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Immune System and Its Relationships with Pathogens: Structure, Physiology, and Molecular Biology

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Abstract

Ticks are of vast medical and veterinary public health importance due to direct damage in livestock by its hematophagous feeding habits and its potential as a vector capable to transmit infectious agents such as Tick-borne diseases. Currently, the knowledge of vertebrates' immune system contributes to the advance in vaccine and drug development, resulting in new drugs that help to control human and livestock pathogens. Unfortunately, very small advances have been achieved in tick's immune system that could help to develop new strategies designated to control tick-borne diseases and other arthropod vectors. On this subject, the study of the mechanisms involved is transcendental as is also the study on molecules, cells, and regulation of immune response involved in signaling pathways in ticks. The progress on the understanding of ticks' physiology represents a necessary advance in molecular approaches related with a tick's immune response, involved in host-vector-pathogen interaction, and, in turn, evolutionary relationships. Current knowledge on tick's immune response to different kinds of pathogens is described in this chapter and the use of modern molecular tools to fill the gaps on different aspects in tick immunobiology that still is unclear or under study.

Keywords: ticks, immune system, pathogens, hemolymph

1. Introduction

Ixodidae, comprising those arthropods commonly called ticks, include nearly 870 acaridae species, and these are obligate hematophagous parasites of terrestrial vertebrates at some part of their life cycle. Moreover, ticks are considered as important veterinary health threat, due to their capacity to cause direct damage to livestock by feeding on blood and transmitting tick-borne pathogens, causing serious animal and human infectious diseases. The pathogenic

diversity of organisms transmitted by ticks exceeds to be found in all other hematophagous arthropods. Tick-borne pathogens include protozoans, the bacteria rickettsia, viruses, and nematodes [1] that in turn evade the tick's immune defense mechanisms, encountered on their route through the tick's body (midgut, hemolymph, salivary glands or ovaries). These immune interactions are very important in tick biology and pathogen relationships. Likewise, some pathogens are also often trans-stadia and trans-ovarian transmitted increasing the vector-pathogen complexity, related with a disease transmission and severity, considering that each tick species is capable to transmit different pathogens [2]. Unfortunately, many metabolic and molecular mechanisms related with a tick's immune response to different pathogens remain unclear. For this reason, in recent years, the study of tick-host-pathogen interface has increased. Currently, we know that tick's innate immunity is carried on by the cellular and innate responses, where the different molecules, enzymes, cells, and proteins are involved in general immune mechanism. On the other hand, we have different molecular and immunological tools, as tick's salivary glands, midgut transcriptome, and proteomics analysis, and the first tick genome project, that contribute to elucidate tick's biological interactions. The immunobiology characterization of the tick-pathogen-host interface dynamic interaction should be exploited as a tool used for development of novel vector and transmission blocking vaccines, targets, and new drug design [3, 4].

2. Immune system in invertebrates

All invertebrates have an immune system, composed of both humoral and cellular response that results as effective defense to different pathogen attack. The cellular immune response is composed of several mechanisms as phagocytosis, nodulation formation, agglutination, and cellular encapsulation, while humoral response involves expression and secretion of different molecules able to kill bacteria, parasites, and other pathogens [2, 4]. The performance of this multifactorial system requires synthesis and regulation of RNA and proteins involved in arthropod protection. Until recently, the investigations of molecular, genetics, and cellular aspects of the arthropods' immune response were scant. One reason behind this paucity is the extremely difficult to control laboratory conditions that allow to maintain the host-parasite interaction in several generations, or different stages of arthropod life cycle [2]. On the other hand, among the invertebrates, the insects have received most attention, compared to arachnids. In this regard, in spite of extensive research, the immune system of ticks is still poorly understood.

2.1. Ticks' immunobiological response

The immune response of ticks as well as arthropods includes both cellular and humoral mechanisms, where the hemolymph and other tissues, such as salivary glands, midgut, hemolymph, and fat body, provide the principal source of molecules and cells involved in the immunological attack of pathogens. In the case of tick's hemolymph, many pathways involved in the immune response still remain unclear [4]. Currently, few reports explain the type of response that ticks have against different infectious agents that, in turn, could be used as target to pest biocontrol.

3. Cellular immune response

3.1. Hemolymph

All tissues in ticks, and other invertebrates, are bathed in a fluid known as the hemolymph, which is the first source of nutrients, osmoregulation, and molecules and hormones transport, and provide protection to pathogen agents to which ticks are exposed [1]. Likewise, hemolymph coagulates at the site of some injury, preventing microbes spreading into the body tissues. The hemolymph consists of protein-rich plasma and different types of cells called hemocytes that play a transcendental role in immune response [2].

3.1.1. Hemocytes

In ticks, the immune cell–mediated response is carried out by hemocytes, cells with free circulation and the major component of the hemolymph. Hemocytes play an important role in the tick's defense against injury as well as microbial infection and increase greatly its population, in response to bacteria, viruses, protozoa, and other pathogen infection; however, the multiplication rates for the hemocyte types in response to a specific pathogen have not been fully clarified [5–8]. The mature hemocytes mediate different events that include phagocytosis, nodulation, and encapsulation. The tick hemolymph can be divided into four cell types based on their function and morphology; however, at the moment, the hemocytes classification is controversial, because it has been observed that population may varied in hard and soft ticks and among species. The prohemocytes are round to oval small cells with a prominent nucleus, numerous mitochondria, and little granular cytoplasm. The cell size is 6–7 μm and represents the stem cells in the hemolymph, from which all cell types can be differentiated and occasionally can be found to be associated with many tissues. The prohemocytes' population proportion varies depending on the species and healthy, wounded, or infected ticks [9, 10]. The granulocytes are large cells with numerous cytoplasmatic granules; some cases have a cytoplasmatic extension called filopodia. In general, granulocytes have a long size about 15–20 μm and are further subdivided into type I and type II, depending on the granule morphology. Type I granulocytes are pleomorphic cells that 6 μm in length, which contain variable electrodense granules and presence of filopodia and lysosomes. The type II granulocytes contain several granules both electrodense and condensing immature granules, located peripherally and at the central cell [11]. Along with granulocytes, the plasmatocytes are the most predominant hemocyte type in hemolymph. These cells have slightly elongated shape, often fusiform and numerous filopodia, with a large variability in size ranging from 8 to 12 and the long axis up to 20 μm. In some species, plasmatocytes have rounded or ovoid shape, with a size about 10–12 μm and containing few vacuoles and granules. The spherulocytes are cells with a size of 11–14 μm and are oval shaped with electron-lucent and fibril-filled granules that fill almost the entire cytoplasm cell. Currently, some studies report the presence of the oenocytoids in limited number of tick species [10]. These cells are 11–18 μm in size and are ovoid shaped with cytoplasmatic granules [12]. However, the oenocytoids' presence in ticks remains controversial [1]. The understanding of functions and pathways involved in the activation of hemocytes could provide elements that help to understand the cells' role in immune

response. In this regard, many groups have studies based on electrophoretic patterns in one and two dimension, obtaining proteomic maps that show proteins related with the hemocytes' pathogen response [13].

3.2. Phagocytosis

Phagocytosis is a complex mechanism that involves the recognition, engulfment, and destruction of pathogens. In this process, the immune cells recognize pathogen-associated molecular patterns (PAMPs) produced by several bacteria and fungi. In all arthropods, phagocytosis is carried out by the hemocytes and represents the first primary defense response to pathogen infection [11, 14]. In ticks, the phagocytosis process has been regulated by granulocytes type I and plasmocytes and sometimes by granulocytes type II, suggesting that differences in hemocytes' population have different roles and contributions to the tick's immune response [15]. In initial steps, the phagocytic cell response is binding receptor-mediated to pathogen cell surface; subsequently, signal transduction pathways are activated and followed by filopodia projections that surround and engulf the bound particle [16]. The particle is internalized by endocytosis into a vesicle, subsequently, with lysosomal compartments that in turn form the phagolysosome. Inside, intracellular enzymes are activated such as acid phosphatases, type c lysozyme, cystatins, and proteases completing the cellular lysis. Little is known about the molecular regulation in tick immune response, some reports suggested that as in insects the most important signal transduction pathways are mitogen-activated protein kinase (MAPK) and FAK/Src pathways that in turn are involved in proPO activation [16]. Moreover, several external factors are capable to enhance this process. Currently, recent evidence indicates that *R. microplus* produces reactive oxygen species (ROS)-mediated oxidative burst modulated by protein kinase C, similar to that found in leucocytes [17].

3.3. Nodulation

Tick hemocytes are capable of expressing lectins on membrane surface involved in pathogen recognition. These molecules can join with lipopolysaccharides (LPS) also present on the pathogen surface. Currently, several lectins involved in the immune response and other mechanisms have been identified in tick hemocytes and different cells [18–21]. In soft ticks, *O. moubata* was described to have a protein called Dorin-M, lectin with high hemagglutinating activity and isolated from hemocytes, and hemolymph plasma [22]; likewise, *I. ricinus* was described to have the Ixoderin A, lectin found in midgut and hemocytes [23]. The protein-carbohydrate interaction confers the ability to hemocyte aggregation that results in the pathogen entrapment and, in turn, the opsonization through lectins that may also cause bacteria aggregates [24].Thus, lectin recognition leads hemocyte recruitment that builds a sticky mass around the bacterial aggregate (nodules), preventing the dissemination of pathogens and eventually digesting it. The formation of this nodule represents a predominant cellular immunity defense mechanism to bacterial challenges [25].

3.4. Encapsulation

Encapsulation is the immunological process whereby the arthropods are capable of attacking pathogens that are very large to eliminate by nodulation or phagocytosis. Other immunological

processes, such as the proteolytic degradation of microbial products (LPS and peptidoglycan), can result in the prophenoloxidase activation. This activation generates phenoloxidase expression that in turn, along with tyrosine metabolism, is directly related to melanin synthesis. In all insects, pathogen encapsulation involves melanization, where hemocytes, mainly type I granulocytes and plasmatocytes, form a capsule of thick layer around the pathogen that leads to asphyxiation and toxic-free radical production, such as quinones and semiquinones [3, 26, 27], with melanin deposition as the final step [10]. In ticks, phenoloxidase is present in *O. moubata* hemolymph [28], in contrast to *A. americanum*, *D. variabilis*, *and I. scapularis*, where there are no reports to phenoloxidase activity [29]. In this regard, in hard tick *D. variabilis*, the simple injection of plastic bead can induce the capsule formation, but without the presence of melanization [30]. These findings suggest that this pathway is present in ticks; however, it has a distinct role in metabolism or immune response. Moreover, genomic analyses in VectorBase indicate the absence of gene homologs for the complete pathway in *I. scapularis* genome sequence [31].

4. Humoral immune response

The humoral factors of the insect and crustacean immune system have been extensively studied. In contrast, in ticks, we know very little of this field. Mostly, the soluble factors are produced by hemocytes and released in the hemolymph, where they are transported to other tissues such as midgut and salivary glands. The humoral factors play an important role in the defense and protection of ticks from microbial invasion. Within these factors, a variety of antimicrobial proteins, such as lectins, proteases, and lysozymes, coagulation factors, proteases inhibitors, antimicrobial peptides, and products related to oxidative stress, are included [3, 32]. These soluble factors are involved in various aspects of the immune protection, such as blood ticks feeding in midgut protection; during migration hemolymph defense; and tissue protection, for example, during pathogen transmission in salivary glands, in all cases during pathogen infestation [2]. The plasma hemolymph represents nearly 90% of total composition, and the proteic soluble component represents approximately 11.5–14.3% of plasma [33]. The knowledge of ticks' hemolymph components is very limited; for this reason, the advance in the understanding is based on other arthropods [33]. For example, electrophoresis assays of two-dimensional gel map obtained of *Drosophila melanogaster* show 160 hemolymph proteins. The results found have been used as basis for comparative studies in other species, including ticks [34].

4.1. Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) represent the most effective humoral immune response, for their ability to kill several pathogens, for their fast response, and for their effectiveness at micronanomolar concentrations. AMPs are small peptides (3–20 kDa), and their action mechanism is based on their capacity to cell membrane or cell wall binding, causing structural disruption that results in loss of pathogen membrane potential. AMPs are secreted mainly by the fat body and hemocytes; however, midgut is capable to produce some peptides [2]. Many authors reported and identified several AMPs in ticks, including microplusin [35], hebraein [36], ixodidin [37], antimicrobial peptide (ISAMP) [38], and some peptides from *Amblyomma hebraeum* [39].

4.1.1. Defensins

Defensins are small cationic peptides (3–6 kDa) with six to eight cystein residues that are folded by three or four disulfide bridges. These bonds help to stabilize and maintain the tertiary structure, called "defensin folds" [40]. Defensin AMPs were found in many arthropods including hard and soft ticks [41, 42]. Defensins may be classified into three major groups: (1) peptides with α-helical conformation, (2) cyclic and open cyclic peptides with cysteine residue pairs, and (3) peptides with overrepresentation of some amino acids [3]. In all cases, the mature peptides present highly conserved regions in contrast with leader regions that show much more variability. Moreover, its sequence contains hydrophobic regions separated from charged regions that enable them to insert into pathogen membranes causing pores that in turn kill the cell [43]. In ticks, the defensin expression is carried out in several tissues such as fat body, hemocytes, salivary glands, and midgut. *In silico* genetic analysis shows the presence of two multigene families of defensin-like peptides. The first family, corresponds to scapularisin-type defensin peptides [44], and the second, the scasin defensin-like peptides, which present low similarity with other defensins; however, they have six conserved cysteine residue characteristics of defensins. Several tick species present multiple defensin isoforms, with regulation tissue-dependent expression. However, *in silico* analysis shows that the protein sequences are very closely related in mature regions. In contrast, three defensins from the hard ticks *Amblyomma hebraeum*, *Haemaphysalis longicornis*, and microplusin from *R. microplus* are the exception of this analysis. Defensin sequence analyses demonstrated four isoforms (A, B, C, and D) present in soft tick *O. moubata*. On the other hand, some assays with several component of bacterial wall, injected into tick's hemolymph showed upregulation of defensin expression by semiquantitative RT-PCR and ELISA [45]. Interestingly, isoforms A, B, and C are overexpressed in midgut, while isoform D is overexpressed in fat body [46]. These results suggested that defensin isoforms are expressed in tissue-dependent fashion. However, the receptors and signaling pathways require more analysis.

4.1.2. Lysozymes

Lysozymes are ubiquitously expressed enzymes with a molecular weight approximately 14 kDa, are involved in digestive processes, and have an antimicrobial activity for their ability to lyse bacteria by hydrolyzing the β-1,4 glycosidic bonds between the N-acetyl-muramic acid and N-acetyl-D-glucosamine residues that form the peptidoglycan walls. In a hard tick *D. variabilis*, the expression of C-type lysozyme, which increases in hemolymph 17-fold, after exposition to *E. coli* at 72 h post-challenge, has been demonstrated [47]. In this case, the level of C-type lysozyme in hemolymph is highest than in midgut and other tissue [48, 49]. In contrast, C-type lysozyme (HI-lysozyme) from *H. longicornis* [50] is detected in all development stages of ticks and in gene expression in fat body, midgut, ovaries, and hemolymph and is upregulated after bacterial challenge. Likewise, the hard tick *O. moubata* expresses a 124 amino acid C-type lysozyme that presents overexpression in midgut, after blood feeding, but not in the tick hemolymph [51]. These results suggest that tick lysozyme is an enzyme with both immune and metabolic functions [51, 52]. Moreover, the lysozymes present in ticks' hemolymph may act synergistically with defensin and other AMPs in the pathogen control by disruption of bacteria cell wall, accelerating the killing action [41].

4.1.3. Other antimicrobial peptides

Currently, in addition to defensins, there exist a large number of antimicrobial peptides identified. In ticks, other types of AMPs have been detected. In *R. microplus*, microplusin, a polypeptide of 10 kDa, is present in hemolymph and presents no sequence similarity with any AMP reported [35]. Structurally, the polypeptide has six cysteine residues, and the gene expression was observed in different tissues such as fat body, ovaries, and hemocytes, suggesting that the mature peptide must be released into the hemolymph. The hebraein is an 11 kDa antimicrobial protein with six cysteine residues and one histidine-rich carboxyl-terminal region. This AMP was isolated from the hemolymph of female *Amblyomma hebraeum* ticks [36]. *In silico* analysis showed similarities and identities between hebraein and microplusin of 73 and 62%, respectively, and this suggests that probably hebraein belongs to the same family and, structurally, has the cysteine motif similar to microplusin. The hebraein is a protein with widespread antimicrobial activity, experimentally demonstrated by different assays, by both recombinant and native proteins against the Gram-positive (*Staphylococcus aureus*) and Gram-negative (*E. coli*) bacteria, and in turn showed antipathogen activity against fungi [53]. Additional experiments demonstrated that histidine-deficient mutants of the protein lack antimicrobial activity. On the other hand, the Ixosin was identified in *Ixodes sinensis*, a peptide of 2.8 kDa isolated from salivary glands, described as the first antimicrobial peptide lacking cysteine residues [53]. Ixosin has an antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi [53]. Additionally, a novel antimicrobial peptide was isolated from the *R. microplus* hemocytes [35]. This peptide was named ixodidin, and demonstrated inhibitory effects against *E. coli* and *Micrococcus luteus* growth [35]; in addition, ixodidin, has a proteolytic inhibitory activity against serine proteinases. This is a first report, the molecule in arachnid with both proteinase activity and bacterial growth.

4.2. Hemagglutination (lectins)

Lectins are proteins whose structure has domains with specific binding sites for carbohydrate [54]. The bacteria membrane or cell wall including the fungi and protozoan pathogens has different carbohydrate moieties that can be recognized by lectins. These proteins exhibit different molecular sizes from 30 to 85 kDa and have been identified in the membrane surface of hemocytes, cell gut, and salivary glands, or synthesized by hemocytes and released in the hemolymph plasma of soft and hard tick species. In invertebrates, lectins are important mediators of immune response. Initially, these molecules are defined by their participation in a hemagglutination process; however, these proteins also bind to pathogens that, in turn, enable hemocytes to recognize and engulf (opsonization). This process includes carbohydrate recognition by ficolins and mannose-binding gal-lectins, among others [55, 56]. Insects, ascidians, crustaceans, and ticks contain molecule type TLP-1 and TLP-2 lectins (*Tachypleustridentatus*), to form molecule clusters that bind and immobilize pathogens [10, 23, 55–57]. The mechanism causes pathogen be trapped and immobilized forming aggregates, which later are surrounded by hemocytes and destroyed by encapsulation or nodulation [58]. Tick lectins are involved in processes of cell adhesion, recognition, opsonization, phagocytosis, and cytolysis of infecting pathogens [59]. The first tick lectin reports were in the papillipes *Ornithodoros tartakovsky* and

O. tholozani [60, 61]. Subsequently, in *O. moubata* hemolymph plasma was identified the lectin Dorin M, lectin to 640 kDa, synthesized in the hemocytes and secreted into the hemolymph plasma, which has a high hemagglutinating capacity [19, 57, 62]. In this regard, molecular structure studies showed that Dorin M lectin has a fibrinogen-like domain related to the ficolin family of proteins that recognizes carbohydrate sequences, especially sialic acid and N-acetyl-D-glucosamine, similar to the tachylectins of *T. tridentatus* [57]. OMFREP is a potential lectin in *O. moubata* and has been identified in hemocytes [23]. The use of the bioinformatics approach complemented with molecular studies results in the identification of fibrinogen-related protein that presents a 65% identity and a similar tissue distribution to Dorin M [23]. Likewise, novel galectin (OmGalec) has a different tissue and stage distribution in *O. moubata* [21]. This protein has galactose-binding properties and consists of tandem repeated carbohydrate recognition domains, where the carbohydrate affinity typical motif is present [21]. In hard tick *I. ricinus*, Ixoderin A is expressed by hemocytes and is present in midgut and salivary glands, while Ixoderin B is only expressed in salivary glands. These findings suggest that the lectin and isoforms have a selective expression in different tissue, plasma, and cells, suggesting that they have specific roles; however, many pathways are still unknown. In salivary glands and the midgut of *Rhipicephalus appendiculatus* was described a lectin that was related to a significant increase of sugar, which inhibits hemagglutination during *Theileria parva* infection, suggesting a decisive role in this process [63, 64]. Recently, it was demonstrated that some lectins are involved in various processes related to feeding [65], ticks' immune cell regulation, and molecule recognition. Interestingly, the cloning and protein expression in ticks' hemocytes and salivary glands of two fibrinogen-related proteins, which present high homology with Dorin M lectin, showed that they are essential in the pathogen transmission [20].

4.3. Proteases and protease inhibitors

4.3.1. Proteases

The feeding mechanisms of ticks involve the presence of midgut, where the blood mead digestion is carried out. In this process, a large variety of cysteine, aspartic, and serine proteases are involved, and many of these molecules also have an important role in mechanisms of immune response. In the lumen, the serine proteases are the most important, which function as hemolytic agents and as cysteine and aspartyl proteases in hemoglobin digestion [66–68]. Various of these proteins are identified; however, in ticks, the regulation, expression, and presence of these molecules still remain unclear. Currently, the protease immune mechanism in insects suggests that metalloproteases may be important in cellular immune defense [69]. In the *D. variabilis* midgut, three metalloproteinases have been identified from cDNA library [70]. In this regard, analysis of sequences showed very little similarity to tick proteases, suggesting that these may be novel metalloproteinases. Likewise, a clip-domain serine proteinase homolog was identified [71]. On the other hand, the arthropod clip-domain family serine proteinases contain two major domains: a trypsin gen-like catalytic domain in the C-terminus and a disulfide knotted regulatory N-terminal domain [72]. In this regard, the *Anopheles* mosquitoes present serine protease overexpression in response to malarial parasites. This mechanism is consistent with the innate immune response generated for the hemolymph [73]. This response is a key factor in the internal control for malaria parasites' number and replication. Similarly,

tick hemocytes are able to responsd with an immune-responsive factor D-like overexpression, in response to Gram-positive challenge [72]. *In silico* analysis showed that immune-responsive factor D-like overexpression has a 54% sequence identity to *Tachypleus tridentatus* serine proteases [74]. It is important to note that the similar domains present in these proteases are found in high invertebrates variety, suggesting the conservation of these molecules [72].

4.3.2. Protease inhibitors

Protease inhibitors are important in tick's pathogen infection as innate immune suppressor of virulence, toxic, and replication factors expressed by microorganisms. Proteases are important virulence factors used in various stages of the infection process, both by prokaryote and eukaryote pathogens. The inactivation of these factors may prevent the pathogen survival in the tick [75].Two major protease inhibitors have been reported in ticks: one called serpins that act as serine proteinase inhibitors and the other α-macroglobulins, large glycoproteins with mostly thiol-ester–containing proteinase inhibitors. Serpins may be found in plasma hemolymph and small cytoplasm granules [76]; however, in *R. appendiculatus* ticks, four serpins in midgut, salivary glands, and other internal tissues have been reported [67]. Moreover, in an *A. americanum* tick, a large number of serpin transcripts were described, many of which were ubiquitously expressed in the midgut (three most strongly expressed); likewise, several transcripts were also expressed in salivary glands and ovary [77]. In reference to immune response, serpins are involved in the fungal or bacterial protease inhibition and protection from several infections. Serpins, containing an active site serine replaced by glycine [47], also are involved in the regulation of several proteases that in turn contribute as cofactors in coagulation and cytokine activation and, most interesting, as a cofactor involved in prophenoloxidase pathway activation [78]. This activation suggests the phenoloxidase (PO) pathway presence in ticks, something that still is controversial. However, serpins could be an antigen target to the development of antitick vaccine or new drugs, since apparently it is related to the tick homeostasis, because of their potential functions as protease inhibitors [79]. The second proteinase inhibitor present in hemolymph ticks is the α-macroglobulins. This protease inhibitor family includes the α-2-macroglobulins, operating by neutralization of pathogen proteases by "entrapping in a molecular cage" through bait region, when protease substrate is recognized [80]. The molecular cage formation activates a proteolytic cleavage, through both the bait region and four thioester bond ruptures that in turn stabilize α-2-macroglobulin complexes, followed by the entrapment and protease transportation to hemolymph, which are degraded by lysosomes of hemocyte phagocyte cells [80]. In hard tick *I. scapularis*, cDNA sequence obtained from the salivary glands shows evidence of the α-2-macroglobulin presence, and in the soft tick *O. moubata*, α-2-macroglobulin present in hemolymph plasma is capable of inhibiting the trypsin action [81, 82]. Another important group of the cysteine protease inhibitors is the cystatins. The cystatins belong to protease family, which are reversible inhibitors of papain-like cysteine proteases, which function as proteolysis mediators, preventing the damage caused by cystein protease release to lysosome. In various species, cystatins are implicated in several functions related to immune response, epidermal homeostasis, antigen presentation, and inflammation [83–85]. In mammals, cystatin C is involved in the defense against pathogen [86]. Currently, cystatin sequence has been found in ticks, from cDNA library obtained from salivary glands of *Ixodes scapularis* [82, 87–90]. The cystatin (sialostatin L) obtained from *I. scapularis* cDNA library

was reported in tick saliva affected by proteolytic activity in infestation sites [88]. Moreover, SI-alostatin L, during tick blood feeding, has an important role as anti-inflammatory and in the inhibition of cytotoxic T-cell proliferation, contributing to feeding and pathogen transmission. On the other hand, cystatin RNAi-mediated silencing assay demonstrated that *Amblyomma americanum* reduced the ability to feed and evade the host immune response [87]. Recently, cystatins were shown to be expressed in ticks' salivary glands and other tissues, where they play an important role in the immune response [89, 90]. Moreover, in hard tick *R. microplus*, cystatin genes show expression in the fat body and ovary and protein expression in salivary glands, fat body, and ovary [89]. However, a possible role of the cystatin in several tissues in ticks still remains unknown. In this regard, novel cystatins from midgut were described in *Haemaphysalis longicornis* that show inhibitory activity against cysteine proteases [90]. In this regard, some assays demonstrate that *Babesis gibsoni* LPS injection is capable to increase the expression in the midgut in adult and larval ticks.

5. Nitric oxide and oxidative stress

5.1. Nitric oxide synthase

The nitric oxide (NO) is an unstable radical, capable to act with a key factor in several physiological and pathological pathways, and it is synthesized by the nitric oxide synthase (NOS) [91]. In invertebrates, including ticks, NO is related with a cytotoxic action against pathogens from hemocytes, derivates to phagocyte process during microbial infection [92]. Now, three NOS isoforms have been described: the classic isoform inducible nitric oxide synthase (iNOS), the endothelial isoform (eNOS), and the neuronal isoform (nNOS) [91, 93]. Currently, the gene that codified for NOS has been identified and cloned from the insects: *Drosophila melanogaster*, *Anopheles stephensi*, *Anopheles gambiae*, and *Rhodnius prolixus*, suggesting the NO activity is present in these arthropods [94–98]. Moreover, the activity of NOS was reported in the salivary gland of hematophagous insect *Rhodnius prolixus*, and the enzyme activity was FAD, NADPH, tetrahydrobiopterin, calmodulin, and Ca^{2+} dependent, suggesting high functionality, similar to NOS enzyme expressed in vertebrates [99]. Likewise, *Litopenaeus vannamei* shrimp is capable of producing nitric oxide, in response to *Vibrio harveyi* inoculation, derivates to NOS activity [93]. In ticks, the activity of eNOS enzyme was reported in *Dermacentor variabilis* salivary glands, and by *in silico* analysis, the presence of NOS gene sequence was demonstrated in *Ixodes scapularis* embryonated eggs [91, 100].

5.2. Oxidative stress and detoxifying protein

In hematophagous arthropods, blood ingestion is the determinant of survival. However, during feeding and digestion, several toxic molecules are produced, such as reactive nitrogen species (RNOS) and reactive oxygen species (ROS) [101]. The protection against nitrosative and oxidative stress is carried out by detoxification agents, produced largely by the midgut epithelial cells. In many insects, enzymes such as peroxiredoxins, catalases, and many members of antioxidant peroxidase family function as antioxidant agents. However, in arthropods, as in

many organisms, the microbial infections are capable to induce oxidative stress. Suppression of pathogen ROS and RNOS induction in midgut facilitates the infection and microbial tissue dispersion [102]. Interestingly, many arthropods have the capacity of enhancing ROS and RNOS against pathogen infection while simultaneously protecting their tissue cells with antioxidants. In this regard, the oxidize enzyme nicotinamide adenide dinucleotide phosphate (NADPH) of *D. melanogaster*, known as dual oxidase (dDuox), is capable to kill and/or inhibit the pathogen proliferation, through the oxidative burst [103]. Moreover, the glutathione S-transferases (GST) family plays an important role during oxidative stress caused by pathogens, through detoxification enzyme reactions and, in turn, removing the formatted ROS and RNOS [104]. In midgut from *D. variabilis* tick, GST isoforms DvGST1 and DvGST2 are upregulated during blood ingestion [105], and during the *B. burgdorferi* infection in tick *I. ricinus*, several GTSs are overexpressed in response to bacterial invasion [106]. In ticks, other detoxification enzymes have been reported, such as glutaredoxins, glutathione peroxidases, phospholipid-hydroperoxidases, thioredoxins, and one superoxide dismutase [107–109]. However, in ticks, the precise role in antimicrobial control of detoxification agents is still unclear.

5.3. Phenol oxidase and melanization

In arthropods, mechanical injury or the presence of foreign objects including pathogens results in melanin deposition around the damaged tissue or around the foreign object that in turn forms a capsule isolating the foreign particle. Melanins are molecules produced in the hemolymph by different types of hemocytes. The key enzyme for the melanization process is the phenol oxidase (PO). The metabolic pathway is initiated by hydroxylation of phenylalanine to tyrosine, followed by a series of reactions, resulting in 5,6-indolquinones, synthesized to phenol quinones, and these quinones polymerize to form melanin. The production of melanin is noticed by a dark and/or blackened color in the arthropod [110–112]. The signaling pathway starts with a hemocyte prophenol oxidase enzyme (PPO) synthesis (PO inactive form) that results in the conversion of the PPO into the active form by serine protease cascade [113]. This molecular system is capable to recognize picomolar of bacterial lipopolysaccharide (LPS), peptide glycans, and fungi β-1,3-glucane. The intermediary components of this pathway, such as semiquinones, ROS, and melanin, are all very toxic to pathogens [114]. On the other hand, the PPO-PO pathway in tick is little known. However, at the present, some studies in *Amblyomma americanum*, *Dermacentor variabilis*, and *Ixodes scapularis* ticks report the presence of genes involved in the PPO-PO pathway; however, the enzymatic activity has not been reported [29]. In the tick *O. moubata*, the PO enzyme has been reported in hemolymph plasma and in the fourth of ecdysiast nymphs [28]. However, currently, the presence of PO in ticks is controversial.

6. Molecular approaches to tick immunology

6.1. Regulation of innate immune system in ticks

The innate immune systems represent one aspect in a generalized response to several pathogens and are composed of individual factors. This variability has a particular behavior in each

tick. The principal components are the hemolymph and hemocytes; however, they are not the only factors. The response depends on the pathogen type, tissue, sex, life cycle phases, and tick species, among others. In this regard, innate immunity starts when membrane receptors recognize component characteristics of bacterial cell surfaces as peptidoglycans or lipoteichoic acid, which leads to synthesis of antimicrobial peptides (AMPs) as defensins, cecropins, attacins, and lysozyme that disrupt the cell wall structure, leading to cell death [2]. Other components in the fungi cell wall are beta-1-3-glucans and beta-1-3 mannose or 2-keto-3-deoxyoctonate LPS, characteristic of Gram-negative bacteria, leading to soluble lectin synthesis [2]. These cell wall components and foreign molecular structures are known as pathogen-associated molecular patterns (PAMPs) [2, 115]. In *D. variabilis* tick, different analyses show 56 genes involved in the immune response; however, these genes do not appear to be regulated. In sexual term, transcriptome analysis in the male reproductive structures of this tick showed seven contigs related to a dual reproductive and immune response [116]. However, the complete role of these peptides is still unknown, but their presence in seminal fluids suggests a role in the clearing of bacteria introduced during mating [117]. In the tissues, the immune response includes several factors such as AMPs, peritrophic membrane, proteases, and protease inhibitors, lectins, detoxificant proteins, and oxidative stress [3]. Transcriptome analyses in *D. variabilis* midgut show 8 transcripts related to the innate immune response, of which one protein (MD-2) is involved in lipid-domain recognition, lectins, and in turn involved in inhibition of macrophage activation [118]. Moreover, transcriptome analyses of tick salivary glands found AMPs, proteases, and protease inhibitors related to innate immune response [119–121]. The synganglion transcriptome of *D. variabilis* contains 0.27–1.15% peptides, depending on the gene ontology, that represent between 4 and 11 genes [122] and includes AMPs, proteases, lectins, protease inhibitors, and regulatory Toll-like proteins [116]. On the other hand, the widespread response has been initiated by hemocyte cell pathogen recognition carried out by the presence of microbe-associated microbial patterns (MAMPs), expressed in pathogen's membrane surface. However, in ticks, the hemocyte receptors to MAMP's recognition are still unknown, but analyses reveal similar receptors to those identified in insects [26]. In this regard, homologs to peptidoglycan receptor proteins (PGRPs), Gram-negative–binding proteins (GNBPs), and gal-lectins have been reported [123]. To identify the pathogen type, the hemocyte cells need to be activated, using specific receptors that result in a specific signaling pathway [116]. The fruit fly *D. melanogaster* has been used as a genetic model to elucidate the activation of the innate immune system, which is an evolutionarily conserved mechanism in eukaryotes. Drosophila has three pathways involved in an immune response: Toll, Imd, and Hop, homologs to TLR, TNFα, and Jak/STAT in mammals [124]. Different components of these three signaling pathways were found in tick's database, such as in Toll pathway, Toll, MyD88 and Pelle. In *Drosophila* model, the fungal pathogens and Gram-positive bacteria activate the Toll cascade, which is composed of different Toll-like receptors (TLRs) capable of recognizing diverse types of PAMPs [2, 115, 125, 126]. After pathogen recognition, intermediate effectors such as myeloid differentiation factor 88 (MyDD88), Tube, and Pelle are activated followed by activation of transcription factor Dorsal (homolog of NF-kB) and Dorsal-related immunity factor (Dif) that translocates into the nucleus and regulates the AMP synthesis [126]. From the Imd pathway, Dredd, Caspar, and Relish have been found. Gram-negative bacteria infection activates the Imd cascade through the recognition of DAP-type peptidoglycan in the membrane protein peptidoglycan (PGRP-LE) [115, 125]

and activates molecules such as TAB2/TAK1, JNK, IKK, and Relish inducing the transcription of AMP [115, 126]. Finally, from the Hop pathway, JAK and STAT have been found [123]. In the absence of infection, a selective repression of this IMD/Toll-dependent AMP pathways is achieved by the home box gene Caudal [127, 128]. Recently, the RNA interference (RNAi) pathway has been described that regulates the immune system in arthropods including ticks. This process is crucial in the innate response to viruses that infect and are transmitted by ticks [129]. In *Drosophila*, the RNAi mechanism is related to a virus penetration and regulation of innate immune response in the midgut. The RNAi pathway in ticks is unknown; however, its components are described in some ticks [130], as RNAi has been used to silence genes involved in several mechanisms, suggesting that RNAi pathway is active in some tick species, which would explain the different capacity of ticks to transfer several viruses [129].

6.2. Advance in molecular, functional genomics and proteomics in tick-host-pathogen interaction

Advances in gene identification and expression in tick tissues are being achieved by the use of expressed sequence tag (EST). The EST analyses correspond to partial sequence of acid nucleic from different random clones included in a cDNA library, obtained from the interest tissue mRNA [131]. The analyses include the translation of EST sequence to amino acid sequence and compared with a public genome database. Interestingly, salivary gland genes of ticks show differential expression during blood ingestion, suggesting that processes are involved in homeostasis, tissue remodeling, immune defenses, angiogenesis, and the facilitation of the transmissible pathogen establishment [132]. The EST library from unfed hard tick larvae of *R. microplus* was the first study reported [133]. However, 234 unique ESTs were identified, and 39% of them were not found in genome database. In *A. americanum*, cDNA libraries showed that 1462 and 480 ESTs (adult and larvae respectively) presented 56% to no-similarity identified in encoded proteins [134]. On the other hand, *R. microplus* gene expression analyses from cDNA library obtained from RNA tissue larvae exposed to different stimuli and infected with *Babesia* showed that 8270 unique sequences were identified to 11,520 total sequenced clones and presented a 44% of shared similarity to database sequences [135]. A meta-analysis was done, which describes the transcripts from salivary glands of several species of ticks, including the salivary gland transcripts from unfed male of *I. ricinus* and *A. americanus*; fed female of *I. pacificus* and *A. variegatum*; unfed female and unfed-fed nymphs of *I. scapularis*; and finally unfed males and fed female of *Dermacentor andersoni*. All tick groups analyzed were from different ages and different feeding times, or unfed. The results showed that the secreted proteins comprised 49% from which 15% were no match with any gene reported *in silico* analyses. Interestingly, transposable elements were found in 0.5% of the transcripts, which suggest gene rearrangements. In the exclusive case of females, differential gene expressions of transcripts were showed. The unfed female showed no change in expression, while fed female showed the highest number of overexpressed variants. All biologically relevant genes are likely redundant and encode antigenic variants, in turn identifying gene families involved in hemostatic deregulation. Other identified genes include cystatins, lectins, cysteine and glycine-rich peptides, and protease inhibitors [119, 132, 136–138]. A very important finding in *R. appendiculatus* tick showed that tick-borne pathogen presence did not modify the gene expression. In this

regard, no significant differences were found in the expressed transcripts of 9162 ESTs from salivary glands of *R. appendiculatus* uninfected, compared with the 9844 ESTs obtained from salivary gland of *R. appendiculatus* infected with *Theileria parva* [139]. Currently, the genome sequence of several arthropod vectors including ticks is under development, and partial results of *I. scapularis* sequencing efforts reveal that deer tick genome is approximately 2.1 Gbp; likewise, the hard tick *R. microplus* genome contains 7.1 Gbp [140]. It is remarkable that cattle tick genome is more than twice the size of the human genome that contains 3–2 Gbp [141]; furthermore, the cattle tick genomes are larger than most insect species genomes.

7. Future directions

The ever-increasing knowledge of the immune system biology of vertebrates represents an important foundation in the research and development of advanced vaccines, new drugs, as well as the search for new targets for chemical or drug treatments of infectious diseases, which have contributed to the control of several human and livestock pathogens. Unfortunately, the immune system of invertebrates, especially, arthropod vectors like ticks, and their relationship with their pathogens, and infectious diseases they transmit, have been little explored. In this regard, the knowledge of mechanisms, molecules, and cells, as well as the regulation of immune response signaling pathways, represents an advance in designing control strategies that will contribute to improve livestock production and animal health. Currently, studies in insects and the molecular tool development help us to advance in the research to arthropod immune system regulation; however, there are many knowledge gaps about the ticks' immune response. Elucidation of the different molecular pathways and their regulation in ticks' immunobiology brings us closer to understand the role in the transmission of various infectious agents. Now, all transcriptome analyses and whole-genome sequencing represent powerful methodologies for understanding the biology, evolutionary relationships, and host-vector-pathogen interaction. The use of DNA/RNA sequencing modern tools could potentiate the discovery of different aspects that remain unsolved in tick biology, for the elucidation of the paradigms that currently remain unknown.

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