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THE ROLE OF SALIVA IN TICK FEEDING

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Abstract

When attempting to feed on their hosts, ticks face the problem of host hemostasis (the vertebrate mechanisms that prevent blood loss), inflammation (that can produce itching or pain and thus initiate defensive behavior on their hosts) and immunity (by way of both cellular and humoral responses). Against these barriers, ticks evolved a complex and sophisticated pharmacological armamentarium, consisting of bioactive lipids and proteins, to assist blood feeding. Recent progress in transcriptome research has uncovered that hard ticks have hundreds of different proteins expressed in their salivary glands, the majority of which have no known function, and include many novel protein families (e.g., their primary structure is unique to ticks). This review will address the vertebrate mechanisms of these barriers as a guide to identify the possible targets of these large numbers of known salivary proteins with unknown function. We additionally provide a supplemental table that catalogues over 3,500 putative salivary proteins from various tick species, which might assist the scientific community in the process of functional identification of these unique proteins. This supplemental file is accessble from http://exon.niaid.nih.gov/transcriptome/tick_review/Sup-Table-1.xls.gz.

2. INTRODUCTION

Ticks are specialized mites in the suborder Ixodida of the order Parasitiformes. They are unique among mites by being larger, having specialized mouthparts and being obligate ectoparasites of terrestrial vertebrates, including amphibians, reptiles, birds and mammals.

Two major families exist among ticks, the Argasidae (soft ticks) and the Ixodidae (hard ticks) [1]. Phylogeny based on 16S rDNA sequences indicated the two families are monophyletic, with predicted divergence time no earlier than the late Jurassic (140 million years ago) [1], although other authors suggest much earlier divergence (late Permian, 245 million years ago) based on the radiation of reptiles [2]. These two families display quite different feeding strategies: Ixodids feed for prolonged periods of time, varying from a few days to over one week, while argasids typically feed for less than one hour. Adult female hard ticks will feed only once, dying after oviposition, while adult soft ticks will feed multiple times.

Ixodidae are further partitioned into metastriate and prostriate ticks. Metastriate ticks, such as members of the Dermacentor or Rhipicephalus genera, have relatively short mouthparts but they secrete copious amounts of a cement or glue that firmly attaches the tick to its host. Prostriates, such as members of the Ixodes genera, have longer, barbed, mouthparts and rely more on this physical mechanism to stay firmly attached to their hosts.

The salivary glands of ticks, in addition to their role in feeding, serve a role in ion and water metabolism. In a tick not attached to its host, certain salivary gland lobes produce hygroscopic saliva that is secreted via the salivarium onto the surface of the hypostome. Atmospheric moisture is absorbed by the highly salty saliva and then sucked back into the body of the tick, thus helping ticks to stay hydrated, sometimes for years, while they wait for a host. In a blood-feeding tick, saliva production is the main mechanism of water excretion [3, 4]. Ticks alternate blood ingestion and salivation, each cycle lasting for 5–20 min at a time [5, 6]. The gut pumps water and electrolytes into the haemocoel, which go back to the host via saliva, while the meal is concentrated in the midgut.

Blood is the only nutritious food taken by ticks. The adaptation to blood feeding involved evolution of a complex cocktail of salivary components that help the parasite to overcome their host's defenses against blood loss (hemostasis), and development of inflammatory reactions at the feeding site that may disrupt blood flow or trigger host-defensive behavior by the sensation of pain or itching. Accordingly, saliva of blood-sucking arthropods contain anti-clotting, anti-platelet, vasodilatory, anti-inflammatory and immunomodulatory components, usually in redundant amounts [7].

3. PROBLEMS TICKS FACE WHEN TAKING A BLOOD MEAL

It is crucial for any hematophagous animal that blood vessels at the feeding site continue to deliver liquid blood to the animal mouthparts despite injury to the vertebrate integument. Vascular injury triggers the phenomenon of hemostasis, which relies on the triad of blood coagulation, platelet aggregation, and vasoconstriction. Hemostasis starts within seconds of tissue injury. Tissue repair mechanisms induce scar formation, a process that starts within the day of injury and proceeds for several additional days. In addition, the immune system may contribute cellular and humoral responses that further modify the tick feeding site of a previously exposed host. Such immune responses may be immediate, as in antigen/antibody/complement reactions, or may take hours to occur, as in cellular reactions that require formation of a leukocyte infiltrate such as the basophilic infiltrate associated with tick rejection reactions in guinea pigs [8]. The following subsections review the vertebrate pathways faced by ticks during the feeding process with an intent to identify targets of salivary components in their adaptive role to facilitate a blood meal.

3.1. Platelets

Platelets, which play a critical role in mammalian hemostasis, are activated by a number of agonists including adenosine diphosphate (ADP), released by damaged cells and by activated platelets (the plasma levels of ADP are normally below 0.1 microM but are over 1 mM within the cytosol), collagen (not found in blood but abundant below endothelial cells (EC)), thrombin (produced by the coagulation cascade), platelet-activating factor (PAF)-acether (produced by activated leukocytes such as neutrophils), and thromboxane A2 (TXA_2) (produced by activated platelets). Of note, ADP and collagen are the primary agonists of platelet aggregation, while the remaining agents are part of a positive feedback loop. These agonists recognize specific receptors and trigger platelet activation by a mechanism involving G protein activation, protein tyrosine kinase activation or calcium influx.

The triggering of these receptor-coupled mechanisms is accompanied by activation of phospholipase C (PLC) and A2 (PLA_2) leading to functional expression of integrin $alpha_{IIb}$ $beta_3$ (“inside-out signaling”). Integrin $alpha_{IIb}$ $beta_3$ is a receptor for fibrinogen, a plasma protein that works as a bridge between activated platelets; it is this fibrinogen bridge that creates the platelet plug. Interaction of fibrinogen with integrin $alpha_{IIb}$ $beta_3$ also induces “outside-in signaling”, which promotes completion of the aggregatory responses leading to

granule release and exposure of phosphatydilserine. Among secreted molecules are ADP and serotonin, two important mediators of platelet aggregation. Serotonin additionally is a powerful and long-lasting vasoconstrictor. Phospholipase A2 activation leads to release of arachidonic acid, which is further metabolized by platelets into the unstable prostaglandin TXA_2, a potent vasoconstrictor and inducer of platelet aggregation. EC, on the other hand, metabolize arachidonic acid into prostacyclin (PGI_2), which is a potent inhibitor of platelet aggregation. Endothelial prostacyclin synthase is important in preventing the platelet plugs' continued growth inside the blood vessel and away from the injury site.

Phosphatydilserine, exposed by activated platelets, is a negatively charged phospholipid critical for amplification of the coagulation cascade through formation of coagulation complexes [9, 10]. Amplification of the clotting cascade results in additional thrombin formation, which not only cleaves fibrinogen to produce fibrin but itself promotes recruitment of activated platelets to the site of injury [9, 10]. The fibrin meshwork formed around the platelets further stabilizes and holds the platelet plug to the injury site.

Activated platelets additionally secrete a number of proinflammatory molecules through the so-called “release reaction” and may contribute to local inflammatory processes by a number of mechanisms [11, 12]. Among these proinflammatory molecules are chemokines such as transforming growth factor beta (TGF-beta), which display pro-apoptotic properties, and interleukin 1β (IL-1β), which activates white blood cells, induces tissue factor (TF) expression, and promotes adherence of neutrophils and monocytes [11, 12]. Platelets are a source of PAI-1, an important inhibitor of fibrinolysis [13]. Platelets also express P-selectin and promote neutrophil–platelet–endothelial cell interactions. Moreover, platelets are a major source of soluble CD40 ligand (CD40L) [14]. CD40L belongs to the tumor necrosis factor (TNF) superfamily of molecules and has multiple actions that may be of significance in microcirculation [14]. These actions include upregulation of cytokine expression on vascular smooth muscle cells and EC, increased expression of surface adhesion molecules on EC, and upregulation of TF on macrophages [15-20].

Platelets are thus initially activated by collagen and ADP. Within 4 seconds of vascular injury, platelet plugs can already be seen. Activated platelets release more ADP, together with serotonin, produce TXA_2 and lead to thrombin formation, all of which are part of an agonist-positive feedback loop to increase platelet aggregation. Serotonin and TXA_2 are also vasoconstrictors. Platelets also produce many other pro-inflammatory mediators. Prostacyclin formation by ECs prevents further spread of the platelet plug containing the hemostatic reaction to the injury site. The importance of platelets to vertebrates can be visualized by the fact that decrease of plasma platelets to less than 5% of normal levels leads to severe bleeding in human patients, even in the presence of normal levels of all clotting proteins.

Crude tick saliva or tick salivary extracts inhibit platelet aggregation *in vitro*. Because platelet aggregation is a redundant phenomena, it is not surprising that inhibitors targeting several of the platelet agonists are found, such as the enzyme apyrase—which hydrolysesADP and adenosine triphosphate (ATP) to adenosine monophosphate (AMP) and orthophosphate—in some ticks (apparently missing in Amblyomma), and secretion of platelet inhibitors such as prostacyclin and disintegrins, which are proteins with the arginine, glycine and aspartic acid (RGD) motif that prevent binding of fibrinogen to platelets and thus prevent activated platelets from being bound by fibrinogen [21-29]. Anti-clotting agents found in tick saliva prevent formation of thrombin that also activates platelets (see below). Triatomine bug saliva contains lipocalins that avidly bind adenosine nucleotides [30, 31]. Such ADP binding lipocalins in ticks are not yet described.

3.2. The blood coagulation cascade and its regulation

3.2.1. Initiation—The tissue factor-dependent activation of coagulation is also known as the 'extrinsic' pathway because TF has been traditionally reported not to be present in the blood [32]; although more recently this view has been challenged (see below in 'Blood-borne TF and microparticles') [33]. TF is abundantly present in the membranes of cells surrounding the vascular bed. Once in contact with the blood, circulating FVIIa binds to TF. The catalytic active complex formed by FVIIa/TF initiates the coagulation cascade through activation of FIX and FX [34-37]. It has been estimated that the complex FVIIa/TF enhances the efficiency of activation of its substrates, FIX and FX, approximately 10^4-fold versus FVIIa alone [10]. After activation, FIXa and FXa may remain associated with the TF-expressing cell or may bind to the membrane of platelets activated by low amounts of thrombin. Thrombin also activates FV and FVIII, and the generated FVa and FVIIIa serve as important co-factors to FXa and FIXa in the so-called intrinsic 'tenase' and 'prothrombinase' complexes, respectively [10]. These complexes assemble on the activated platelet membranes, which display negatively charged phospholipids such as phosphatidyl serine [9] (figure 1A).

3.2.2. Amplification—The prothrombinase complex activates prothrombin to thrombin in the presence of FVa, phospholipid surface and Ca^{2+}, while the main function of the intrinsic Xnase is to convert FX to FXa in the presence of FVIIIa and Ca^{2+} [9, 10, 38]. Both complexes convert several thousand substrate molecules per minute. In contrast, free FXa and FIXa are inefficient, indicating that many blood coagulation reactions are localized and occur on the surface of activated cells (*e.g.* activated platelets), not during the fluid phase [39]. For comparison, the amount of thrombin produced by the prothrombinase complex in one minute would require six months if produced by an equivalent concentration of FXa alone acting on 1.4 μM prothrombin solution [10]. It has been estimated that the fully assembled prothrombinase complex catalyzes thrombin generation at a rate that is at least 300,000 times more efficient than FXa acting alone. Notably, the assembled intrinsic Xnase complex on a membrane surface increases the rate of FXa generation approximately 10^9-fold more than that of FIXa alone [10, 32].

It should be noted that FVIIa/TF complex activates limited quantities of FX and FIX [40]. With the generation of FXa, the inhibitory effect of the protein named tissue factor pathway inhibitor (TFPI) takes place, preventing further production of FXa and FIXa by FVIIa/TF [35, 40]. Additional FXa can be produced only through the alternative pathway involving the intrinsic Xase complex, which helps to consolidate the coagulation cascade initiated by FVIIa/TF. This is achieved by FXI, which is efficiently activated to FXIa by thrombin when bound to the activated platelet surface [41-43]. FXIa generates FIXa, which triggers formation of sufficient amounts of FXa to propagate coagulation. Accordingly, the assembly of enzymes and co-factors on the phospholipid surface is a prerequisite for the propagation of the coagulation system and also counteracts regulation by anticoagulant mechanisms [9, 10]. Finally, thrombin is critical for formation of the fibrin clot, which is stabilized by thrombin-activated FXIII (FXIIIa), a transglutaminase that catalyses covalent crosslinkage of fibrinogen [44] (figure 1A).

A relatively large number of tick-derived anti-clotting agents have been reported in the past 20 years [45-63]. These include members of the Kunitz family of protease inhibitors [64], which may have single or multiple Kunitz domains. Multiple Kunitz domains allows interaction of the inhibitor with two or more partners, usually the Xase or the prothrombinase complexes, such as shown for Ixolaris [53, 59] in *I. scapularis*. Novel protein families have also been reported, such as the Salp9 and Salp14 of *Ixodes scapularis*

[62], showing the redundant recruitment of different protein families toward the same function, possibly a mechanism to achieve immune evasion.

3.2.3. Blood-borne tissue factor and microparticles—Recently, low but detectable levels of tissue factor antigen were found in normal plasma [65]. It has been proposed that TF in blood, so-called 'blood-borne' TF, contributes to clot propagation [33] and is associated with microparticles (which are membrane remnants) derived from EC, platelets, and leukocytes. Recruitment of microparticles to the propagating clot is dependent on endothelial P-selectin and microparticle P-selectin glycoprotein ligand-1 (PSGL-1) [66-69]. Because microparticles contain TF and phosphatidyl serine, which respectively generate FXa and thrombin, they presumably contribute to formation of a fibrin meshwork, platelet accumulation, and inflammation *in vivo* at sites of endothelium activation [70-73]. Microparticles are also increased under conditions associated with increased thrombotic risk [74, 75].

In addition to the salivary anti-clotting proteins discussed above, tick salivary gland extracts have been shown to modulate host adhesion protein expression, such as P-selectin [76].

3.2.4. Tissue factor and inflammation—TF is increasingly recognized as the interface of blood coagulation and inflammation and viewed as a critical player in mounting and sustaining an inflammatory response [15-20, 77, 78]. In fact, coagulation factors activate protease-activated receptors (PAR), which are typical seven-transmembrane, G-protein-linked receptors activated by a unique mechanism. Thrombin cleaves the aminoterminus of the PAR, allowing the internal ligand to autoactivate [79]. Of the four mammalian PAR, PAR1, PAR3, and PAR4 are activated by thrombin, while PAR2 can be activated by coagulation proteases FVIIa and FXa but not thrombin [79-81]. PAR connects coagulation proteases to cellular responses and represents one mechanism by which coagulation might affect inflammation [15, 75, 82-84]. In fact, PAR activation in a variety of cell types in and around blood vessels is associated with production of pro-inflammatory cytokines, expression of adhesion molecules and cell activation.

Tick salivary anti-clotting proteins that prevent formation of thrombin and factor Xa would prevent PAR activation. It remains to be determined whether inhibitors of the PAR receptor are present in tick saliva. Tick salivas also have various proteins that can bind and thus inhibit host inflammatory cytokines [85-90].

3.2.5. The 'contact' pathway—The 'contact' pathway is initiated when FXII changes conformation and is activated *in vitro* after interactions with macromolecular constituents of the subendothelial matrix such as glycosaminoglycans and collagens, sulfatides, soluble polyanions, or nonphysiologic materials such as kaolin [91]; however, the *in vivo* activator of the contact pathway is not known. Activated FXIIa, in a reaction involving high-molecular-weight kininogen (HMWK), converts plasma pre-kallikrein to kallikrein [91, 92]. Kallikrein acts on HMWK releasing bradykinin, a kinin involved in vascular permeability and inflammation. Importantly, bradykinin is also a powerful agonist of pain. Kallikrein itself is a potent activator of FXII, allowing for autocatalytic amplification of the initial stimulus. Additionally, kallikrein has chemotatic activity and also directly converts plasminogen to plasmin and C5 to C5a, which increases vascular permeability [92, 93].

FXIIa additionally activates FXI, which in turn activates FIX [41-43]. FIXa in the presence of FVIIIa and Ca^{2+} and PS is critical for FXa generation, as described above [9, 10]; however, the physiologic importance of the contact system in coagulation has been questioned, because deficiency of FXII [94-96], prekallikrein [97, 98], and HMWK [98, 99]

is not associated with bleeding diathesis. More recently, a role for FXIIa in stable thrombus formation has been reported in mice [100].

The contact pathway, which was previously wrongly thought to be an important component in the initiation of clotting, is, however, important for the activation of the kallikrein/kinin system leading to bradykinin and resulting pain. Although no inhibitors of Factor XII or kallikrein have been described thus far, the saliva of *I. scapularis* has a powerful enzyme that selectively destroys plasma bradykinin [28, 101].

3.2.6. Endogenous anticoagulant regulators of the clotting cascade—There are three major anticoagulant mechanisms in blood: TFPI, anti-thrombin III (ATIII), and protein C//thrombomodulin/activated protein C. TFPI is a bivalent, Kunitz-type inhibitor that prevents FVIIa/TF complex [102-104], and acts in a two-step manner. In the first step, TFPI inactivates FXa to form a TFPI/FXa complex. The TFPI within this complex then inactivates TF-bound FVIIa as the second step. Because the formation of the TFPI/FXa complex is a prerequisite for the efficient inactivation of FVIIa, the system ensures that some FXa generation occurs before FVIIa-mediated initiation of the coagulation system is shut down (figure 1B).

Antithrombin inhibits thrombin, FXa, and other activated clotting factors but these reactions are slow in the absence of heparin [102-104]. With heparin (presumably with heparan sulphate *in vivo*), the rate of inhibition is accelerated about 1,000 fold [105]. Heparin binds to antithrombin *via* its high-affinity pentasaccharide sequence and, by altering the conformation of the reactive center loop of antithrombin, renders the protease trap more accessible to target enzymes [106] (figure 1B).

Thrombomodulin is a thrombin receptor found on the endothelium. Once bound to thrombomodulin, thrombin undergoes a conformational change at its active site that converts it from a procoagulant enzyme into a potent activator of protein C (PC) [105, 106]. Activated protein C (APC), in the presence of its cofactor (protein S), serves as an anticoagulant by proteolytically inactivating FVa and FVIIIa, thereby attenuating thrombin generation [107, 108]. Protein S binds to the endothelial cell membrane and to PC, forming a cell-surface-bound complex [109]. Thrombomodulin is primarily located on the endothelial surfaces of capillaries within the microcirculation, but it is not present in the vessels of the brain [110]. On the other hand, the EC PC receptor (EPCR) is principally located on endothelial surfaces of larger vessels on small arteries and arterioles [111, 112]. APC is also known for its anti-inflammatory properties [113-116]. The PC system is physiologically very important, as is most clearly demonstrated by the severe thromboembolic disease associated with homozygous deficiency of PC [108, 117-119] or protein S [120]. Finally, heparin cofactor II is a protein that uses certain glycosaminoglycans such as dermatan sulphate as a cofactor in the inhibition of thrombin [121] (figure 1B).

Tick-derived products interfering with or mimicking antithrombin, protein S, protein C, heparin or thrombomodulin have not been described.

3.2.7. Fibrinolytic system—Fibrinolysis is initiated by plasminogen activators that convert plasminogen to plasmin. Plasmin in turn, degrades fibrin into soluble fibrin degradation products [122, 123]. Two distinct plasminogen activators are found in blood: tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA). Both plasminogen activators are synthesized and released from EC and bind to uPA receptor (uPAR). Plasminogen activation is targeted to fibrin because plasminogen and tPA bind to—and the enzymatic activity of t-PA is enhanced by—fibrin [122, 123] (figure 1C). The plasminogen activation system is also involved in extra-cellular matrix remodeling, cell

adhesion, migration, chemotaxis, mitogenesis and signal transduction [122, 123]. It is noteworthy that basophils, the predominant cell type that infiltrates the site of tick bites in the skin of hosts that do not permit efficient blood feeding [124], express uPAR (CD87) and are recruited by uPA [125]. Also of interest is the finding that IL-16 activates the plasminogen-plasmin system and promotes migration into extracellular matrix of eosinophils, another cell type that is conspicuous at the site of tick bites. [126].

The fibrinolytic system is regulated at two levels: PAI-1 blocks tPA and represents ~65% of the antifibrinolytic component of plasma. Regulation of PAI-1 activity is also important, and this is achieved primarily through its interaction with vitronectin, a cofactor that helps to stabilize PAI-1 molecules [13]. Another modulator of fibrinolysis is thrombin-activatable fibrinolysis inhibitor (TAFI), a carboxypeptidase that cleaves off the carboxyterminal lysines from fibrin. These lysines are important for the binding of fibrinolytic enzymes to fibrin, and TAFI activation results in inhibition of fibrinolytic attack (figure 1C). At last, two plasmin inhibitors modulate fibrinolysis, namely alpha$_2$-antiplasmin and to a lesser extent by alpha$_2$-macroglobulin [13, 127].

While tick proteins targeting the enzymes above have not been described, *I. scapularis* saliva has abundant metalloprotease activity with fibrin and fibrinogenolytic activities [128].

3.3 Angiogenesis and wound healing

Angiogenesis, the formation of new blood vessels, occurs as a result of the growth of capillaries by vascular sprouting from preexisting vessels [129]. Upon growth stimulation, quiescent EC can enter into the cell cycle, migrate, degrade the underlying basement membrane, and form a lumen. Angiogenesis is required for a variety of physiologic processes such as embryonic development and wound healing. Wound healing involves a dynamic and changing process that has been conveniently divided into three phases—inflammatory, proliferative, and remodeling [130] (figure 2). This process is continuous; the phases overlap, and different mechanisms occurring at different times trigger the release of chemical signals that modulate orderly migration, proliferation, and differentiation of cells and the synthesis and degradation of extracellular matrix (ECM) proteins. Of note, angiogenesis is generally considered to be a phenomenon that occurs during the proliferative phase of wound healing [129, 130]. This phase is critical for formation of granulation tissue, a hallmark of wound healing characterized by proliferation of EC, fibroblast accumulation, and collagen synthesis. Accordingly, granulation tissue provides nutrition, oxygen, and physical support for the tissue in repair [131].

Temporally, the first process that takes place as a reaction to a wounding is prevention of local hemorrhage. This is attained via platelet aggregation and activation of the hemostasis cascade. The resulting blood clot first contributes to stopping the bleeding and then functions as a provisional matrix for the wound healing that already begins approximately four days after injury. New capillaries endow the neostroma with its granular appearance. Leukocytes, fibroblasts, and blood vessels move into the wound space and each contributes to the wound healing process. The macrophages provide a continuing source of cytokines, which are necessary to stimulate fibroplasia and angiogenesis, a process in which new blood vessels are formed from pre-existing ones. During angiogenesis, the vascular basement membrane and the fibrin or interstitial matrix are degraded by ECs, upon which these cells start to migrate into the matrix and to proliferate by forming new capillary-like tubes [132]. Fibroblasts are important for the production of a new ECM, which is necessary to support the additional cell ingrowth [133]. Blood vessels play an important role in sustaining cell metabolism by providing oxygen and nutrients. The integrity of the granulation tissue depends on the presence of biological modifiers (*e.g.* lipid mediators and growth factors), the activity of target cells, and the environment of the extracellular matrix (ECM) [134].

These processes are regulated by growth factors that may come from the plasma but can also be released by activated platelets in the wounded areas. Infiltrated peripheral blood monocytes and macrophages are also sources of the synthesis and release of growth factors. In addition, growth factors can be synthesized and secreted by injured and activated parenchymal cells. The newly formed temporary fibrin matrix also promotes granulation tissue formation. Once fibroblasts and ECs express the proper integrin receptors, they invade the fibrin/fibronectin-rich clot in the wound space and start synthesizing a permanent ECM [reviewed in [131]].

It is important to recognize that angiogenesis is a complex process, in which ECs are stimulated by growth factors, such as VEGF or FGF-2, to proliferate and migrate into the ECM to form new capillaries. The invasion of the ECM by EC is controlled by proteolytic enzymes of the plasminogen activator/plasmin system and the matrix metalloproteinases [135]. The cells proliferate and elongate, and vessel stabilization is finally achieved by interaction with pericytes and reconstitution of the basement membrane [129, 130]. Integrins such as alpha$_{pi\ b}$ beta$_{3,}$ alpha5beta1, alpha vbeta 3, and alpha vbeta5, among others, play an important role in wound healing and angiogenesis by facilitating binding of EC to ECM proteins such as fibrin(ogen), vitronectin, and fibronectin [136].

Tick saliva has anti-angiogenic properties [137, 138]. This is a neglected area of tick research where many host targets abound. It is possible that some growth factors may be targets for salivary binder proteins, as occurs with host cytokines and chemokines.

3.4 Ticks and immunity: an overview

To complete their blood meal, ticks remain attached to their hosts for periods that can vary from minutes to a few hours in the case of soft ticks (Argasidae) or several days in the case of hard ticks (Ixodidae). It is reasonable to assume, therefore, that different feeding strategies profoundly influence the host immune response upon infestation. Although these strategies evolved in different ways for these arthropods, ticks have to deal with innate immunity (inflammation) in primary infestation and with both innate and adaptive immunity during secondary or subsequent infestations. The immunologic basis of tick resistance manifested by decreased yield, weight, fecundity, and/or fertility of the parasites has long been recognized and well demonstrated in some mammalian species infested with ixodid ticks, although it is difficult to demonstrate in fast-feeding argasid ticks.

The challenge to the immune system begins even before contact with host blood, in the moment that tick mouthparts are inserted into the skin, causing the breaking of this physical barrier. Resident leukocytes of epidermis and dermis, such as mast cells, eosinophils, dendritic cells, and macrophages, as well as keratinocytes are the first to make contact with tick mouthparts and saliva. These cells release pre-formed mediators in addition to producing chemoctatic factors to recruit inflammatory cells such as neutrophils to the attachment site. Subsequent infestations may activate adaptive responses involving T cells and B cells by production of antibodies and sensitization of mast cells and basophils that, in conjunction with eosinophils, are predominant cells in the tick attachment site. Whether this response will confer host resistance will depend on such factors as host genetic background, health status, and host and tick species involved. The role of cells—the mediators involved in the immune response during tick infestation—as well as that of ticks ability to circumvent such immunity is further discussed.

3.4.1. Mast cells—Mast cells are distributed throughout the body and are present in connective tissues, especially beneath epithelial surfaces exposed to the external environment, such as skin. These cells present granules with a range of pre-formed mediators, such as vasoactive amines, proteoglycans, serine proteases, sulfatases, and

cytokines. Upon activation triggered by degranulation process, they are also able to produce newly synthetized mediators such as growth factors, chemokines and lipid mediators that recruit inflammatory cells to the injury site [139].

Histologic examinations of *Dermacentor variabilis*-infested skin of BALB/c mice revealed increased numbers of mast cells during secondary and tertiary infestations but not in primary infestation [140, 141]. Furthermore, a higher number of degranulated mast cells are observed in *I. ricinus*-infested rabbits and *Hyalomma anatolicum anatolicum*-infected rabbits and cattle upon reinfestation rather than in primary infestation [142-144]. Nevertheless, the role of mast cells in the development of tick resistance is still unclear. Mast cell-deficient mice are able to develop some degree of resistance after repeated infestations with *D. variabilis*, as are their wild-type counterparts [141]. Contradicting these results, mast cell deficiency was found to abrogate resistance to *Haemaphysalis longicornis* [145], while intracutaneous injections of cultured mast cells in these mice recover their ability to reject ticks [146]. In accordance, zebuine cattle breeds, known as highly resistant to *Rhipicephalus (Boophilus) microplus*, have more dermal mast cells than taurine breeds, and infested F_2 crossbred cattle present acquired resistance to tick infestation associated with increased mast cell numbers in the dermis [147]. Whether the importance of mast cells for anti-tick immunity is a universal truth or not, remains to be determined. It is important to associate the fact that histamine release by mast cells produce pruritus and triggers scratching host behavior that might dislodge the tick from the feeding site. The importance of histamine to tick feeding is underlined by the possibly universal existence of histamine-binding tick salivary lipocalins, that efficiently sequester this biogenic amine away from their receptors [148-151].

3.4.2. Eosinophils—Eosinophil blood levels are generally quite low in the absence of parasitic infection or allergic reactions such as atopic dermatitis. However, most of the eosinophil population is found in the tissues, predominantly at the surfaces of the body that interact with the external environment, such as skin and mucosal surfaces of the gut, respiratory and reproductive systems. Eosinophils are the source of several cytokines, chemokines and lipid mediators and the most important source of indoleamine 2,3 deoxygenase (IDO), an enzyme induced by interferon gamma that inhibits the T helper 1 (Th1) subset of lymphocytes [152]. Furthermore, their granules are rich in cytotoxic granules containing eosinophil peroxidasse (EPO), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and major basic protein (MBP). This last one is a mast cell (and probably basophil) degranulation factor, displaying a positive feedback in the responses associated with these cells [153]. Finally, eosinophils are a very important source of molecules involved in tissue repair and inflammation, such as tenascin, TGF-alpha and TGF-Beta1 [154, 155].

In guinea pigs, resistance to repeated infestation with hard ticks is associated with accumulation of eosinophils and basophils in the attachment site. In fact, high numbers of eosinophils were found after repeated infestation of guinea pigs with *Amblyomma americanum* [156, 157] and *Rhipicephalus appendiculatus* [158], but were virtually absent in the dermis of *Ixodes holocyclus*-infested guinea pigs in the primary and secondary feeding [159]. Soft ticks also induce a strong eosinophil response in guinea pigs [160]. Other species, such as cattle [161], bovines [162], rabbits [142], dogs [163], mice [164], and even capybaras [165] present similar eosinophil infiltration in the attachment site upon repeated hard-tick infestation. An anti-eosinophil serum conferred only a partial lack of resistence in tick-sensitized guinea pigs [166]. However, the absence of studies employing infestation of IL-5-deficient, or anti-IL-5 treated animals (which lack eosinophil response) does not permit a definite role to be assigned to eosinophils in tick resistance.

3.4.3 Dendritic cells—Dendritic cells are professional antigen-presenting cells that initiate the adaptive immune response to invading pathogens, and are found in two distinct functional states. Immature dendritic cells are located in non-lymphoid tissues, such as skin and mucosa, and their primary function is to uptake antigens. In contrast, mature dendritic cells are poorly phagocytic but highly efficient stimulators of naïve T cell responses [167].

Dendritic cells do not cause direct damage to ticks but seem to be important in the generation of the acquired immunity which, in turn, leads to resistance against the tick. Evidence for interactions of tick saliva with dendritic cells was demonstrated in studies where epidermal cell population containing Langerhans cells (the immature dendritic cell population of skin) were incubated *in vitro* with tick saliva. These cells, loaded with salivary antigens, were able to stimulate proliferation of T lymphocytes obtained from the lymph node of bite-sensitized, tick-resistant guinea pigs [168]. This indicates that dendritic cells are presumably capable of priming an immune response *in vivo* during tick infestation. That assumption is supported by an earlier study that identified the presence of antigens from ticks' salivary glands associated with Langerhans cells [169]. Another study showed that during primary infestations with *Dermacentor andersoni*, there is a decrease in the number of these cells around sites of tick attachment [170]. This suggests that Langerhans cells probably migrate to lymph nodes, after contact with components from saliva, to activate T cell responses. In addition, the acquisition resistance was abrogated with ticks fed on ultraviolet-irradiated skin, presenting a marked reduction in ATPase-positive Langerhans cells [171]. More recently, tick saliva has been shown to affect several dendritic cell functions [172-174]. These results are consistent with the concept that dendritic cells are active components in immune response to saliva and potentially modulate tick resistance.

3.4.4. Macrophages—Most resident macrophages in tissues originate from circulating bone marrow-derived monocytes. Macrophages are found in different organs, such as liver (Kupffer cells), lung (alveolar macrophages), nervous system (microglia), epidermis (Langerhans cells), reproductive organs, serosal cavities, gut lamina propria and the interstitium of the heart, pancreas, and kidney. In response to inflammatory and immune stimulation, additional monocytes are recruited in increased numbers to the site, displaying different phenotypes from originally resident macrophages [175]. Studies of the differentiation antigens and surface receptors expression have shown that macrophages become markedly heterogeneous and express very different phenotypes, reflecting specialization of function within particular microenvironments. Thus, macrophages differ in terms of receptor expression, cytokine production, effector function and chemokine repertoires [176].

Unfortunately, most of the studies describing the histologic features during tick infestation have focused on the neutrophil, eosinophil, basophil and mast cell infiltration in the tick-bite site [160, 162, 177]. Macrophages are usually classified as mononuclear cells [144, 178-180], which also include lymphocytes, making difficult a precise analysis of the participation of these cells in the inflammatory infiltrate that follows tick attachment and of their putative role in resistance to tick infestation. However, few studies described the presence of macrophages (and monocytes) in both feeding cavities and the area around the lesion of the first tick infestation. In general, macrophages and monocytes were found to be slightly increased in the feeding cavities, but not in the adjacent tissue during the two or three first days of infestation with larvae, nymphs and adults of *A. americanum* [181, 182].

Wikel et al. [183] examined the specific *in vitro* proliferative responses to tick salivary antigens in lymph node cells from guinea-pigs undergoing primary and secondary tick infestations. At that time, it was assumed that macrophages were acting as the antigen-presenting cells that elicited a potent proliferative response observed during secondary

infestation. In fact, macrophages were shown to have at least the same ability in presenting salivary antigens to lymphocytes as enriched populations of Langerhans cells [168]. Despite the fact that this role is nowadays recognized as being that of dendritic cells, macrophages remain an important resident cell producing cytokines and chemokines that attract inflammatory cells to the tick-bite site.

3.4.5. Neutrophils—Neutrophils are highly motile phagocytic cells that constitute the first line of defense of the innate immune system. Neutrophils engulf and degrade microorganisms and produce several chemokines—as well as major pro-inflammatory cytokines— indicating that, in addition to killing microorganisms, these cells are important in influencing early cell trafficking and activation during pathophysiologic processes. In a general line, neutrophils are stimulated by the aetiological agent to produce chemokines that subsequently serve an instrumental role in recruiting other leukocyte types during the early phases of infection [184].

Some earlier studies suggest that the feeding lesions of ticks result from extra-oral digestion, through the action of cytolysins in their salivary secretions [185]. However, an accurate study of the feeding lesions caused by *R. microplus* suggested that tissue damage is caused by the host response [186]. In fact, both collagen destruction beneath the tick mouthparts and the intense neutrophil infiltrate associated with this lesion were prevented by treatment of *R. sanguineus*-infested dogs with nitrogen mustard, a drug used to deplete neutrophils [187]. Later, it has been reported that tick salivary gland extract is not chemotactic by itself, but generates a neutrophil chemoctatic factor by the cleavage of C5 [188]. In fact, neutrophils are the most abundant cells in the acute inflammatory infiltrate induced by the primary infestation but not in subsequent infestations of all species of hard ticks studied [143, 158, 159, 179]. Despite a recognized role of neutrophil against pathogens and their extensive presence in the primary infestations, it is not known whether their absence would affect host-tick resistance. Saliva of *I. scapularis* was shown previously to inhibit neutrophil aggregation, granule release and phagocytosis of *B. burgdorferi* [189], but the salivary molecules determining these actions remain to be identified.

3.4.6. Basophils—Basophils are the third cell type derived from the granulocytic lineage that also includes neutrophils and eosinophils. Normally confined to the circulation and not found in normal tissues, the role played by basophils in immunity against parasites is surrounded by controversy. Despite the similarities with mast cells in terms of morphology and the classical IgE-dependent activation, basophils are much more restricted with respect to the mediators stored relative to the mast cells and their ability to kill invading pathogens is also diminished in comparison with other granulocytes [190]. Basophils, however, have long been documented as the predominant cell type that infiltrates the tick-bite site in the skin [191] and recognized as important effectors in tick rejection [192], mostly in guinea pig models and in bovines [124, 142-144, 166, 191].

The migration of these cells and the resulting basophil-rich responses in the tissue are known as cutaneous basophil hypersensitivity (CBH). The degranulation of basophils and local release of mediators is believed to represent an essential part of the resistance response to ticks [193]; histologic studies have suggested that CBH responses are associated with immune skin rejection of blood-feeding ticks. When ticks attempt to feed on the skin of sensitized animals, an intense CBH response is usually elicited that contains a massive infiltrate of basophils. This reaction ultimately causes rejection and killing of ticks by hosts that present significant levels of histamine in their body; this rejection is reduced by *in vivo* administration of H_1 and H_2 receptor antagonists [194]. Of interest, guinea pigs develop resistance to tick feeding after a single tick exposure and basophils are found to be present in their skin upon infestation with virtually all hard tick species studied up to date [195]. For

soft ticks, basophil infiltration was also demonstrated in *Ornithodoros parkeri*-infested guinea pigs [160], *Argas polonicus*-infected pigeons [196], and *Argas verpertilionis*-infested bats [197].

Tick resistance can be conferred by adoptive transference of serum and/or peritoneal exudate cells to naïve animals and, in both cases, CBH responses are present after transference [179, 198]. In addition, anti-basophil serum treatment of *Amblyomma americanum*-sensitized guinea pigs abrogated tick rejection [166]. For some time, mice were reported to be lacking basophils [192] but actually these cells are rare and difficult to identify in these animals [199]. Murine tick resistance was also demonstrated after repeated infestations and it was initially associated with mast cell degranulation on the attachment site [140]. Nonetheless, mast cell-deficient mice also developed tick resistance and the presence of basophils in the skin reactions of these animals was demonstrated by electron microscopy, suggesting that basophils must also contribute in the pathogenetic mechanisms in these hosts [177]. Unfortunately, since basophil-specific developmental pathways are not well understood [200], none of the currently available gene-deficient animals are likely to elucidate the protective role of basophils on tick-infested hosts.

Of interest is the fact that tick salivary glands express an insulin-like growth factor-binding lipocalin [201]. IGF-1 has been shown to be a selective chemotactic factor for basophils [202] and skin gamma-delta T lymphocytes are an important source of this chemokine, which also regulates proliferation of keratinocytes [203].

3.4.7. T cells—T cells derive from lymphoid progenitors in hematopoietic tissue of bone marrow and undergo differentiation in the thymus. Mature T cells are then seeded to the peripheral lymphoid tissue that virtually covers the whole body (lymph nodes, spleen, mucosal and gut-associated lymphoid tissues, and others), and also to the recirculating pool of lymphocytes. Based on their T cell receptor (TCR), these cells are subdivided into alpha/beta and gamma/delta cells, alpha/beta being the majority of them. Among alpha/beta T cells, two subpopulations are described according to the co-receptor molecule expressed in their surface, $CD4^+$ T cells and $CD8^+$ T cells, which differ in how they recognize antigens and mediate effector functions. According to the cytokine profile they secrete, $CD4^+$ T cells can differentiate into Th1 and Th2, associated with cellular (e.g. macrophage activation) and humoral (e.g. antibody production) specific immune response, respectively. On the other hand, $CD8^+$ T cells can develop into cytotoxic T-lymphocytes (CTLs) capable of lysing target cells. Despite T cell role in the adaptive immunity, TCR does not recognize antigenic determinants on intact, undenaturated molecules, and T cells themselves do not initiate the specific immune response. Instead, T lymphocytes are activated by interaction with major histocompatibility complex(MHC)/peptides on the surface of antigen presenting cells. Thus, T lymphocytes may represent the principal sensory arm of the adaptive immune system [204].

Acquired resistance can be transferred from tick-resistant donors to normal recipients with viable lymph node cells, suggesting that T cells are involved in resistance, which is associated with cutaneous basophil hypersensitivity reactions to tick antigens [205]. In fact, cutaneous basophil hypersensitivity reactions could be inhibited by anti-T-cell serum in guinea-pigs [206]. Maximal *in vitro* proliferative response to tick salivary antigens was found in lymph node cells from guinea pigs undergoing secondary tick infestation, when tick resistance had been acquired [183]. In agreement, column purified lymph node cells (depleted of adherent cells) from resistant, but not from control, guinea pigs presented specific proliferation when incubated with Langerhans cell-enriched population or macrophages in the presence of salivary gland extract [168]. Langerhans cells trap tick salivary gland antigens in the skin and function as antigen presenting cells for T-

lymphocytes [168, 170]. Anti-tick immunity, however, is regional and only cells from the lymph nodes which drain the tick attachment site are able to proliferate *in vitro* in the presence of tick antigens [207, 208]. An immunohistochemical analysis of mouse skin at 72 h after *Ixodes ricinus* attachment revealed that $CD4^+$ and $CD8^+$ T cells are present in a proportion of 2.2:1 in the primary, 3.2:1 in the secondary and 4.7:1 in the tertiary infestations. These histological findings together with the IL-2, IL-4 and IFN-gamma mRNA expression are in accordance with the mild cutaneous delayed-type hypersensitivity observed in tick-infested mice [209].

3.4.8. B cells—B cells originate from the same lymphoid progenitor as T cells, but their development follows distinct steps. Short lived immature B cells migrate to the spleen by the bloodstream, where they develop into long-lived mature B cells. At the end of this process, mature B lymphocytes expressing immunoglobulins (or antibodies) in their surface, which are the B cell receptor (BCR), recirculate among the lymphoid follicles of spleen and lymph nodes. Upon activation, these cells differentiate into antibody-secreting cells [210].

Antibody production is one of the host's acquired responses to the salivary secretion introduced into the feeding site. Despite the absence of B cells in the skin of infested hosts [209], lymph nodes draining infestation sites are increased upon primary and secondary infestations in comparison with lymph nodes from non-infested animals [211] and present germinal centers [156], suggesting proliferation and differentiation of B cells. In agreement, the lymph node cell response to *in vitro* lipopolysaccharide (LPS) stimulation is more pronounced in infested mice than in controls, suggesting again an increase in either the proportion or activity of B cells of these mice [208, 212]. In fact, cyclophosphamide treatment in a dosage reportedly selective for B lymphocyte depletion, has been shown to partially block guinea pig resistance to *D. andersoni* [213]. Regardless of the uncertain role of antibodies in the tick resistance, rabbits infested with *H. leporispalustris* were shown to produce homocytotropic antibodies [214]. Mast cell and basophil degranulation observed in the secondary (and subsequent) tick infestations are dependent upon sensitization of these cells with antibodies after the primary contact with these parasites. However, the acquired resistance of rabbits infested with *I. ricinus* adults was not directly related to the levels of anti-tick antibodies in these animals [215]. Studies on the passive transfer of resistance by serum range from no resistance [216] to significant [178, 198, 217, 218] and strong resistance [205]. It has been suggested that variations in the guinea pig strain, serum dose, or route of administration would explain such differences.

Some of the studies demonstrating serum-associated tick resistance also show the cutaneous basophil response that accompanies this immunity. In some cases, immune serum-treated guinea pigs present a predominant mononuclear cell infiltrate, weaker basophil infiltrate and no eosinophil changes in comparison with actively sensitized animals, which infiltrate is rich in basophils and eosinophils [178]. In others, immune resistance and strong cutaneous basophil responses were transferred to naïve animals by IgG1 antibody fraction from guinea pigs sensitized with *A. americanum* larval ticks [219], but not *R. appendiculatus* [220]. For both species, however, resistance was demonstrated to be dependent on the host Fc receptors on the cell surfaces, perhaps skin mast cells and blood basophils [220, 221].

Tick infestation can affect or modulate antibody production by their hosts. After heavy tick infestations in bovines of a tick-resistant breed, levels of IgG1 and IgG2 anti-saliva antibodies remained unchanged relative to levels produced during less intense infestations, but decreased significantly in a tick-susceptible breed [222]. Cattle heavily infested with *R. microplus* show diminished responses to an extraneous antigen when compared to controls [223] Saliva from the tick *I. ricinus* inhibits secretion of IL-10 by activated murine B cells as well as proliferation of B cells stimulated with LPS or *Borrelia burgdorferi* outer surface

protein [224, 225]. These findings suggest that the humoral response is suppressed by tick saliva, at least in mice and in genetically susceptible bovine breeds. The fact that saliva from the male tick contains immunoglobulin G-binding proteins [226] indicates that the antibody response is an important immune effector mechanism against ticks. It is noteworthy that, in contrast to IgG, an IgE-binding protein has not been described in ticks. Indeed, heavy tick infestations are followed by an increase in anti-tick saliva IgE in genetically susceptible, but not in resistant bovine breeds [222], indicating that IgG is the important isotype in anti-tick humoral responses.

3.4.9. Molecular communication between the different cell types: complement, cytokines and chemotactic factors—The ability of the immune system to recognize and respond to the presence of foreign antigens and tissue damage, as it happens during tick feeding, depends on the production and release of several mediators, including those produced by the activation of the complement system, and by cytokines.

The complement system, like the clotting system, is a proteolytic cascade comprised of at least 35 different proteins (including enzymes and their regulators) that can be initiated by four different mechanisms and converge to the assembly of a lytic complex, usually on the surface of a foreign organism [227-230]. The classical pathway is initiated by complement component C1q that binds to specific subclasses of antibodies bound to their antigens (immunocomplexes), or to C-reactive protein; the alternative pathway is initiated by the assembly of the C3 convertase on usually foreign carbohydrate surfaces, assisted by factors B and D; the lectin pathway is initiated by binding of lectins (such as mannan binding lectin or MBL) or ficolins to foreign carbohydrate surfaces leading to the activation of the MBL-associated serine protease (MASP), and the fourth pathway, recently discovered, is initiated by activation of C5 by thrombin [231]. These different processes converge to the formation of the C5 convertase, generating two C5 convertases, C4b2b3b and C3bC3bBb. These convertases can cleave C5 producing one C5b and one smaller C5a anaphylatoxin molecule discussed below. Association of C5b with C6, C7, C8 and C9 ultimately leads to the generation of the lytic membrane attack complex (MAC) that might kill the pathogen.

The smaller cleavage peptides of C3 and C5, C3a and C5a, are known as the anaphylatoxins, having inflammatory, edema producing, and chemotactic properties [232, 233]. The alternative pathway of complement activation was shown before to be important in tick rejection reactions by guinea pigs [234, 235], perhaps by the production of the inflammatory anaphylatoxins. In fact, the cleavage of host's complement protein C5 by component(s) of *D. variabilis* saliva has been demonstrated, generating chemoctatic fragments for neutrophils [236]. The first characterized molecule displaying such activity was an *I. scapularis* anticomplement protein (Isac) [237], but several other proteins sharing homology with this former one were identified constituting a large family of anticomplement molecules [238]. From those, Salp20 [239], and the *I. ricinus* orthologues IRAC I and IRAC II [240] were recently cloned and characterized. On the other hand, soft ticks have a lipocalin that binds and inhibits the C5 convertase thus inhibiting both the classical and the alternative pathways [241]. Ticks also evolved mechanisms to neutralize histamine released during basophil and mast cell degranulation, and other activities triggered by complement-derived peptides. For exemple, *I. scapularis* saliva inhibits neutrophil function and aggregation [189] and also has an anaphylatoxin inactivator, possibly an yet unidentified carboxypeptidase [242]. The first histamine blocking activity found in ticks was demonstrated in *R. sanguineus sanguineus* salivary gland homogenate [150, 151]. This spectrum has been amplified by the discovery of new members of the lipocalin family in several genera of hard and soft ticks with putative and demonstrated histamine-binding activity [148].

Phagocytic cells such as macrophages have receptors for complement components, thus pathogens with complement molecules bound to them are avidly phagocytosed. These complement receptors also triggers signalling cascades when bound to their ligands, affecting their physiological status [243].

Cytokines are proteins released by cells that affect the behavior of other cells that bear receptor for them. They represent an important group of molecules that regulate all aspects of the inflammation and adaptive immunity acting in an autocrine manner (affecting the cell type that produced them), paracrine manner (affecting adjacent cells) or endocrine manner (affecting distant cells, sometimes at systemic levels) [244]. These molecules act in conjunction with chemotactic factors, which recruit specific leukocytes to the “right place” and activate these leukocytes at the “right time”. It is currently accepted that cell migration in response to chemoattractants features many sequential interactions involving adhesion molecules and glycosaminoglycans (GAGs) as well as the chemoattractant ligands and their G protein-coupled receptors (GPCRs) [245]. The adhesion molecules will not be discussed in this text, and a more detailed view on this subject can be found elsewhere [246, 247]. In fact, they seem to be important for tick feeding, as ticks are found to down-modulate their expression [76, 248]. To date, no work has been conclusive about the complex network of inflammatory and immune processes or the relative importance of each cell type during the tick feeding and resistance development. However, based on the activities of saliva and salivary gland extracts on the host immunity, and on the role of salivary molecules that are now being faster identified, some clues about the cell migration and its participation in primary and subsequent tick infestations may be inferred.

Upon primary infestation with several hard-tick species, the cellular infiltrate observed is typically constituted by more than 50% of neutrophils. Taking *A. americanum*-infested guinea pigs as a representative example, significant presence of neutrophils is found in the feeding cavities as early as 12 hours after tick attachment. These cells peak in the feeding cavity at different time points, depending on the stage of development, typically 24 h for larvae, 48 h for nymphs and 96 h for adults [181, 182]. Other cell types have also been found to be increased in the cavity, but far less than neutrophil population, in the following order: eosinophils, monocytes/macrophages and mast cells. Few or no lymphocytes are detected in the skin upon primary infestation.

The cellular infiltrate following secondary and subsequent infestations is contrastingly different. Keeping the *A. americanum* infestation as the example, one may observe in the site of secondary infestation only a small number of neutrophils (less of 5% total). Eosinophil and mononuclear cells absolute numbers are increased in comparison with the primary infestation, but represent approximately the same percentage (~ 20% each) of the whole cell infiltrate. Basophils, however, which are virtually not present in the first days of primary infestation are the predominant cell type (more than 50%) in the secondary infestations [179]. This latter response is associated with resistance against ticks. Since tick salivary gland extracts presented no intrinsic chemotactic activity, this pattern suggests an unspecific inflammatory reaction in the primary infestation and, possibly, an immune-driven cellular infiltrate in the subsequent infestations.

Chemokines are a superfamily of small proteins that function as chemotactic factors by directing cellular migration. The term was coined by the fusion of *chemoattractant* and *cytokine*, which describe the properties identified for many of these molecules. The cellular migration occurs along chemokine gradients, and changes in chemokine-receptor expression by leukocytes are a key mechanism for regulating the homing of these cells to various tissues. Specificity patterns of responsiveness to different chemokines have been identified, determined by the expression of the suitable chemokine receptors [259]. Despite the

extensive chemokine and their receptors nomenclature standardized few years ago [260-262], there is now some reliable knowledge about the expression of each receptor on the surface of most cell types [176]. Classically, IL-8 (CXCL8) is the major mediator of neutrophil migration while eotaxins (CCL11, CCL24 and CCL26) are implicated in eosinophil chemotaxis. Mast cells and basophils also present receptors for these chemokines [263-265]. Given the overlap in the chemokine receptors expressed by these cells, it is not surprising that they share recruitment pathways, as observed in the tick attachment site (mostly for eosinophils, mast cells and basophils). Neverthless, some of these pathways are selective and distinct [266], which explains the chronological differences in the presence of each cell type observed in histologic studies of the tick attachment site. Given the localization of neutrophils, mast cells, basophils and eosinophils within inflamed tick bite sites, it is important to understand how these cells are preferentially recruited and concentrated in the tissues. Unfortunately, there are no studies about the behavior of ticks in gene-knockout hosts for chemokines or their receptors. However, the presence of Evasin-1, the first chemokine-binding protein characterized in *R. sanguineus* [88], and anti-chemokine activity for IL-8 (CXCL8), MCP-1 (CCL2), MIP-1α (CCL3), RANTES (CCL5), eotaxin (CCL11) in the *D. reticulatus*, *A. variegatum* and *I. ricinus* salivary gland extracts [85] strongly suggest the importance of the chemokines in the host-tick interface.

The ultimate control of the inflammation and immune response is the modulation of the cytokine network involved in the tick immunity. In fact, *in vivo* and *in vitro* modulation of the cytokine production by tick saliva or during tick infestation has been demonstrated. Macrophage production of the inflammatory cytokines IL-1 and TNF-alpha was decreased in the presence of salivary gland extracts from different tick species [267, 268]. Dendritic cell production of IL-12 and TNF-alpha was also strongly decreased in the presence of tick saliva [172, 174]. Lymphocyte production of IL-2 and IFN-gamma were diminished in the presence of *D. andersoni* salivary gland extracts [267]. Tick saliva inhibited Con A-induced [174] and OVA-induced IL-2 production by T cells [172]. In addition, splenocytes from mice infested with *I. scapularis* nymphs presented decreased production of IL-2 and IFN-gamma and increased production of IL-4 and IL-10 when stimulated with concanavalin A [269, 270], suggesting that tick infestation polarizes adaptive immune response to a Th2 profile [271]. Despite the extensive literature on the tick modulation of cytokine production, the characterization of molecules controlling such activities is still in the beginning, but an increasing number has been characterized in the last few years. For example, an *I. ricinus* immunossupressor (Iris) has been shown to inhibit the production of several inflammatory cytokines [272]. An IL-2 binding protein was described in *I. scapularis* saliva, which decreases the availability of this cytokine and affects T-cell proliferation [90]. Salp15, another salivary protein described in *I. scapularis* is a ligand of CD4, preventing the activation of these cells [273]. Prostaglandin E_2 (PGE_2) found in tick saliva inhibits maturation of dendritic cells and, consequently, their effector function in activating T cell proliferation [172].

3.4.10. Cytokines, pain, and itching responses—It must now be clear to the reader that the artificial compartmentalization of immunity and inflammation is not followed in nature. The classical signs of inflammation, redness and heat (vasodilation), turgor (edema), pain and loss of function (by excessive edema and/or pain) involves components artificially defined as hemostasis, inflammation and immunity, and can be produced by both humoral and cellular responses. A successful tick should properly maintain the feeding cavity otherwise it might become a pus cavity, or contain only a relatively protein-poor edema. Additionally, the successful tick should be invisible or unapparent to its host, because tick rejection reactions often are assisted by host behavior, which is triggered by pain or itching at the site of the infestation.

To the tick, the inflammatory vasodilatation is actually beneficial, ticks themselves secreting vasodilators such as PGE_2 and PGF_{2alpha} [274-276]. The edema can be brought about by humoral reactions, such as activation of the kallikrein-kinin system and the complement system (in the form of C3a and C5a), and by cellular systems through serotonin and histamine released by platelets, basophils and mast cells, as well as leukotrienes produced by neutrophils, mast cells and basophils. All these mediators increase vascular permeability (by changes in the capillary EC that allows fluid to filter from inside the capillary to the skin interstitial matrix. Whether mast cells and basophils are going to degranulate or not, will depend on whether they are sensitized with the proper Ig subclass. Vasodilators such as PGE_2 potentiate the edema because it increases the perfusion pressure at the capillary level. Accordingly, a purely “liquid” edema may occur. However, activated ECs produce receptors that increase adhesion of leukocytes that can then migrate throughout the spaces between ECs and accumulate in the skin matrix, thus creating a cellular component of the edema as well. All the chemokines mentioned in the above sub-chapter will play a role in this system, determining the cellular composition of the infiltrate. Tick salivary proteins can bind and neutralize several cytokines and chemokines, as indicated above. No tick leukotriene inactivator has been found thus far.

The fifth and last component of inflammation to be considered, loss of function, is associated with the conscious perception of the affected site that can be perceived as itching, burning or pain. To the tick, or any blood-feeding arthropod, this can be a most challenging reaction. Indeed behavioral responses of scratching can determine the success or failure of a successful infestation. Suffice to say that the experimental model of infestation of lice in mice requires the amputation of one of the rear legs of the rodent, allowing successful infestation of the ear on the same side of the amputation, and that a rabbit collar is normally used in the laboratory to allow ticks to successfully feed in the rabbit's ear. One of the first mediators of pain is ATP, released by injured cells. Serotonin and histamine, released by platelets and mast cells, are also inducers of pain and increased vascular permeability, as is bradykinin, which is produced following activation of factor XII by tissue-exposed collagen [277]. Activated factor XII converts prekallikrein to kallikrein, which hydrolyzes blood kininogen to produce the vasodilatory peptide, bradykinin. Note that many of the hemostatic mediators are linked to pain production in inflammation. Perhaps evolved to also reduce pain, saliva of ticks can contain enzymes that destroy ATP [22, 24, 28], lipocalins that sequester serotonin and histamine [149, 150], and peptidases with high specificity for bradykinin [101, 242].

Polymorphonuclear cells and monocytes are important mediators of inflammation, including pain. ATP, released by injured cells, activates neutrophils that accumulate and degranulate at the injury site [278, 279]. Activation of neutrophil and other cell types is also accompanied by generation of prostaglandins that potentiate the pain induced by bradykinin [277]. Pain is also induced by cytokines such as IL-1 generated by neutrophils. Therefore, several molecules work in concerted manner to generate pain [280]. In this regard, bradykinin induces TNF-alpha release from neutrophils [281, 282], which in turn stimulates the release of IL-1^{-} and IL-6 from various cell types including those of the phagocyte mononuclear system. These cytokines contribute to the phenomenon of increased sensitivity to pain, or hyperalgesia, that accompanies inflammation. Cytokine-mediated inflammatory hyperalgesia is accompanied by production of cyclo-oxygenase products and IL-8 released by monocytes, macrophages, and ECs, stimulating the production of sympathomimetic mediators also involved in increased pain reception [283, 284]. All these cytokines and mediators, therefore, could be targets of tick salivary molecules playing an adaptive function in tick feeding. Indeed, anti-TNF and anti-IL-8 activities, among other cytokine-binding activities, have been described in tick saliva [85, 86, 89, 285]; *I. scapularis* saliva contains a kininase enzyme [28, 101].

4.0. TOWARD THE TICK SIALOVERSE

Transcriptome and proteome advances of the past six years have opened an unprecedented window into the variety of tick salivary transcripts and proteins. Three main surprises arose with this approach. The first was that saliva has a much broader complexity than anticipated, having hundreds of different proteins, many of which are novel, in the sense that they produce no similarities to other proteins in large databases, such as the nonredundant (NR) database of the National Center for Biotechnology Information (NCBI). Another surprise is that the most abundant tick salivary proteins are members of multi-gene families. For some of these protein families, it is known that they are differentially expressed as feeding progresses, thus, at the last day of feeding the tick is producing a different family member in saliva than that produced at the first day and may thus be evading the host immune response. The third surprise, and challenge, is that we cannot anticipate at all the function of the majority of the tick salivary proteins. Indeed, for any tick species with a known transcriptome, less than 5% of the proteins have been expressed and their function verified. Whole protein families await functional identification.

Several tick sialotranscriptomes and proteomes have been described [238, 286-289], and many expressed sequence tag (EST) collections are present at the NCBI DBEST site deriving from tick salivary glands. To attempt an ample view of these salivary proteins, we have assembled the EST collections and extracted their open reading frames for the ticks A. americanum, A. cajennensis, A. variegatum, R. microplus and *R. appendiculatus,* which are present at NCBI, plus all other tick salivary proteins deposited at GenBank, including the ticks *I. scapularis, I. ricinus, I. pacificus*, and the soft ticks *O. parkeri, O moubata, A. monolakensis,* as well as a few additional proteins from Hyalomma and Haemaphysalis genera. This database can be downloaded from http://exon.niaid.nih.gov/transcriptome/tick_review/Sup-Table-1.xls.gz. This supplemental table containing 3,454 putative secreted salivary proteins from ticks is organized in an annotated and hyperlinked Excel spreadsheet which we attempted to curate by gene families, as follows:

4.1. Glycine-rich, or proline-rich, collagen-like Superfamily

This superfamily, including a total of 446 sequences in Supplemental table S1, has the following distinct subdivisions: Probable cuticle proteins, Ala-rich cuticle proteins, collagens, small GGY peptides, large GGY peptides (further subdivided into several subfamilies), GYG-RLVP metastriate family, rhipicephalus GYG family (with two divisions), rhipicephalus large GYG expansion, rhipicephalus super large GYG family, metastriate spider-like cement protein, Ixodes-specific collagen-like small peptides, metastriate and argasidae proteins distantly related to Ixodes collagen-like proteins. The cuticle and true collagens included in this group may actually be functioning as housekeeping proteins, as part of the extracellular matrix of the salivary glands and as part of the tracheolar system. The remaining proteins appear to be associated with tick-cement function, or immunity. In this regard, metastriates and prostriate hard ticks differ in their attachment strategy, metastriates having shallower mouthparts and producing an abundant cement protein cone that spreads and attaches the tick on the host skin, while prostriate ticks have longer mouthparts that mechanically attach the tick into the host dermis, and have less abundant cement production [290-293]. Perhaps reflecting these differences in attachment strategies, there are specific metastriate families of cement proteins, which tend to be relatively larger in molecular weight, including the very large spider-silk-like proteins, unique to metastriates. Other proteins are similar to host epidermal proteins, such as loricrin, suggesting a role in evasion mechanisms. Several of these metastriate cement proteins have been characterized and shown to be immunogenic, and were proposed as vaccine candidates [294-299]. On the other hand, the Ixodes genus contains a large expansion of collagen-like

smaller peptides, rich in proline and possibly hydroxyl-proline, as previously noted [238, 286, 300, 301].

Included in this superfamily are a number of proteins with GGY and GYG repeats, including relatively short (< 10 kDa) polypeptides that are similar to worm peptides identified to have antimicrobial activity [302]. Their similarity to cuticle and cement proteins may reside in the GY richness. Perhaps the tyrosine residues in cuticle proteins allow for ample opportunity for crosslinking by phenoloxidases. It is also interesting that cement cones from hard ticks have been shown to have antimicrobial activity, but whether this activity derived from the cement components themselves or to embedded antimicrobials was not demonstrated [291, 292]. Thus far, no insect or tick peptide containing GY-rich domains have been characterized for antimicrobial activity.

4.2. Mucins

Mucins are serine- and/or threonine-rich proteins that are potentially added to N-acetylgalactosamine residues [303, 304]. They may function in tick feeding by coating the chitinous feeding mouthparts or the feeding lesion. Several of these proteins have a distinct chitin-binding domain in addition to the Ser/Thr-rich domain. Supplemental table S1 presents 58 such proteins, divided into three groups containing two families and an assemblage of Thr/Ser-rich proteins lacking sequence identity to characterize a common ancestor.

4.3. Antigen 5 (AG5) protein family

AG5-related salivary products are members of a group of secreted proteins that belong to the CAP family (cysteine-rich secretory proteins; AG5 proteins of insects; pathogenesis-related protein 1 of plants) [305]. Members of this protein family are found in the salivary glands of many blood-sucking insects and ticks [306-314]. Most of these animal proteins have no known function; in the few instances to the contrary, they diverge from proteolytic activity in Conus [315], to smooth muscle-relaxing activity [316-318] in snake venoms, to salivary neurotoxin in the venomous lizard *Heloderma horridum* [319]. Supplemental table S1 presents 17 proteins of this family from hard and soft ticks. None have been functionally characterized thus far.

4.4. Ixodegrin superfamily

This cysteine-rich family was named after identification of *I. pacificus* and *I. scapularis* predicted proteins with a RGD or lysine, glycine, aspartic acid (KGD) domain indicative of proteins that interfere with fibrinogen binding to platelets and thus act as an antiplatelet inhibitor [286]. Anti-platelet RGD-containing peptides named variabilin, from *D. variabilis* [25], and savignygrin [51] from Ornithodorus were previously described. The Ixodes ixodegrins differ in structure from variabilin by having cysteines flanking the RGD motif, lacking in variabilin, while savygnigrin is a non-canonical RGD peptide inserted into a Kunitz fold. The Ixodegrin family I (exclusive of the genus Ixodes) were shown to be similar to the short neurotoxin family found in elapid snakes [286]. Many members of the ixodegrin superfamily lack the RGD or KGD motif and may have a non-canonical disintegrins function, or another function altogether. Many Ixodegrin members contain the prokineticin motif of PFAM, mostly due to the conserved cysteine framework. Supplemental table S1 lists 37 such proteins from soft and hard ticks subdivided into five groups. None of these proteins have been characterized functionally.

4.5. Ixostatins

The ixostatins thus far are found only in hard ticks, with a larger representation and lineage expansion in the genus Ixodes, where 24 such proteins are known, while only four are recognized from the genera Dermacentor and Amblyomma (table S2). Most family members possess the SMART ACR domain (ADAM cysteine-rich domain), which is the domain found in metalloproteases that interact with matrix components, suggesting the function for this protein family, from which no members have been thus far characterized.

4.6. Families containing protease inhibitor domains

4.6.1. Kunitz domain containing proteins—The majority of the sequences having this domain inhibit proteases of the S1 family. The domain itself has nearly 50 residues with few secondary structures [64]. The fold is constrained by three disulphide bonds, but some of the tick Kunitz fold may lack one or more disulfide bonds and possibly create a more flexible fold. This is one of the larger protein families expressed in tick saliva, several members of which have been functionally characterized as anti-clotting agents. Multi-Kunitz domain proteins occur and they may target, as indicated above, enzyme complexes such as the Xase and prothrombinase complexes. Supplemental table S1 presents 297 proteins with Kunitz domains, or related to proteins with Kunitz domains.

The Monolaris family of single Kunitz proteins (145 proteins in table S1) contains the anti-thrombin inhibitor savignin [52], as well as the anti-Xa peptide of *O. moubata* [63] and *O. savignyi* [320]. Table S1 also lists ten proteins containing a Kunitz domain with or without the RGD disintegrin domain typified by savignygrin [51] and disagregin [29], which is a platelet inhibitor lacking the canonical RGD domain.

The Bilaris/Ixolaris group has two Kunitz domains and is typified by Ixolaris, the TF pathway inhibitor of *I. scapularis* [53, 54, 59], the anti-thrombin of *A. hebraeum* [50] and the as yet unpublished *R. microplus* anti-thrombin named boophilin (gi|17529566). Table S1 lists 59 such proteins, only three of which have been functionally characterized.

Table S1 additionally lists 33 proteins containing from three to nine Kunitz domains, only one of which, Pentalaris, with five Kunitz domains, has been characterized thus far as a TF pathway inhibitor [58].

Finally, 49 proteins with modified Kunitz folds are listed in table S1, one of which has been characterized as a tryptase inhibitor [321] and another as a thrombin inhibitor from *O. moubata* named ornithodorin [322]; this last one having two modified Kunitz domains but, interestingly, these do not interact with the target enzymes—different protein regions having been co-opted for protein interaction.

4.6.2. Serpin domain family—The protein domain Serpin (for Serine Protease Inhibitor) is ubiquitously expressed in animals, and plays an important role in controlling serine protease cascades such as the clotting system of vertebrates [323] and the prophenol oxidase activating system of invertebrates [324]. Both intracellular and extracellular serpins exist. Serpins have been recruited to the saliva of blood-sucking insects to achieve factor Xa inhibition, as in the case of the mosquito *Aedes aegypti* [325]. In ticks, these proteins have been studied primarily with an aim to produce anti-tick vaccines [326-330]. The *I. ricinus* serpin named IRIS has immunosuppressive properties in cellular assays [272] and relatively weak anti-hemostatic activity [331]. Interestingly, immunization against this predicted intracellular serpin produces antisera that recognizes a salivary protein of the expected serpin size [272]. These results indicate the possibility that intracellular proteins may reach the tick saliva or that a different but similarly antigenic serpin may be present in the saliva of

I. ricinus. This last possibility is enhanced by the similarity of IRIS to other *I. ricinus* salivary serpins that have a signal secretion peptide. Supplemental table S1 displays 49 Serpin sequences, including fragmented sequences, and shows IRIS clusters with other tick metastriate and prostriate serpins lacking a signal secretion peptide. Except for the putative intracellular IRIS Serpin, no other salivary Serpin has been functionally characterized.

4.6.3. Cystatins—According to the SMART database [332], the cystatin family primarily functions as cysteine proteinase inhibitors. They mainly inhibit peptidases belonging to families C1 (papain family) and C13 (legumain family). The cystatin family includes the Type 1 cystatins, which are intracellular cystatins that are present in the cytosol of many cell types but can also appear in body fluids at significant concentrations. They are single-chain polypeptides of about 100 residues, which have neither disulphide bonds nor carbohydrate side chains. Type II cystatins are mainly extracellular secreted polypeptides synthesized with a 19–28 residue signal peptide. They are broadly distributed and found in most body fluids. Type 3 cystatins are multidomain proteins. The mammalian representatives of this group are the kininogens. There are also unclassified cystatins found in a range of organisms: plant phytocystatins, fetuin in mammals, insect cystatins, and a puff adder venom cystatin that inhibits metalloproteases of the family M12 (astacin/adamalysin). Also, a number of the cystatin-like proteins have been shown to be devoid of inhibitory activity against proteases.

Two secreted type II salivary cystatins form *I. scapularis* have been characterized functionally (named sialostatin L and sialostatin L2) and determined to have anti-inflammatory and immunosuppressive functions [333, 334], possibly due to its inhibitory activity against cathepsins L and S, which are important in matrix degradation by fibroblasts, and intracellularly for protein cleavage by antigen-presenting cells. These two cystatins show differential tissue expression, one of them having abundant transcripts in unfed tick salivary glands while the other is abundantly expressed in gut tissues, the pattern reversing as time of blood feeding progresses [333]. These cystatins also show limited antigenic cross reactivity. Vaccination using these cystatins or RNAi disruption of the cystatin gene expression, impaired tick feeding [333], as happened previously with RNAi disruption of an uncharacterized cystatin of *A. americanum* [335]. Other tick cystatins, not necessarily salivary or type II cystatins, have also been characterized [336-338].

Supplemental table S1 presents 16 cystatins of soft and hard ticks, including the two from *I. scapularis* described above. It additionally displays nine cystatin-like proteins from *R. microplus* that are clearly derived from a gene expansion. However, because these sequences were deducted from whole body ESTs, we cannot be sure if they are secreted in saliva.

4.6.4. Thyropin family—The SMART TY motif identifies Thyroglobulin type I repeats, which are found to inhibit cysteine proteases and are binding partners of heparin. Seven proteins in table S1 contain this domain, five of which have 21 to 27 kDa of mature weight and contain two such domains. The sole described member of this family derives from an unpublished product of a gut library from *O. moubata*, with a single TY domain; the remaining proteins are all from the metastriates *R. microplus, R. appendiculatus, A. cajennense* and *A. variegatum.* Their function is unknown.

4.6.5. TIL domain-containing peptides—The TIL domain stands for Trypsin Inhibitor Like domain found in protease inhibitors, antimicrobial peptides and venom toxins. Forty-eight such peptides are listed in table S2, including the only one thus far characterized named ixodidin, which has anti-trypsin and anti-elastase properties, in addition to antimicrobial activity [339].

4.6.6. Hirudin-like/Madanin/Variegin superfamily—Madanins are relatively small (~6 kDa mature peptide) anti-thrombins formerly isolated from the salivary glands of the tick *Haemaphysalis longicornis* [340]. Supplemental table 2 shows additional sequences from this family deriving from *Dermacentor andersoni*. It appears that this protein family is specific to metastriates.

Hirudin-like salivary proteins were proposed, but not characterized, in *Amblyomma variegatum* [341]. The sequence coding for a protein of this name was deposited in GenBank in 2004 (GI:50284523) but has no published paper associated as yet. The predicted mature protein should have nearly 200 amino acids (aa) and 21.3 kDa. However, a related peptide of only 32 aa named variegin [49] was isolated from *Amblyomma variegatum* salivary homogenates and shown to be a thrombin inhibitor. Interestingly, the larger protein has several variegin repeats, thus appearing that the larger protein may originate several anti-thrombin peptides by post-translational proteolysis. Supplemental table S1 displays nine protein sequences of this family, from *A. americanum, A. cajennense,* and *R. appendiculatus.* These hirudin-like proteins, in addition to having sequence similarity to variegin, also display similarity to madanins, indicating that possibly the Haemaphysalis and Dermacentor madanins (of ~6 kDa) are shorter relatives of the hirudin-like proteins of Amblyomma and Haemaphysalis. This superfamily has no known prostriate or argasidae members.

4.6.7. Basic tail and 18.3-kDa superfamily—Basic tail proteins were so named because most family members have a very basic carboxy terminus or tail [238, 301]. Conversely, some members have an acidic tail instead, and other members lack the tail altogether [238]. The basic tail may help interaction with anionic phospholipids, expressed in the surface of activated platelets and mast cells, that function as scaffold surfaces for assembly of the Xase and prothrombinase complexes [342]. This protein family is ubiquitously found in soft and hard ticks but appears more expanded in the prostriates than metastriates and argasids. Table S2 lists 102 members of this family, most of which have a distinct pattern identified by the PFAM domain TSGP1. Eight additional members from metastriates are distant members of this family, and yet another five members have only basic or acid tails but no recognized additional family pattern. A few members of this large family have been characterized as anti-clotting in *Ixodes scapularis* [62]. The *O. parkeri* protein gi|149286982 has a uniquely RGD domain flanked by cysteines and could act as a platelet aggregation inhibitor.

The 18.3-kDa family is a distinct family within this superfamily, 27 proteins being listed in table S2 from both hard and soft ticks. No protein from this family has been functionally characterized. One member of this family (Rh_micro-2791) has a carboxyterminus RGD domain flanked by cysteines and could act as a disintegrin.

4.6.8. Carboxypeptidase inhibitor family—Thus far, this is an exclusive metastriate family first identified in *R. bursa* [343], the proteins have been crystallized [344], and homologs from *Ha. longicornis* also characterized [345]. Supplemental table S2 shows additional family members from *D. andersoni and R. microplus* (identified by Psiblast). These inhibitors may affect fibrinolysis by inhibiting plasma carboxypeptidase N and plasma carboxypeptidase B, also known as thrombin-activable fibrinolysis inhibitor [343].

4.7 Lipocalins

Lipocalins are ubiquitously distributed proteins remarkably diverse at the sequence level, yet have highly conserved structures characterized by a repeated +1 topology beta-barrel [346]. Their barrel structure makes them versatile structures to carry hydrophobic ligands inside its

cavity. Remarkably, they are abundantly expressed in the salivary glands of ticks [238, 286, 289] and triatomine bugs [347-349], but not in blood-feeding Diptera or fleas. Triatomine lipocalins were found that carry nitric oxide [350, 351], bind adenosine nucleotides [30], histamine [352], serotonin [353], negatively charged phospholipids [342] and are anti-clotting [354-356] ; this last function is clearly unrelated to binding of small ligands. In ticks, their function has been associated similarly as binders of histamine and serotonin [149-151], but also with anti-complement activity [241], immunoglobulin binding [357] and with toxic properties [358]. Supplemental table S2 lists 307 proteins belonging to this family, organized into ten major groups. Note that many of these groups are lineage-specific gene expansions. Only a handful of these proteins have been functionally characterized thus far. Several of these uncharacterized proteins have RGD domains that may indicate functions other than agonist sequestration. Possible functions yet to be discovered for these proteins are binding of inflammatory lipids such as PAF and leukotrienes, as well as adenosine nucleotides.

It is interesting to consider that to achieve efficient binding and sequestration of serotonin or histamine, a local concentration of near 1 microM needs to be achieved by the sequester protein, because the histamine or serotonin receptor saturates at sub-micromolar concentrations. Accordingly, 1 microM (or 0.5 microM if the protein sequesters two amines) of a 20-kDa protein needs to be present in the tick feeding cavity to sequester the 100 Dalton agonist. Although the cost effectiveness of such an endeavor appears inefficient, it actually occurs in nature. Histamine and/or serotonin binding proteins are among the most abundant salivary proteins in triatomine bugs [347], ticks, mosquitoes (role played by the D7 protein family) [359], and sand flies (role played by the Yellow protein family) (Andersen, unpublished). A still more cost-effective solution would occur for agonists that saturate their receptors at much lower concentrations, such as occurs for PAF or leukotrienes, for example, where ten times less protein would be needed in comparison to histamine or serotonin sequestration.

4.8 8.9-kDa polypeptide family

This polypeptide family, with 60 members in table S2, is thus far uniquely found in hard ticks. The proteins fall into three main groups, one being common to prostriates and metastriates, the second containing only metastriate proteins, and the third represented by two larger *R. appendiculatus* sequences that have two 8.9-kDa domains. The function of each of these proteins is unknown.

4.9. 23-kDa family

This is a uniquely tick family containing ten known members in table S2 originating from soft and hard ticks. Its function is unknown.

4.10. 13-kDa family

This, too, is a unique tick family containing five known members in table S2 originating from individual soft and hard ticks species, suggesting this protein possibly originated from a non-duplicated gene family. No function is known for this protein family.

4.11. 12-kDa family

This is another uniquely tick family containing three known members in table S2 originating from individual soft and hard ticks species. No function is known for this protein family.

4.12. PGFG repeat family

Four members of this family are reported in table S2, constituting basic proteins varying in mature weight from 14 to 24 kDa and having Pro Gly Phe Gly repeats in their carboxytermini region. These repeats are also found in Drosophila proteins, and it may be possible that this family codes for a housekeeping function yet unknown.

4.13. IS4 family

Three polypeptides from Ixodes and Rhipicephalus characterize this group, one of which has a weak PFAM histamine- binding domain. The mature weights vary from 16 to 20 kDa, which is within the range for the lipocalins; accordingly, these proteins may belong to the lipocalin superfamily.

4.14. Cytotoxin-like family

Eight proteins from soft and hard ticks characterize this group that produces similarities by Blast to pesticidal crystal protein cry15Aa and other bacterial cytotoxins. Proteins from this family have also been found in sialotranscriptomes of kissing bugs of the genus Triatoma [348]. Their function is unknown.

4.15. 16-kDa family

This family consists of 14 proteins, 13 of which derive from *I. scapularis* and *I. pacificus* and one that derives from a distantly related *D. andersoni* protein. They code for mature proteins varying from 9.2 to 12.1 kDa. These are Cys-rich proteins that have distant similarity to tick antithrombins. Their function has not been evaluated.

4.16 Enzymes

Several enzymes have been found in the saliva of blood-sucking arthropods that appear to assist blood feeding. These include the ubiquitously found apyrase activity that hydrolyse ATP and ADP released by broken cells that activate platelet and neutrophils [360]. Tick saliva has been shown to have apyrase activity [22-24, 28], as well as esterases and glucosidases [361], phospholipase A_2 [362-364], kininase and anaphylatoxin-inactivating enzymes (exercised by a dipeptidylpeptidase) [101] and metalloproteases that have specificity toward fibrin and fibrinogen [128, 365]. This last group of enzymes has been previously indicated to have abundant salivary expression resulting from a large gene expansion.

Supplemental table S1 lists 110 metalloproteases from hard and soft ticks, 34 trypsin-like serine proteases, 13 serine carboxypeptidases, 2 prolyl carboxypeptidases, 20 carboxy esterases, 20 chitinases, 7 lipases, 7 phospholipase A_2, 8 sphyngomyelinases, 1 leukotriene hydrolase, 14 5′nucleotidases/apyrases (fragments only), 1 ectonucleotide pyrophosphatase/phosphodiesterase, 2 multiple inositol phosphatases, 9 dipeptidyl peptidase (kininase), 3 alkaline phosphatases, 4 ribonucleases, 1 epoxy hydrolase, 1 pyrophosphatase, and 8 endonucleases. It is possible that some of these enzymes may actually be lysosomal and not secreted in saliva, or may have yet another housekeeping function. Several of the enzymes described above have not been described in tick saliva before, such as endonuclease, chitinase and serine proteases. Endonuclease was found previously in the mosquito *Culex pipiens quinquefasciatus* saliva and may assist diffusion of pharmacologic components through the host dermis by decreasing the viscosity of the skin matrix [366]. Chitinases may exert an anti-fungal activity or may be related to some housekeeping function associated with tracheolar or cuticular structures. Serine proteases may possibly affect the protein C or fibrinolysis pathways in their hosts, or be part of the prophenoloxidase anti-pathogen pathway.

4.17. Immunity-related products

Salivary transcriptomes of blood-sucking insects and ticks consistently show the presence of immune-related transcripts, including antimicrobial compounds such as cecropins, lysozyme or defensins, as well as pattern recognition molecules such as lectins, and serine proteases which may be part of the phenol oxidase activating system or the invertebrate complement system. Table S2 presents 13 families of tick proteins that might play a role in immunity.

4.17.1. Pattern recognition proteins

4.17.1.1 Ficolins/Ixoderins: This protein family has the Ficolin motif from Kog and the Fibrinogen_C domain from the PFAM database, and belong to a lectin family named the Ixoderins, as proposed by [367]. They may represent pattern-recognition molecules associated with the activation of the invertebrate complement system, which are terminally represented by the thioester-capable proteins similar to vertebrate complement C3. These proteins were ubiquitously found in hard- and soft-tick transcriptomes.

4.17.1.2. Peptidoglycan recognition proteins: These pattern recognition proteins are also important in the pathogen recognition and activation of anti-microbial enzyme complexes in invertebrates. A single truncated protein from *I. scapularis* and two from *R. microplus* are represented in table S1.

4.17.1.3. Galactose-binding protein: A single truncated galactoside-binding protein is represented in table S1.

4.17.1.3. ML domain containing proteins: The ML domain from the SMART database indicates proteins involved in the recognition of lipids important in innate immunity and lipid metabolism. Proteins of this family are also associated with mite allergens, and with the major epididymal protein Kog domain. Seventeen proteins from hard and soft ticks are represented in table S2, including two that have a smaller, truncated version of the ML domain.

4.17.2. Thioester/alpha2 macroglobulin family—Various (13) fragmented coding sequences expressing members of this family are shown in table S1, from both hard and soft ticks.

4.17.3. Antimicrobial peptides and proteins—Insect and tick salivary transcriptomes have revealed abundant expression of antimicrobial peptides that might prevent microbial growth in the ingested blood and, in the case of hard ticks, prevent contamination of the feeding cavity with bacteria or fungi. Seven protein families are tentatively represented under this classification, as follows:

4.17.3.1. Lysozyme: Five lysozyme protein sequences are shown in table S1, from Dermacentor, Rhipicephalus and Ornithodoros ticks. To our knowledge, there is only one paper that identified a possible lysozyme in tick salivary secretions [292]

4.17.3.2. Defensins: Defensins are ubiquitous antimicrobial peptides, several of which have been described in ticks [368-387], but only a few of these studies demonstrated salivary expression of these peptides [381, 383, 384]. Tick salivary transcriptomes reveal abundant expression of these peptides in both hard and soft ticks, 44 of which are shown in table S1. Note that some of these peptides are highly divergent and only a Cys framework and their conserved size reveals their possible membership in the family.

4.17.3.3. Microplusin family and other histidine-rich peptides: Fifteen peptides from metastriate and argasides show similarity to this novel antimicrobial peptide family, which includes the original microplusin peptide [376], and hebreain [388], both of which have been functionally characterized. This family also includes the protein encoded by gi|111559370, which is annotated as a neutrophil elastase inhibitor, but the work is still unpublished. It may be possible that this peptide family has multiple functions, similar to some serine protease inhibitors of the TIL family described above which possess antimicrobial activity [339]. Note that both microplusin and hebrein have a histidine rich domain, which has been indicated before to exercise antimicrobial activity possibly due to sequestration of zinc, a microbial growth factor [389].

Another subset of the microplusin family includes ten proteins from metastriate and prostriate ticks, but none have been characterized functionally. Table S1 also lists 11 other proteins that do not produce high similarities to microplusin members, but have histidine-rich repeats that might indicate an antibacterial function.

4.17.3.4. 5.3-kDa family: This expanded protein family with six conserved cysteines has been previously identified in transcriptomes of *Ixodes* ticks [286, 301]. A few members of this family were shown upregulated in sialotranscriptomes of *I. scapularis* nymphs infected with *B. burgdorferi* [238], suggesting their function in immunity. Expression of one of the members of this protein family did indeed demonstrate antimicrobial activity (Unpublished). Interestingly, a protein from *I. holocyclus* deposited in GenBank (gi| 54401697) is annotated as holocyclotoxin and belongs to this family. It had been deposited in 2004 and has no associated publication as yet. *I. holocyclus* is known to produce a paralytic toxin of veterinary and medical concerns [390]. Table S2 identifies 29 members of this family, divided in one group of soft ticks and prostriates, and one smaller group of Amblyomma and Haemaphysalis proteins.

4.17.3.5. Y-rich GR peptide family: This is a tick ubiquitous family of small peptides having in common richness of tyrosine and ending in Gly-Arg. They are similar to the Gly-Tyr peptides from *Caenorhabditis elegans* that have been shown to have antimicrobial activity [302]. None of the ten peptides shown in table S2 have been characterized.

4.18. Metastriate-specific families

Thirty-five families of proteins totaling 196 sequences were found exclusively on metastriate genera or as expansions in a single metastriate species or genus. Most of these do not have any other close relatives to other arthropod proteins, as identified by Blast searches. Only two proteins on two different families of these 35 families where functionally characterized, namely the Da-p36 protein and Evasin.

The *D. andersoni* Da-p36 protein has been characterized as an immunosuppresand protein [391], and it is found in all metastriate genera included in this analysis, 22 of which were obtained by Psiblast. A distinct Amblyomma-specific clade is observed within this expanded protein family.

Evasin is a salivary protein from *R. sanguineus* that selectively binds three CC chemokines, namely CCL3 and CCL4 and the closely related chemokine CCL18, [88]. Evasins have a typical cysteine pattern C-x(15,31)-C-x(3)-Cx(12,13)-x(18,22)-C-x(4)-C-x(4)-C-x(7,11)-C, and a longer conserved pattern C-x(12,17)-G-x(2)-T-[DN]-x(3)-C-x(4)-L-x(10,26)-C-x(3)-C-x(12,13)-C-Lx(17,21)-C-x(2)-G-x(1)-C-x(4)-C-x(8,12)-C-x(2)-P that helped to identify 11 additional evasins on the genera Amblyomma, Dermacentor and Rhipicephalus.

Of the remaining 33 metastriate families, a few have domain identities or similarities that may hint to a function: A group has hormonal similarities, and perhaps are housekeeping proteins, three other families each have either a fibronectin, an endostatin, or an insulin growth-factor binding domain, and a fifth family has a three domain structure of Immunoglobulin, Insulin growth factor binding and a Kazal domain. There remain 27 metastriate families for which we do not have a clue for its function.

Six proteins coding for homologues of previously identified hormones were identified, as follows: *R. microplus* encodes an orcokinin precursor, which belongs to a neuropeptide family of invertebrates [392, 393]. An insulin precursor from *A. variegatum* and a protein with similarites to the adipokinetic hormone found in *R. appendiculatus* were also found. Two related proteins from *R. microplus* code contain similarities to granulins, which are conserved growth factors. These five proteins may actually be playing a housekeeping role. More interesting is the expression of a protein with similarity to the adrenomedulin peptide of vertebrates, found in the sialotranscriptome of *O. parkeri*. Adrenomedulin homologues are not known in invertebrates, and in vertebrates they have vasodilatory, angiogenic and immunomodullatory properties [394-396]. It is possible that *O. parkeri* adrenomedulin was obtained by horizontal transfer.

Five proteins that are unrelated by Blast similarities have weak fibronectin type-3 domains that may indicate a function targeted to the ECM or receptor interactions. Also possibly targeting the ECM are four small proteins (6 kDa) from *A. americanum* that have a weak endostatin motif, which are collagen-derived anti-angiogenic factors [397]. Three related proteins from Rhipicephalus and Amblyomma genera contain a 3-domain structure of an insulin growth-factor binding domain, followed by a Kazal domain, and finally followed by an immunoglobulin domain. These proteins are similar to previously described antigens of mites, which contain the same three domain structures.

Among the 27 remaining families of metastriate proteins, we call attention to a Pro-Ala-rich family that is abundantly expressed in Rhipicephalus and Amblyomma ticks, and to an expansion of Cys-rich proteins in *A. americanum*, named 19-kDa Amblyomma expansion in table S1.

Table S1 also presents an additional 19 protein families from metastriate ticks that are classified as orphans since they cannot be grouped with any other known protein family. These proteins include members with low complexity including proline rich families.

Many other individual orphan proteins from individual metastriate tick species are listed in table S1, as follows: 33 for *A. americanum,* 44 for *A. cajennense*, 86 for *A. variegatum*, 52 for *D. andersoni*, 3 from the genus Haemaphysalis, 491 from *R. appendiculatus* and 208 proteins from *R. microplus.*

4.19. Prostriate-specific families

Six protein families and one group of orphan proteins, all deriving from the Ixodes genus are shown in table S1. Only one of these groups had a few members characterized functionally, namely the Isac protein family (with 18 proteins in table S1) for which the salivary anti-complement of *I. scapularis* and *I. ricinus* have been described [237, 239, 257]. The proteins of this family prevent activation of complement by the alternative pathway of complement, which was shown before to be important for tick rejection reactions [234, 235]. The remaining 101 proteins in five groups or families have not been investigated functionally.

4.20. Argasidae-specific families

Five protein families and two groups of orphan proteins, representing a total of 57 proteins, are thus far unique to the genus Argas or Ornithodoros (table S1). Their function is unknown.

4.21. Secreted conserved proteins

Ten groups of proteins, totaling 126 sequences, all containing a signal peptide indicative of secretion and displaying conservation of sequence beyond Ixodidae are shown in table S1. This class contains many calreticulins, most of which have an ER retention signal, but may be secreted in saliva and actually have a function in blood feeding. Indeed anti tick calreticulin has been used as an immunological marker of tick exposure [398-400], and calreticulin has been shown to have extracellular physiological activities [401]. Other conserved proteins included in this group include metastriate selenoproteins, which may be involved in anti-oxidation reactions, possibly associated with high oxidative stress of the salivary glands undergoing arachidonic acid conversion to prostaglandins, and growth factor-like families such as multiple coagulation deficiency-like, hematopoietic stem/progenitor cell protein-like, and serum amyloid A like protein, among other conserved proteins. Their function as housekeeping proteins or in feeding is not clear.

4.22. Possible housekeeping proteins

Table S1 additionally presents 4,531 sequences coding for proteins that probably have a housekeeping function and will not be further considered in this work. Their sequences may, however, help to identify novel secreted protein families if identified in proteome experiments, and in the annotation of tick genomes and proteomes. Among this set of proteins are several associated with oxidant metabolism including the antioxidant salp25, which was implicated recently in the successful transmission of *B. burgdorferi* from mammals to ticks[402], suggesting some of these “housekeeping” proteins are secreted in saliva.

4.23. Non-peptidic salivary components

Bioactive lipidic salivary components are known to exist in the saliva of hard ticks, which abound in the vasodilatory prostaglandins PGE_2 and PGF_{2alpha} [28, 274-276, 403-406]. PGE_2 was also demonstrated to be the main dendritic cell inhibitor in the saliva of *I. scapularis* [172]. Cannabinoids have also been described in Amblyomma ticks, where they might serve analgesic and anti-inflammatory functions [407]. It may be possible that other lipidic agonists, perhaps the anti-inflammatory lipoxins [408], or analogs, could be produced by ticks. In this regard it is interesting to point out that one of the human prostaglandin D_2 synthase enzymes is a member of the lipocalin family [409].

4.24. Evolutionary considerations

It is becoming clear that the sialomes of ticks are much more complex than originally proposed. In the case of soft ticks their sialomes might possess 100-200 secreted proteins of which approximately 60% could be confirmed as being synthesized at a level amenable to detection by current proteomic methods [410, 411]. For hard ticks the latest count extended the number of possible secreted proteins well beyond 500, although no estimate has been obtained on how many of these are in fact abundant or present in the glands at a given time point [238]. This is further confounded by the possible differential expression of proteins in salivary glands of larvae, nymphs and adult hard ticks at various time points during feeding. This makes comparative analysis of hard and soft tick sialomes problematic in regard to obtaining global insights into the evolution of salivary gland complexity. Even so, some general conclusions can be made in this regard.

Comparative analysis indicates that while the major families of proteins seem to be conserved in different tick genera, the number of orthologs that can be assigned with confidence is limited [410]. In many cases, proteins assigned to well known enzyme or inhibitor families are found haphazardly in the species analyzed in this review. This is most probably due to three main factors. The first factor is extensive gene losses among lineages and our current small set of sialome data for representative species that we have for each genus. The second factor is the fairly high level of divergence that we observe for secretory proteins. This leads to an under-estimation/assignment of sequences to protein families and may lead to an artificial inflation of novel families observed in ticks. The third factor is the extensive gene duplications observed within species or genera and the lack of experimental data on whether these duplicates represent proteins with novel functions or whether they are paralogous groups that share the same function. The completion of more sialomes with larger representation of tick species should address the shortcomings of these confounding factors and provide better evidence for conservation of function and protein families expressed in salivary glands among related species and genera.

What we can conservatively conclude is that the genome of the ancestral tick lineage encoded a large number of the currently described protein families, as these are present in both hard and soft tick salivary glands [410]. This is especially true for the abundant families such as Kunitz/BPTI, lipocalins, BTSP and metalloprotease families. Phylogenetic analysis of these protein families shows, however, that gene duplications occurred as lineage specific expansions, i.e. duplications occurred within species or genera after divergence of the main tick families [410]. As such, a number of gene duplicates with novel functions evolved after the divergence of the main families or even genera. In this regard, many proteins involved in tick-host interactions differ between hard and soft ticks [412]. This would imply that the ancestral lineage possessed a fairly small number of protein members per family before adaptation to a blood-feeding lifestyle. It also indicates that gene duplication played a major role in the evolution of novel function in ticks during their adaptation to a blood-feeding environment [410].

When protein family members are compared between major lineages (Argasidae, Prostriata and Metastriate) it is clear that major gene duplication events occurred in most tick lineages. In the case of the Metastriata this is particularly striking. In addition the Metastriata shows the highest number of novel protein families (33 unique families) confined to a specific lineage. While the current differences might be due to our lack of adequate species/genus coverage it should be noted that the increased degree of diversity observed in the Metastriata correlate with a larger genome size compared to the Argasidae and Prostriata [413]. The sequencing of Prostriate, Metastriate and Argasid genomes will eventually show whether the differences seen in gene and protein family numbers is due to gene duplication alone, or whether whole genome duplication played a significant role in this process [238, 412, 413].We foresee that once sialome coverage for more species is obtained, the evolutionary pathways for the acquisition of novel protein families in different tick lineages, genera and species will become more evident. Larger datasets will also facilitate the assignment of highly divergent proteins currently described as orphan families, into more defined protein superfamilies.

It is possible that the diversity of salivary proteins found in tick saliva reflects a fast evolutionary scenario driven by an arms race between the tick proteins and the adaptive immunity of their hosts. Gene duplications initially may confer an advantage by creating an increased tissue expression of the gene product. Later on, diversion of function, or acquisition of new functions may occur with the duplicated gene [414, 415]. The fast diversification of the salivary protein families could be the result of acquisition of novel functions, as might be the case with the main lipocalins families, but they may also reflect

maintenance of function with diversification in epitopes, as may be the case with the Ixodes family of anticomplement proteins [237, 240, 381], or in the more closely related lipocalins subfamilies in the same species [238]. These different genes may also be expressed at different stages or times after host attachment, thus helping evasion of these molecules by the host immune response.

5.0. CONCLUSIONS AND PERSPECTIVES

It is becoming clear that tick saliva contain an extraordinary array of salivary proteins that allows disarming of host hemostatic, inflammatory and immune reactions that would otherwise deter tick feeding. The advent of transcriptomics allowed uncovering of salivary proteins of novel folds that potentially interact with both known and yet unknown vertebrate proteins, thus increasing the complexity of the study, but providing for exciting discoveries. Recombinant expression of these proteins and attempting to identify their vertebrate targets by using plasmon resonance-based methods or “pull-down” assays may lead to relatively fast functional identification of these pharmacologically active proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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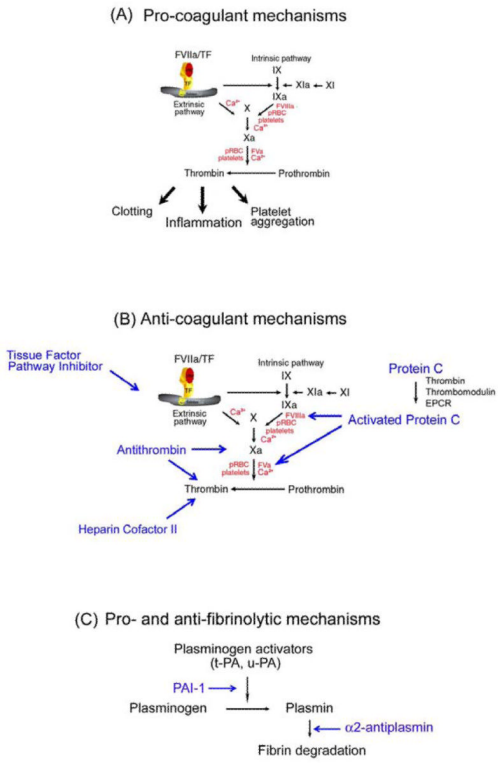

Figure 1. Coagulation cascade and its regulation

A) Pro-coagulant mechanisms. TF: a critical initiator of coagulation. Formation of a complex with Factor VIIa (FVIIa) leads to activation of FIX and FX. FXa in the presence of phosphatidyl serine and Ca^{2+} (prothrombinase complex) amplifies the coagulation cascade through conversion of prothrombin to thrombin, resulting in platelet aggregation, fibrin formation, and inflammation. Thrombin also activates FXI to XIa, which activates FIX to FIXa. FIXa in the presence of phosphatidyl serine and Ca^{2+} converts FX to FXa, consolidating the coagulation cascade. pRBC, parasitized red blood cells. B) Anticoagulant mechanism. TF pathway inhibitor (TFPI) binds to FXa and inhibits FVIIa/TF complex. Protein C is activated by thrombin (in the presence of thrombomodulin and EPCR), and APC inhibits the coagulation cascade through cleavage of cofactors FVa and FVIIIa. Antithrombin in the presence of heparin sulphate specifically interacts with and inhibits FXa and thrombin. Heparin cofactor II (in the presence of dermatan sulphate) inhibits thrombin. C). Pro- and anti-fibrinolytic mechanism. PAI-1, plasminogen activator inhibitor-1. The zymogen plasminogen is converted to the active serine protease, plasmin, primarily through the action of two-chain tissue plasminogen activator (tc-tPA) or two-chain urokinase (tc-uPA). Both tPA and uPA can be inhibited by plasminogen activator inhibitor-1 (PAI), while plasmin is inhibited by its major inhibitor, α -antiplasmin, and to a lesser extent by α_2-macroglobulin (not shown).

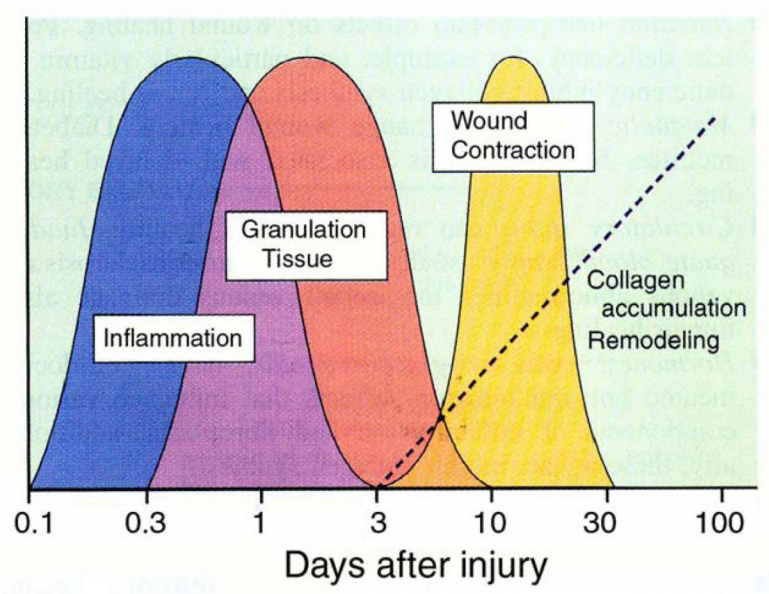

Figure 2. Orderly phases of wound healing

Wound healing is divided into three phases: inflammatory (inflammation), proliferative (granulation tissue), and remodeling (wound contraction) phases. Granulation tissue is a critical step in this process and is characterized by an intense proliferation of endothelial cells, fibroblast accumulation, and collagen synthesis. The process provides nutrition, oxygen, and physical support for growing tissue. Modified from Clark, R. A. 1991. p. 577. *In* L. A. Goldsmith (ed.), Physiology, biochemistry and molecular biology of the skin, 2nd ed., vol. I. Oxford University Press, New York, NY.