C-terminal contains an identical sequence and our results show that the subunit is incorporated into the spider clot. Thus, the C-terminal could potentially be released in the clot and express antimicrobial activity here, however it was not detected in the peptidome analysis.

The 42 kDa factor D protein is among the larger antimicrobial substances in the horseshoe crab [4]. It is a serine protease homolog with antimicrobial activity [58], and the homolog is incorporated into the *A. geniculata* clot (Table S1). Vitellogenin is present in relative high concentration in the spider clot displays anti-bacterial activity in insects [59]. These different findings indicate that the clot not only traps pathogenic intruders; they further suggest that AMP, as well as antimicrobial proteins, are incorporated. Furthermore, the activation of the coagulation cascade and the subsequent processing of the involved zymogens, most likely also releases an antimicrobial response in the spiders. This assumption is based on the identification of several Factor B and proclotting enzyme-encoding sequences in the *A. geniculata* transcriptome [31]. Like in the horseshoe crabs, these serine proteases contain a CLIP-domain in the pro-region of the zymogens. This domain is similar to the AMPs defensins and the released CLIP domain display antimicrobial activity in other arthropods [60,61].

4.4. Coagulation factors could mediate the conversion of hemocyanin to PO enzymes

Spider transcriptomics data, as well as analyses of various arachnid genomes, fail to identify a sequence homologous to the horseshoe crabs' coagulogen [26]. Consequently, the end product of spider coagulation is most likely not coagulin. In the horseshoe crab, Factor B and the clotting enzyme are similar both in primary sequences and in function. In addition to cleavage of the proclotting enzyme and coagulogen, respectively, they also convert hemocyanin subunits to active PO enzymes [27]. This conversion of hemocyanin might be one of the primary functions of the coagulation cascade-related enzymes in the arachnids. The finding that hemocyanin becomes covalently bound to the clot during coagulation supports this notion. In addition, it has previously been shown that *E. californicum* (Mygalomorph species) hemocyanin's express PO activity following limited trypsin proteolysis [28] indicating that trypsin-like clotting factors, potentially mediate this conversion in vivo. However, studies have also demonstrated hemocyanin-associated PO activity independent of proteolytic cleavage. It was shown that the conversion was be mediated by, i) the zymogen of the proclotting enzyme [27], ii) SDS [62], iii) lipoproteins [30], where, based on our findings, vitellogenin-1 could be a potential candidate, and by, iv) the horseshoe crab AMP tachyplesin [29], which is similar to the *A. gomesiana* gomesin [56]. An alignment of two full-length proclotting enzyme sequences from the *A. geniculata* transcriptome and the horseshoe crab's proclotting enzyme demonstrated that the three active site residues were not conserved in one of the *A. geniculata* sequences (L22103_T1/1_T_WB) suggesting that the enzyme is inactive. The corresponding protein was present both in hemolymph [31] and in the clot (Table S1), but apparently it expresses another function than proteolytic activity. Taken together, more studies, for example on purified clotting factors and hemocyanin are needed to unravel the molecular details of the conversion of hemocyanin and the coagulation factors' function in the arachnids.

4.5. Spätzle-, fondue-, and coagulogen- like proteins are not part of the spider hemolymph clot

The lack of identification of coagulogen in the spider clot is most likely not due to an incomplete reference sequence database, since state of the art next-generation sequencing was employed to generate the *A. geniculata* transcriptomics sequence database, used as reference database in the present proteomics study [31]. The database contains approximately 36.000 annotated protein sequences, which also indicates a very deep sequencing covering almost all genes. In addition, as previously mentioned, none of the published arachnids genomes, including a spider [31], predicts a gene that is homologous to the horseshoe crab coagulogen [26], supporting the lack of coagulogen-identification in the *A. geniculata* transcriptome and proteome. Coagulogen displays structural similarity with the spätzle protein in *Drosophila* [63]. Although sequences with weak similarity with spätzle are present in the arachnid genomes [26], our data do not suggest that

these spätzle-like proteins are part of the clot (see Table S1), or part of the *A. geniculata* hemolymph proteome [31]. In addition, we find no evidence of the presence of homologous of the *Drosophila* clot-protein fondue, neither in the *A. geniculata* transcriptome and the characterized arachnid genomes [26] nor in the hemolymph clot. Instead, the present study revealed a complex clot composition in *A. geniculata* with hemolectins and hemocyanin as main components, and due to the genetic similarity this could also be the case for the other arachnid species. In contrast, the horseshoe crab clot is speculated to be more homogenous with one protein (coagulogen) constituting the majority of the clot, similar to fibrin in mammals. However, the protein composition of the natively formed horseshoe crab hemolymph clot has not been analyzed in details.

5. Conclusion

Although central to understanding the evolution and function of the innate immune system in arthropods, the clot composition of a chelicerate species has never been addressed. Here, we present a global analysis of a spider hemolymph clot. The newly published next-generation sequencing-based *A. geniculata* transcriptome [31], combined with state-of-the-art mass spectrometry equipment, facilitated an in-depth analysis of the clot-proteome focusing on the covalently incorporated proteins. While spiders and horseshoe crabs both are chelicerates, the present study indicates that the spider clot resembles insect clots more than the horseshoe crab clot, since lectin-like and vWF-like proteins are part of the spider clot, but coagulin homologs apparently are absent. This indicates an ancient origin of lectin-like and vWF-like proteins in the clot, most likely predating the split between chelicerates and the other arthropods. Furthermore, it suggests that coagulogen has evolved in the horseshoe crabs. In contrast to the resulting clot, the spiders' bacterial recognition, as well as the coagulation cascade, seems to be more similar to that of the horseshoe crab than of insects.

The findings in the present study and in previous work [26,31], suggest that hemolymph clot-formation in spiders is a multifaceted process involving, i) polymerization of VWF-like proteins, ii) covalent incorporation of hemocyanin, and consequently PO activity and melanization in the clot, iii) complement involvement and antimicrobial activity in the clot, iv) transglutaminase-mediated cross-linking, and, v) perhaps formation of NETs. With respect to the innate immune system, the other arachnids are genetically similar to the spiders [26], and the described complex clot-formation scenario might broadly apply to all arachnids.

The clot proteome revealed that hemocyanin constitutes more than half of the *A. geniculata* hemolymph clot suggesting that this multifunctional protein plays a role in coagulation. The proteins are also involved in innate immunity and in general we identify many innate immune system-related proteins in the clot (Table 2). Thus, the presented clot proteome substantiates and underlines that crosstalk between coagulation and the innate immune system exist.

Taken together, our study shows that clot-formation in spiders differs from previously described arthropod coagulation mechanisms underscored by the finding of hemocyanin in high concentration in the clot. Although unique, the study also demonstrates that spiders exploit functional clotting elements that are known from other arthropods, including, i) coagulation factors with similarity to the horseshoe crab, ii) lectin-like and VWF-like proteins, as in the insects, and iii) vitellogenin, as in the crustaceans.

The supplementary table is an Excel document that presents the analysis of the mass spectrometry studies, as described below.

The mass spectrometry proteomics data and the transcriptomics-based *A. geniculata*-sequence database (FASTA-format) have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002500 (http://www.ebi.ac.uk/pride/archive/). Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.bbapap.2015.11.004.

Transparency document

The Transparency document is associated with this article can be found, in the online version.

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