A FAMILY OF TICK (*IXODES SCAPULARIS*) SALIVARY PROTEINS THAT INHIBIT COMPLEMENT

Katharine Rose Tyson

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology.

Chapel Hill
2008

Approved by:

Aravinda de Silva, MPH, Ph.D.

Robert Bourret, Ph.D.

Christopher Elkins, Ph.D.

Jeffrey Frelinger, Ph.D.

Michael Roe, Ph.D.

ABSTRACT

KATHARINE ROSE TYSON: A family of tick (*Ixodes scapularis*) salivary proteins that inhibit complement
(Under the direction of Aravinda de Silva)

Ixodes scapularis, the blacklegged tick, is an ectoparasitic bloodsucking arthropod that transmits multiple pathogens, including *Borrelia burgdorferi*, the agent of Lyme disease. *I. scapularis* ticks secrete numerous salivary anti-hemostatic, anti-inflammatory, and immunosuppressive compounds into the host to inhibit host responses that could interfere with feeding. *I. scapularis* anti-complement protein (Isac) specifically inhibits the alternative complement pathway by destabilizing the C3 convertase. Here, we report on the identification of several tick proteins that are paralogues of Isac and members of the Isac-like protein (ILP) family, which contains at least 49 unique proteins. In this work we have characterized the biochemical and functional activities of multiple ILPs, their mechanisms of complement inhibition, and their roles in facilitating tick feeding.

We expressed recombinant ILPs (rILPs) that possessed several N- and O-linked glycans and inhibited the alternative complement pathway by destabilizing C3 convertases, similar to Isac. rILPs specifically bound properdin, a positive regulator of the alternative complement pathway, causing its removal from C3 convertases and accelerating decay of the convertases. This mechanism of complement regulation is distinct from any characterized negative regulators of the alternative complement pathway, which mediate decay acceleration by interacting with Bb or C3b.

As *I. scapularis* ticks likely co-express multiple ILPs during tick feeding, we evaluated various properties of individual ILPs that possibly justify their co-expression. When multiple rILPs were added together, no synergistic effects were observed, indicating the proteins acted individually. Individual rILPs also inhibited the alternative complement pathway from different animal hosts comparably, indicating rILPs did not display host specificity. We believe that individual *I. scapularis* ILPs likely display antigenic variation, justifying their co-expression during tick feeding.

Expression of the ILP gene family is likely essential for successful tick feeding as ILPs suppress host innate immune responses. RNAi mediated gene silencing of the ILP genes and the generation of antibodies against rILPs in immunized mice had no effect on successful *I. scapularis* nymphal tick feeding. However, RNAi may have not revealed a phenotype because of redundancy in the ILP genes. Similarly, ILP specific antibodies may have showed no phenotype because of antigenic variation between different ILPs and redundancy in ILP functions.

ACKNOWLEDGEMENTS

I first want to thank my mentor, Aravinda de Silva, for providing me with a wonderful opportunity to learn and develop as a scientist. Besides his continued support and encouragement, Aravinda has taught me how to properly design experiments, interpret the outcomes, and think of future directions to pursue. I also want to thank Chris Elkins, who taught me the importance of appropriate controls, for mentoring me while Aravinda was on sabbatical. I would also like to thank my committee members, Bob Bourret, Chris Elkins, Jeff Frelinger, and Mike Roe, for their continued suggestions and guidance. I also want to thank Kathy Deponte, David Klapper, Nandhini Ramamoorthi, Dan Sonenshine, and Ruth Silversmith all assisted for their assistance with various experiments presented in this work.

Both past and present members of the de Silva lab have made my graduate career an extremely enjoyable experience. Everyone has been enormously supportive and especially tolerant of my talkativeness, playfulness, and "colorful" phrases! The encouragement and support of my friends and family have also made this work possible and I want to thank them for always listening to me and helping in any way that they can. Finally, I especially want to thank my husband, David, who always remained positive and encouraging throughout this period in my life. His love and support helped me reach this achievement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CAT	chloramphenicol acetyltransferase
DAF	decay accelerating factor
dsRNA	double-stranded RNA
ELISA	enzyme-linked immunosorbent assay
fB	factor B
fD	factor D
fH	factor H
fI	factor I
IC_{50}	concentration of inhibitor that inhibits 50% of the assay
ILP	Isac-like protein
Irac-1	*I. ricinus* anti-complement protein-1
Irac-2	*I. ricinus* anti-complement protein-2
Isac	*I. scapularis* anti-complement protein
kDa	kilodalton
MAC	membrane attack complex
MBL	mannose-binding lectin
NHS	normal human serum
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RbE	rabbit erythrocyte
RNAi	RNA interference

Salp9	*I. scapularis* salivary protein 9
Salp15	*I. scapularis* salivary protein 15
Salp20	*I. scapularis* salivary protein 20
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SGE	salivary gland extracts
siRNA	small interfering RNA
S20L2	Salp20-like clone 2
S20L12	Salp20-like clone 12
S20NS	Salp20 with C-terminal V5-epitope and 6X-histidine tags
TBS	Tris-buffered saline
TSR	thrombospondin type I repeat

CHAPTER 1

Background and Significance

1.1 Introduction

Ticks are obligate parasitic blood-sucking arthropods that feed on a wide variety of vertebrate hosts including mammals, birds, reptiles and some amphibians (22, 138). Ticks are distributed on virtually every continent, excluding Antarctica, where they are persistent pests of livestock and wildlife (63, 137). Tick infestations of livestock can cause severe toxic conditions, including tick paralysis, toxicoses, allergic reactions, and severe blood loss, which can lead to major economic losses in several countries. In addition to causing several severe pathogenic conditions, ticks are also important vectors of numerous viral, bacterial, and protozoan pathogens that cause various diseases in animals and humans including babesiosis, ehrlichiosis, tularemia, Rocky Mountain spotted fever, tick-borne encephalitis, Crimean-Congo hemorrhagic fever, and Lyme disease, the most prevalent vector-borne disease in the United States and Europe (22, 137, 138).

Approximately 850 species of ticks exist worldwide, which are separated into two major families, the Ixodidae (hard ticks) and the Argasidae (soft ticks) (6, 22, 42, 137). A third family exists, the Nuttaliellidae, containing only a single species. The life cycle of ticks consists of three developmental stages (instars), the larva, nymph, and adult (138). Larval ticks have 3 pairs of legs like insects, while nymphal and adult ticks possess 4 pairs of legs, resembling arachnids, which are comprised of ticks, mites, spiders, and scorpions (93, 137,

138). Tick mouthparts consist of three main appendages: the chelicerae, which are toothed organs used for cutting, ripping, and tearing skin; the palps, which are sensory organs used for host attachment; and the hypostome, which is the barbed mouthpart inserted into the skin during feeding (Fig 1.1A) (137, 138). Ixodidae, or hard ticks, possess a tough, sclerotized plate, the scutum, on their dorsal body surface, which acts as an attachment site for many vital muscle groups (Fig 1.1B) (42, 137, 138). Argasidae, or soft ticks, lack a scutum but possess a tough leathery cuticle that is highly folded, allowing ample growth during feeding (Fig 1.1B). Due to their feeding and molting characteristics, ixodid and argasid ticks both exhibit long life spans, lasting several years. Even though they share many common features, drastic differences exist between argasid and ixodid ticks.

Argasidae

Argasid ticks can be subdivided into four main genera, *Argas*, *Carios*, *Ornithodoros*, and *Otobius*, where *Argas*, *Ornithodoros*, and *Otobius* ticks are important medical and veterinary pathogen transmission vectors (22, 63). As mentioned previously, argasid ticks lack a scutum, containing only a tough, leathery cuticle with many folds, giving rise to the term “soft ticks” (42, 137, 138). The mouthparts of argasid nymphs and adults are found at the anterior end of the body, but are covered by the body and cannot be seen from the dorsal view (Fig 1.1B). Argasid ticks are typically found in environments very close to their hosts, such as bird nests, bat caves, burrows and dens of various animals, or dilapidated huts and cabins of humans (99, 138). When ready to feed, argasid ticks attach to hosts by cutting into the skin with their chelicerae and inserting their barbed hypostome (6, 42, 93, 99, 137, 138). Once attached, they begin to secrete saliva into the host and suck blood from the host. Argasids only feed for short periods of time, usually several minutes. As they feed, their

highly folded body cuticle stretches to accommodate the incoming host blood meal, which is approximately 5-10 times their body weight. In order to maximize blood consumption, the tick concentrates the blood as it feeds by secreting a colorless fluid consisting of water and salts extracted from the blood from pores in the body.

Argasid ticks typically have a multi-host life-cycle (22, 42, 93, 99, 137, 138). Larvae emerge from eggs and attach to a host, feed to repletion, drop off, and molt into nymphs. Nymphs then feed on the same or a different host, drop off, and molt. Nymphs usually feed several times on multiple hosts and undergo 3 to 5 molts before becoming adults. Once adults, females may mate before or after feeding away from a host. After feeding to repletion, adult females drop off the host and lay several hundreds of eggs. Argasid females, like nymphs, are also capable of feeding numerous times and laying multiple batches of eggs several times during their lifetime.

Ixodidae

Ixodid ticks, which account for approximately 80% of all tick species worldwide, are subdivided into seven genera including *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Rhipicephalus*, and *Boophilus* (63). Ixodid tick species within each of the seven genera are important medical or veterinary pathogen transmission vectors (22). Ixodid ticks possess a scutum, or a tough, sclerotized plate, on their dorsal surface giving them the name “hard ticks” (6, 42, 137, 138) In adult males, the scutum covers the entire dorsal body surface, while it only covers a portion of the dorsal body surface in nymphs and adult females. As ixodid ticks are sensitive to environment humidity and temperature conditions, most of the body in nymphs and adult females, excluding the scutum, is covered by a waxy, tough, dense cuticle that prevents desiccation (138). The mouthparts of ixodid

ticks are very similar to argasid ticks and located at the anterior end of the body. However, unlike argasids, the body does not cover the mouthparts in ixodids and can be readily seen from the dorsal view (Fig 1.1B) (6, 42, 137, 138). Ixodid ticks are usually found in brushy, wooded, or weedy areas populated by medium or large sized mammals (42, 138).

The life-cycle and feeding characteristics of ixodid ticks are different from argasid ticks. Ixodid ticks display a two- or three-host life-cycle, as opposed to argasid ticks, which display a multi-host life-cycle (6, 42, 93, 137, 138). Larvae emerge from eggs and attach to small animals for their first blood meal. After feeding for several days to repletion, larvae drop off the host and molt into nymphs. In some ixodid species, larvae remain on the host after feeding and molt into nymphs. Nymphs then either seek a new host to feed or feed from the same host if the larvae remained on the host after the initial feeding. After the second feeding, the nymphs drop off the host and molt into either female or male adults. Adult ixodid ticks attach to a new host and begin feeding, and mating typically occurs during feeding. After feeding to repletion, the mated female adult drops off the host, lay thousands of eggs, and dies. When larvae emerge from the eggs, the cycle repeats itself. Unlike argasid ticks, ixodid ticks only ingest three blood meals and molt three times in their lifetime. Since ixodid ticks do not undergo multiple nymphal molts like argasid ticks, the ixodid life-cycle is typically shorter, usually 1-2 years depending on environmental humidity and temperature conditions, than the argasid tick life-cycle.

Feeding Characteristics of Ixodid Ticks

Ixodid ticks exhibit unique feeding characteristics, distinguishing them from argasid ticks and other blood feeding arthropods including flies, fleas, mosquitoes, and mites. Unlike

most blood sucking arthropods, ixodid ticks feed for several days on a single host, consuming large quantities of blood.

The majority of ixodid tick species find suitable hosts using a strategy termed "questing" (6, 22, 42, 137, 138), which consists of climbing blades of grass, weeds, or bushes and waiting for a host to brush against them. While questing, ticks remain hydrated by moving from their perch to the humid environment of the leafy ground cover and secreting a hygroscopic, salty saliva solution onto their hypostomes that adsorbs water from the surrounding atmosphere. Once rehydrated, the ticks climb back up the vegetation and continue waiting for a host. Ixodid and argasid ticks sense approaching hosts by several factors including shadows, vibrations, and odors. Ticks are especially attracted to carbon dioxide, which is found in host breath, and ammonia, which is found in host urine. When they finally sense a host, ticks stretch out their forelegs and cling to the hair or clothing of the host. Some species of ixodid ticks, in particular species of *Hyalomma*, actively hunt their hosts (22, 138). These ixodid ticks bury themselves in sand or dirt, preventing desiccation. When they sense a nearby host, they emerge from the ground and run towards the host.

Upon finding an appropriate host, ticks use the sensory organs in their palps to locate an attachment site (137, 138). Once an attachment site is found, the chelicerae of the tick begin slicing into the skin in an outward motion, creating a small hole (6, 42, 99, 137, 138). The tick then inserts the barbed hypostome into the hole and secretes a proteinaceous cement compound. The cement covers the hypostome and host skin, acting as an adhesive to anchor the hypostome in place. Since argasid ticks only feed for a few hours rather than several days, they do not secrete any cement upon attachment. In ixodid ticks, the process of attachment may take hours to days, but once firmly attached, the ticks are hard to remove.

After the tick is firmly cemented in place, blood feeding begins from a pool created by tissue and blood vessel damage during attachment (6, 93, 99, 138). For the first few days, feeding proceeds slowly as new cuticle is synthesized to allow expansion of the ixodid tick with the incoming blood meal. Once the new cuticle has been synthesized, feeding proceeds rapidly and the tick may increase its weight 10 (larvae and nymphs) to 100 (adult mated females) times its prefed weight (93, 99, 137, 138). Since adult ixodid males are covered by the rigid scutum and cannot expand easily, they usually do not ingest large amounts of blood when feeding. Feeding is discontinuous, characterized by periods of blood sucking alternating with periods of tick salivation (99, 138). In order to maximize blood consumption, portions of ixodid tick salivary glands function as water secreting compartments that remove excess water and salts to concentrate the blood meal (93, 99, 137, 138). When the tick is replete, it will drop off the host and either molt (larvae or nymphs) or lay eggs (adult mated females).

1.2 Host Responses to Tick Feeding

Ticks acquire a meal by sucking blood from a pool created during attachment, when the mouthparts cut into the skin, lacerating numerous small blood vessels and causing tissue destruction at the feeding site (93, 99, 145). The host is normally capable of detecting and repairing wounds through the processes of hemostasis and inflammation. Hemostatic responses prevent blood loss, potentially making the acquisition of a blood meal difficult for the tick (93, 120, 133, 145). Inflammation results in redness, swelling, and irritation at the feeding site, allowing the host to sense the presence of the tick, which potentially leads to host grooming and tick removal (99, 120, 133). In order to counteract the hemostatic and

inflammatory responses of the host, ticks produce multiple anti-hemostatic and anti-inflammatory mediators that are secreted into the host through the saliva during feeding.

In addition to hemostatic and inflammatory responses, other host immune responses may also be triggered during tick attachment and feeding. Host immune responses likely mediate recognition of multiple tick antigens, eventually resulting in rejection of the feeding tick (120). Tick rejection is normally characterized by a reduction in fed tick weights, altered feeding times, a reduction in the viability and number of ova produced, impaired molting, and death of the feeding tick (120, 133, 162). In addition to secreting anti-hemostatic and anti-inflammatory mediators, ticks also secrete immunosuppressive molecules in their saliva to prevent host immune recognition and rejection (93, 99, 120, 133).

1.3 Anti-Hemostatic Tick Salivary Components

Hemostasis, a host response generated during tissue injury, prevents blood loss from damaged tissues through platelet aggregation, vasoconstriction, and blood coagulation (7, 16, 93, 120, 133, 145). In order to obtain a complete blood meal successfully by avoiding host hemostatic responses, many blood sucking arthropods, including mosquitoes, flies, lice, fleas, mites, and ticks, secret salivary anti-hemostatic components into the host during feeding (7, 16, 17, 41, 120, 141). Argasid, and in particular ixodid ticks, which feed on a host for periods of several days, secrete a wide variety of anti-hemostatic molecules including platelet aggregation inhibitors, vasodilators, and anti-coagulants, which all aid the tick in overcoming host hemostasis.

Platelet Aggregation Inhibitors

Tissue damage leads to the exposure of various agonists, including adenosine diphosphate (ADP) and collagen, which cause platelet aggregation. In order to prevent platelet aggregation, many blood feeding arthropods secrete salivary apyrases, which are enzymes that hydrolyze adenosine triphosphate (ATP) and ADP into adenosine monophosphate (AMP) and inorganic phosphate. Eliminating ADP potentially prevents activation and aggregation of platelets (16, 93, 99, 133). Apyrase activity has been identified in the saliva or SGE of the soft ticks *Ornithodoros savignyi*, *Ornithodoros moubata*, and *Argas monolakensis*, as well as the hard tick, *Ixodes scapularis* (Table 1.1) (88, 89, 93, 99, 118, 120, 121). *O. moubata* also expresses moubatin and tick adhesion inhibitor (TAI), salivary gland proteins that specifically inhibit collagen induced platelet aggregation or adhesion, respectively (Table 1.1) (67, 70, 156).

Once platelets are activated by various agonists, they express glycoprotein IIbIIIa (GPIIbIIIa) on their surfaces. This integrin binds fibrinogen or von Willebrand's factor, resulting in platelet cross-linking and aggregation (7, 93, 145). In addition to apyrases, ticks have also developed salivary components that block GPIIbIIIa, preventing fibrinogen binding and platelet aggregation. *Dermacentor variabilis*, *O. moubata*, *O. savignyi*, and *A. monolakensis*, produce salivary gland proteins that bind GPIIbIIIa, inhibiting fibrinogen binding or displacing bound fibrinogen from the receptor (Table 1.1) (66, 88, 91, 92, 155). These activities either prevent platelet aggregation or cause aggregated platelets to disaggregate. *I. scapularis* saliva is also capable of causing platelet disaggregation through the proteolysis of fibrinogen (30). The presence of multiple platelet aggregation inhibitors in a variety of ticks indicates the necessity to inhibit this host response during feeding.

Vasodilators

Many blood feeding arthropods employ a variety of strategies to promote vasodilation, which counteracts vasoconstriction induced by platelet aggregation and increases the rate of blood flow to the feeding site (16). In order to maintain blood flow during their extended feeding periods, ixodid ticks produce and secret numerous salivary prostaglandins. As argasid ticks feed for much shorter periods, prostaglandins have not been detected in their saliva. Ixodid salivary prostaglandins are lipid molecules that promote smooth muscle relaxation, causing vasodilation (7, 12). Prostaglandin E_2 has been identified in the saliva of many ixodid ticks including, *Boophilus microplus*, *Haemaphysalis longicornis*, *Amblyomma americanum*, *Ixodes holocyclus*, and *I. scapularis* (Table 1.1) (3, 13, 23, 57, 119, 130). In addition, *I. scapularis* also produces prostaglandin I_2, while *A. americanum* produces prostaglandin F_2 (3, 119, 122) . Besides their primary functions in vasodilation, prostaglandins also display immunosuppressive effects and prevent platelet aggregation (12, 130).

Tissue damage and platelet activation and aggregation lead to elevated levels of Ca^{2+}, which potentially triggers vasoconstriction (7). Several ixodid tick species secrete salivary calreticulins, which bind Ca^{2+}, potentially preventing vasoconstriction (Table 1.1) (29, 37, 59, 128, 163). Similar to prostaglandins, calreticulins may also display anticoagulant and immunosuppressive functions in addition to vasodilatory activities as Ca^{2+} is required for a variety of host cell responses (14, 16, 59).

Anticoagulants

Blood coagulation is initiated through two different pathways, either the intrinsic or the extrinsic pathway (16, 93, 145). Both pathways converge at the step of factor X (FX)

activation into FXa. FXa activates prothrombin into thrombin, which then cleaves fibrinogen into fibrin. Fibrin then polymerizes, ultimately resulting in clot formation, which prevents blood loss from damaged tissues. Numerous blood feeding arthropods, including argasid and ixodid ticks, secrete anticoagulants into the host during feeding, inhibiting blood coagulation. Interestingly, single ixodid tick species typically express several anticoagulants, as opposed to argasid ticks, likely because of their prolonged feeding times. Most tick salivary anticoagulants inhibit either FXa or thrombin. Several species of *Ixodes*, *Hyalomma*, *Rhipicephalus* and *Ornithodoros* ticks secrete various salivary proteins that directly bind FXa, preventing it from activating prothrombin (Table 1.1) (5, 39, 55, 64, 86, 100, 101, 157). Additionally, species of *Ixodes*, *Boophilus*, *Haemaphysalis*, *Hyalomma*, *Amblyomma*, and *Ornithodoros* also secrete salivary proteins that directly bind thrombin, preventing it from cleaving fibrinogen (Table 1.1) (5, 19, 49, 50, 54, 58, 72, 88, 90, 97, 103, 148, 165, 166). Ticks also produce salivary components that inhibit blood coagulation prior to the activation of FX. *I. scapularis*, *Dermacentor andersoni*, and *O. savignyi* secrete salivary proteins that inhibit the extrinsic, or tissue factor pathway, and *H. longicornis* secretes an intrinsic pathway inhibitor (24, 31, 32, 43, 69). Presumably, inhibition of blood coagulation by various secreted salivary components is essential for successful argasid or ixodid tick feeding.

1.4 Anti-Inflammatory Tick Salivary Components

Tissue damage, platelet activation and aggregation, and activation of blood coagulation all trigger the induction of inflammation, a host response resulting in pain, itch, redness, and irritation at the site of tissue damage (120). Inflammation can lead to host

grooming, resulting in removal of a feeding tick (145). In order to obtain a blood meal without premature host removal during their extended feeding periods, ixodid ticks secrete an array of anti-inflammatory proteins including histamine-binding proteins, kininases, and anaphylatoxin inhibitors.

Histamine-binding proteins

Histamine and serotonin are essential mediators of inflammation that cause itching sensations, edema, and erythema by increasing vascular permeability (99, 133, 145). As ixodid ticks feed for several days, they have developed histamine-binding proteins (HBPs) to inhibit the activities of histamine and serotonin and prevent inflammation during feeding. *Rhipicephalus sanguineus* SGE inhibit the activity of histamine, and *Rhipicephalus appendiculatus* was found to express three salivary HBPs (18, 107). Homologues of these proteins have been identified in *I. scapularis* and *A. americanum* (Table 1.2) (11, 147). Additionally, *Dermacentor reticulatus*, secretes a salivary protein that binds both histamine and serotonin (Table 1.2) (129). These proteins are likely essential for successful tick feeding as RNAi knockdown of *A. americanum* HBPs prevented successful tick feeding (4).

Bradykinin Inhibitors

Another important mediator of inflammation is bradykinin, which is generated during activation of the intrinsic coagulation cascade. Bradykinin acts similarly to histamine, promoting pain, itch, and edema by increasing vascular permeability (99, 133, 147). *I. scapularis* secretes a carboxypeptidase that degrades bradykinin, while *B. microplus* secretes a serine protease inhibitor that inhibits kallikrein, preventing the formation of bradykinin (Table 1.2) (123, 140). Recently Ribeiro et al. demonstrated that sialostatin L and sialostatin L2 from *I. scapularis* saliva bind and inhibit cathepsin L, a protease implicated in kinin

generation, preventing inflammation (75, 76). Sialostatin L and sialostatin L2 also display immunosuppressive functions.

Anaphylatoxin Inhibitors

Anaphylatoxins are inflammatory mediators released during complement activation that induce vascular permeability, cause histamine release, and recruit inflammatory cells to sites of tissue damage (96, 145, 150, 151). *Ixodes ricinus* and *I. scapularis* secrete salivary proteins that inhibit the alternative complement pathway, preventing the production of anaphylatoxins (Table 1.2) (20, 116, 124, 143, 146). *O. moubata* also produces a complement inhibitor that binds C5, preventing its cleavage into the anaphylatoxin C5a (Table 1.2) (127). By preventing the generation of anaphylatoxins, ticks inhibit the induction of inflammation allowing them to feed successfully.

1.5 Immunosuppressive Tick Salivary Components

The host innate and adaptive immune responses are likely activated when blood sucking arthropods attach and begin feeding. These immune responses can lead to the induction of inflammation and the activation and production of various components that result in host immune recognition and rejection. Numerous blood sucking arthropods have evolved various salivary components to inhibit host innate and adaptive immune responses. As ixodid ticks uniquely feed on hosts for longer periods than most blood sucking arthropods, they have developed large families of salivary proteins that inhibit multiple host innate and adaptive immune mechanisms.

Inhibitors of endothelial cell adhesion molecule expression

During tissue damage or antigen stimulation, activated macrophages release cytokines that promote endothelial cells to express various surface adhesion molecules. These molecules allow circulating leukocytes to attach to endothelial cells and eventually traverse the blood vessel wall, migrating to sites of tissue damage. SGE from *D. andersoni* and *I. scapularis* significantly reduce the expression of several endothelial cell adhesion molecules *in vitro* and *in vivo* (Table 1.3) (87, 94). In addition, expression of adhesion molecules on leukocytes is also reduced by *I. scapularis* saliva *in vitro* (95). Expression of leukocyte adhesion molecules by endothelial cells and leukocytes is likely down-regulated during tick feeding, potentially preventing the initiation of innate immune responses and inflammation.

Inhibitors natural killer cells, neutrophils, and macrophages

Activated natural killer (NK) cells are important lymphocytes in innate immunity as they are cytotoxic cells and release interferon-γ (IFN-γ), a cytokine that activates macrophages (1, 7, 14). Neutrophils are polymorphonuclear leukocytes (PMN) that are activated during tissue damage and phagocytose invading organisms or cellular debris, inducing the production of reactive oxygen intermediates (ROIs) and releasing various granular constituents, including lysozyme. Macrophages are phagocytic cells important in innate and adaptive immunity. In innate immunity, macrophages function to phagocytose and kill invading microorganisms and secrete proinflammatory cytokines.

When SGE of *D. reticulatus*, *Amblyomma variegatum*, *Haemaphysalis inermis*, and *I. ricinus* were incubated with natural killer cells, the activity of the cells was substantially decreased (Table 1.3) (73, 79, 80). *I. scapularis* saliva also inhibited the activity of neutrophils, preventing the phagocytosis of *Borrelia burgdorferi* (95, 125). SGE of *I.*

ricinus, *R. sanguineus*, and *D. andersoni* inhibited the killing of intracellular parasites and *Borrelia afzelii*, a causative agent of Lyme disease in Europe, by activated macrophages *in vitro* (27, 81, 114). These inhibitory activities possibly prevent the activation of detrimental innate immune responses allowing successful tick feeding and facilitating efficient pathogen transmission.

Antioxidants

ROIs generated by activated neutrophils and macrophages promote inflammation, tissue damage, and killing of invading microorganisms (1). Even though ixodid ticks have rigid, sclerotized mouthparts that are likely not sensitive to ROIs, antioxidants, which inhibit the activities of ROIs, have been detected in the SGE of *I. scapularis* and *H. longicornis* (Table 1.3) (21, 142). These antioxidants potentially aid the tick during extended feeding periods by preventing inflammation and activation of innate immune responses and by possibly protecting tick gut tissue.

Cytokine and chemokine regulators

Cytokines and chemokines are vital for the initiation and development of innate and adaptive immune responses. Cytokines mediate various inflammatory responses and stimulate the activation and proliferation of lymphocytes and effector cells, i.e. macrophages, while chemokines are chemoattractants for circulating neutrophils, basophils, lymphocytes, and monocytes, recruiting them to sites of tissue damage and inflammation (14, 99). Since they are likely exposed to various immune components during their extended feeding periods, ixodid ticks have developed multiple strategies to inhibit the effects of cytokines and chemokines that include limiting their production and directly binding them to prevent their functions.

Multiple components in the saliva or SGE of *I. ricinus*, *I. scapularis*, *Ixodes pacificus*, *D. andersoni*, *R. appendiculatus*, and *R. sanguineus* reduce the expression and secretion of various proinflammatory cytokines, including IFN-γ, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6, thereby limiting the activation of inflammation and cell mediated immunity (T_H1 immune responses) (Table 1.3) (27, 28, 36, 44, 74, 77, 78, 85, 98, 111, 113, 131, 132). Interestingly, the production of immunosuppressive cytokines, such as IL-4 and IL-10, is usually unchanged or enhanced by the saliva or SGE from these same tick species. The production of IL-4 and IL-10 induces a T_H2, or antibody-dependent, immune response. In addition to producing factors that inhibit cytokines important for innate immunity, *I. scapularis* saliva also affects adaptive immunity by preventing secretion of IL-2, a cytokine important for T-cell and B-cell proliferation from activated T-cells, and by expressing a salivary protein that directly binds IL-2 (40, 144). Besides limiting the production and action of cytokines, components in saliva and SGE from *A. variegatum*, *D. reticulatus*, *H. inermis*, *I. ricinus*, *R. appendiculatus*, and *R. sanguineus* also bind and inhibit the activity of multiple chemokines, preventing the recruitment of immune cells to sites of tissue damage (Table 1.3) (33, 45, 46, 149).

Inhibition of antigen presenting cells

Dendritic cells (DCs) and macrophages are important antigen presenting cells (APCs) in adaptive immunity that stimulate antigen specific T-cells through antigen presentation. DCs and macrophages normally reside in peripheral tissues in an inactive state. Upon antigen or cytokine stimulation, activated DCs and macrophages engulf antigens and migrate to draining lymph nodes where they present antigens to $CD4^+$ T-cells. Saliva of *R.*

sanguineus inhibited the activation and migration of DCs *in vitro*, while *I. scapularis* saliva prevented $CD4^+$ antigen T-cell stimulation *in vitro* (15, 106, 130).

Inhibition of T-cell and B-cell proliferation

T- and B-cell activation is essential for the generation of adaptive immune responses, specifically cell-mediated immunity and antibody responses. APCs present antigens to $CD4^+$ T-cells through MHC class II complexes, causing activation, proliferation, and differentiation of the T-cells. Upon activation, $CD4^+$ T-cells secrete multiple cytokines and stimulate B-cell proliferation and maturation, which results in the generation of antibody secreting plasma cells (1). *I. scapularis* saliva contains a protein, Salp15, which inhibits the activation of $CD4^+$ T-cells by directly binding the CD4 coreceptor and inhibiting signaling pathways, resulting in a reduction of IL-2 production and inhibition of T-cell activation and proliferation (Table 1.3) (8, 38, 65). Recently, Salp15 homologues have been identified in *I. ricinus* and *I. pacificus*, and recombinant Iris, an immunosuppressive protein from *I. ricinus*, was found to inhibit T-cell proliferation *in vitro* (53, 85). *D. andersoni* and *R. appendiculatus* saliva also contains components that inhibits T-cell activation and proliferation *in vitro* (Table 1.3) (2, 9, 10, 27).

B-cell proliferation, which eventually leads to the generation of antibody secreting plasma cells, is also inhibited by ixodid tick saliva. Since IL-2 secreted by $CD4^+$ T cells is important for B-cell proliferation, the IL-2 inhibitory activities of *I. scapularis* saliva likely inhibit B-cell proliferation (8, 40). Proteins isolated from the saliva of *I. ricinus* and *Hyalomma asiaticum* inhibited lipopolysaccharide (LPS)-induced proliferation of B-cells *in vitro* (47, 164). Furthermore, *D. andersoni* and *R. sanguineus* ticks reduced antibody responses in tick infested animals, suggesting salivary secretions from these ticks suppress

antibody production, possibly through inhibition of B-cell activation and proliferation (56, 161). Interestingly, *B. microplus* saliva altered the isotype of antibodies produced in susceptible hosts (68). Ticks successfully avoid immune recognition by limiting T-cell and B-cell responses. In addition, inhibition of adaptive immune responses also potentially facilitates the transmission of multiple pathogens.

Immunoglobulin binding proteins

Besides limiting the activation and proliferation of B-cells, which ultimately prevents the production of antigen specific antibodies, ixodid also secrete salivary proteins that directly bind immunoglobulins. Immunoglobulin binding proteins were detected in the hemolymph and SGE of *R. appendiculatus*, *A. variegatum*, and *Ixodes hexagonus* (Table 1.3) (152-154). These proteins are speculated to be an important defense mechanism of the tick, allowing excretion host antibodies that may be detrimental to tick midgut tissues from the tick during feeding through the tick saliva.

1.6 Host Complement Pathways

Activation of the complement pathways is an important host innate immune response generated during tick feeding. Complement activation results in the production of anaphylatoxins, C3a and C5a, phagocytosis of opsonized invading organisms, and formation of the MAC in the outer surface of invading microbes leading to lysis of the organism (96, 150, 151). As the mouthparts of ticks are large, tough, rigid, sclerotized structures, they are unlikely to be affected by opsonization and MAC complexes. However, MAC complexes generated from host complement activation may form in the membranes of tick midgut cells during ingestion of a blood meal, potentially disrupting feeding and causing lethality.

Anaphylatoxins are important for the recruitment of inflammatory mediators to sites of tissue injury and subsequent immune responses, potentially causing detrimental effects on feeding ticks. Complement activation occurs through three different pathways, the MBL pathway, the classical pathway, and the alternative pathway (96, 150, 151). Activation of the alternative complement pathway likely mediates tick rejection (160), either through the induction of inflammation or immune responses, as ixodid ticks specifically produce salivary components that inhibit this pathway (116).

Classical and Mannose-Binding Lectin Complement Pathways

The classical complement pathway is activated when IgG or IgM bind specific epitopes on the surfaces of invading organisms (Fig 1.2) (96, 150, 151). C1q, a multimeric protein with globular head domains and a collagenous stalk, then binds the Fc protein of IgG or IgM. Two molecules of C1r and C1s in the presence of Ca^{2+} subsequently bind C1q between the globular head domains. After autoactivation, C1r cleaves and activates C1s, and C1s consequently cleaves C4 into C4a and C4b. C4b covalently binds to amino or hydroxyl groups on activating surfaces through an exposed thioester group. In the presence of Mg^{2+}, C2 then binds to C4b and is cleaved by an adjacent C1s into C2b and C2a. C2a remains bound to C4b, forming the classical pathway C3 convertase (C4b2a). The C3 convertase then cleaves C3 into C3b and C3a (Fig 1.2).

The MBL pathway is initiated when MBL, a multimeric molecule with globular domains and a collagenous stalk resembling C1q, binds mannose and N-acetyl glucosamine residues on the surfaces of invading microorganisms (Fig 1.2) (35, 96, 150, 151). After attaching to surfaces, MBL-associated serine protease-1 (MASP-1) and MASP-2 interact with MBL. Upon autoactivation, MASP-2 cleaves C4 and C2, generating the classical

pathway C3 convertase (C4b2a). The classical and MBL complement pathways converge with the alternative complement pathway at the step of C3 cleavage by the C3 convertase (Fig 1.2).

Alternative Complement Pathway

The alternative complement pathway is initiated when C3b covalently binds hydroxyl and amino groups on activating surfaces through an exposed thioester group (96, 109, 150, 151) (Figure 1.3). C3b is generated by the cleavage of C3 into C3a and C3b. C3 is a large protein, composed of two chains, α and β, held together by a disulfide bond. When C3 is cleaved, a small portion of the α-chain (C3a) is removed, leaving the remaining α-chain fragment bound to the β-chain (C3b). Once C3b is covalently attached to a surface, fB then binds C3b in an Mg^{2+} dependent manner (Fig 1.2). fD cleaves bound fB, releasing Ba and generating the alternative pathway C3 convertase (C3bBb). The C3 convertase, either C4b2a or C3bBb, cleaves additional molecules of C3 into C3b and C3a, amplifying the complement cascades. C3b then covalently binds more surfaces, forming more C3 convertases or causing opsonization. C3b also binds to C3 convertases to form C5 convertases (C3bBbC3b or C4bC2a3b).

Small of amounts of C3 are constantly hydrolyzed in biological fluids. In order to generate C3b for alternative pathway initiation, hydrolyzed C3 ($C3(H_2O)$), which functionally resembles C3b, binds fB in solution (96, 110). fB is then cleaved by fD and the resulting complex, $C3(H_2O)Bb$, cleaves C3, producing C3b that covalently attaches to activating surfaces. Activation of the classical and MBL pathways also leads to the generation of C3b, which initiates the alternative pathway.

The late steps of complement activation result in the formation of the MAC. C5 convertases assembled from the classical, MBL, or alternative pathways cleave C5 into C5a and C5b (Fig 1.2) (96, 150, 151). C5b, which is still attached to the C5 convertase, then binds C6. Upon binding C6, C5 undergoes a conformational change exposing a membrane and C7 binding site. When C7 binds to C5b, the resulting complex, C5b67, is released and associates with membranes. C8 then binds C7, causing the complex, C5b-8, to insert into the membrane and form a small pore. Multiple C9 molecules then bind the complex causing the formation of a large pore, the MAC, and lysis of the cell.

Regulators of the Alternative Complement Pathway

The alternative complement pathway is both positively and negatively regulated by various factors. Properdin is the only characterized natural positive regulator of the alternative pathway. Properdin is a soluble plasma protein that directly binds both C3b, which increases its affinity for fB, and C3bBb, increasing its half-life more than ten-fold (26, 51, 96). Recent evidence has also demonstrated that properdin binds activating surfaces independently of C3b, thereby acting as a platform to recruit C3b and fB to the activating surface and initiate assembly of the C3 convertase (139). Kimura et al. have established that serum from properdin deficient (properdin$^{-/-}$) mice is incapable of alternative pathway complement activation in response to bacterial LPS or lipooligosaccharide (LOS) (71). Furthermore, Crry-deficient erythrocytes, which are susceptible to the alternative complement pathway *in vivo*, were not cleared when injected into properdin$^{-/-}$ mice. The findings of these studies support an essential role for properdin in the initiation of the alternative complement pathway, in addition to its established role in C3 convertase stabilization.

Several negative regulators of the alternative complement pathway are characterized, including fH, DAF, complement receptor 1 (CR1), and membrane cofactor protein (MCP) (96). fH, a soluble plasma protein, directly interacts with C3b to cause displacement of Bb in the C3 convertase, resulting in destabilization and decay of the C3 convertase (108, 126, 158, 159, 167). fH also acts a cofactor for the serine protease fI, which cleaves C3b resulting in the formation of inactive C3b (iC3b) (Fig 1.3) (108, 126, 158, 159, 167). Interestingly, when bound to C3b, properdin protects C3b from fH/fI mediated cleavage (96). fH binding to C3b is primarily determined by the surface to which C3b is bound. Deposition of "tickover" C3b is constantly occurring on host cell surfaces, but complement is not activated because negatively charged host cell surfaces rich in sialic acid promote binding of fH to C3b rather than fB (Fig 1.3). Therefore, C3b is degraded when bound to host cell surfaces. If C3b binds to the surface of invading microbes, which are deficient in sialic acid and are not negatively charged, fB favorably binds C3b rather than fH, promoting activation of the alternative complement pathway (96, 150).

DAF, a membrane anchored protein found on most cells including endothelial cells, erythrocytes, and leukocytes, protects the cells from the destructive effects of complement activation by directly binding the C3 convertase and accelerating its dissociation (34, 48, 52, 102, 108). CR1, a membrane anchored protein found on erythrocytes, monocytes, macrophages, lymphocytes, neutrophils, and eosinophils, acts as a decay accelerating factor, similar to DAF, and mediates fI degradation of C3b, similar to fH (25, 108, 126). MCP, a membrane anchored protein found on circulating cells including platelets, granulocytes, lymphocytes, NK cells and monocytes, specifically binds C3b and acts as a cofactor for fI mediated degradation of C3b, similar to fH (96, 134, 135). These negative regulators of the

alternative complement pathway serve important roles in protecting host cells from damage generated by inappropriate complement activation.

1.7 Anti-complement Tick Salivary Proteins

Activation of the alternative complement pathway is an important host innate immune response triggered during tick feeding. Ixodid and argasid ticks have evolved various mechanisms to inhibit complement activation, preventing the recruitment of inflammatory mediators and the induction of an inflammatory response. *I. scapularis* saliva was originally found to specifically inhibit the alternative complement pathway as measured *in vitro* by the lysis of rabbit erythrocytes in the presence of NHS (116). Additionally, Ribeiro also determined that C3b deposition on activating surfaces and C3a release were inhibited in the presence of *I. scapularis* saliva. Several years later, Valenzuela et al. purified and characterized Isac from pooled SGE, which inhibited the alternative complement pathway (146). Recombinant Isac (rIsac) expressed in transformed monkey kidney cells (COS-7) inhibited the alternative complement pathway by preventing the assembly of C3 convertases (C3bBb) on activating surfaces and dissociating preformed C3 convertases. In addition to the anti-complement activity present in *I. scapularis* saliva, Lawrie et al. demonstrated that other ixodid tick saliva, specifically *I. ricinus, I. hexagonus*, and *I. uriae* saliva, also inhibited the alternative complement pathway (83). *I. ricinus* SGE prevented the deposition of C3b on activator surfaces, the generation of C3a, and the cleavage of fB, suggesting inhibition of C3 convertase formation (83, 84). Recently, Daix et al. identified and expressed two members of a large family of anti-complement proteins in *I. ricinus* that share homology to Isac (20).

These proteins, Irac-1 and Irac-2, inhibit the alternative complement pathway by preventing the formation of C3 convertases and dissociating preformed convertases, similar to Isac.

Das et al. identified *I. scapularis* Isac homologues, Salp20 and Salp9, by screening a tick salivary gland cDNA library with serum from tick immune guinea pigs. Salp20 and Salp9 have predicted masses of 20 kDa and 9 kDa, respectively, and share 83% and 50% identity to Isac at the amino acid level (21). Together with Isac, Salp20 and Salp9 potentially comprise a family of *I. scapularis* anti-complement proteins, the ILP family. In support of this observation, Soares et al. and Ribeiro et al. have identified several other potential members of this family that share sequence homology with Isac (117, 136).

The argasid tick, *O. moubata*, also expresses a salivary lipocalin, OmCI, which inhibits the classical and alternative complement pathways as measured by erythrocyte lysis (104, 127). Recombinant OmCI produced in yeast cells contained N-linked glycosylations and prevented the generation of C5a in the presence of C5 convertases. Nunn et al. further demonstrated that OmCI directly bound C5 preventing its cleavage by the C5 convertase. OmCI shares no homology with ILP family members or characterized regulators of complement.

1.8 Saliva Activated Transmission of Pathogens

Not only do the anti-hemostatic, anti-inflammatory, and immunosuppressive components of tick saliva facilitate the tick during feeding, but they may also mediate saliva activated transmission (SAT) of various pathogens. SAT is defined as the promotion of tick-borne pathogen transmission through the action of tick salivary components on the host (105). As the tick is salivating during feeding, it is secreting its anti-hemostatic, anti-

inflammatory, and immunosuppressive components into the host and creating an immunosuppressed environment in which the pathogen enters. Several tick-borne viruses and bacteria, including various species of *Borrelia*, have been shown to display enhanced infectivity in the presence of tick SGE (60-62, 82, 112). In fact, *I. scapularis* Salp15 directly binds outer surface protein C (OspC) of *B. burgdorferi*, enhancing transmission of the pathogen to a murine host (115). Currently, researchers are trying to characterize various tick salivary components with the goal of ultimately developing anti-tick vaccines that target different salivary components, potentially preventing successful tick feeding and pathogen transmission.

1.9 Overview of thesis

A large family of *I. scapularis* salivary proteins related to Isac, the ILP family, which includes Salp20, has been discovered. The function of additional related ILP family members and the necessity for such a large family of proteins during tick feeding are currently unknown. As Salp20 and other ILP family members share substantial homology with Isac, it is likely they also display anti-complement activity. Since ticks produce multiple members of this family, individual proteins likely display either structural or functional variability. In order to address these possibilities, we have expressed several members of the ILP family and determined their functions. In Chapter 2, we describe the expression and characterization of Salp20, which shares significant amino acid identity with Isac. We also examine the potential role of this protein in the protection of various *Borrelia* species, suggesting a role for this salivary protein in pathogen transmission. In Chapter 3, we determine the specific mechanism of complement inhibition used by Salp20. We next

determine the functional activities and mechanisms of complement inhibition for additional ILP members in Chapter 4, with the goal of elucidating any differences that may exist between these family members. We attempt to determine the purpose of producing multiple ILP family members. Finally, in Chapter 5, we examine the necessity and importance of ILP family members during tick feeding. We discuss the significance of these studies and the future goals of this project in Chapter 6.

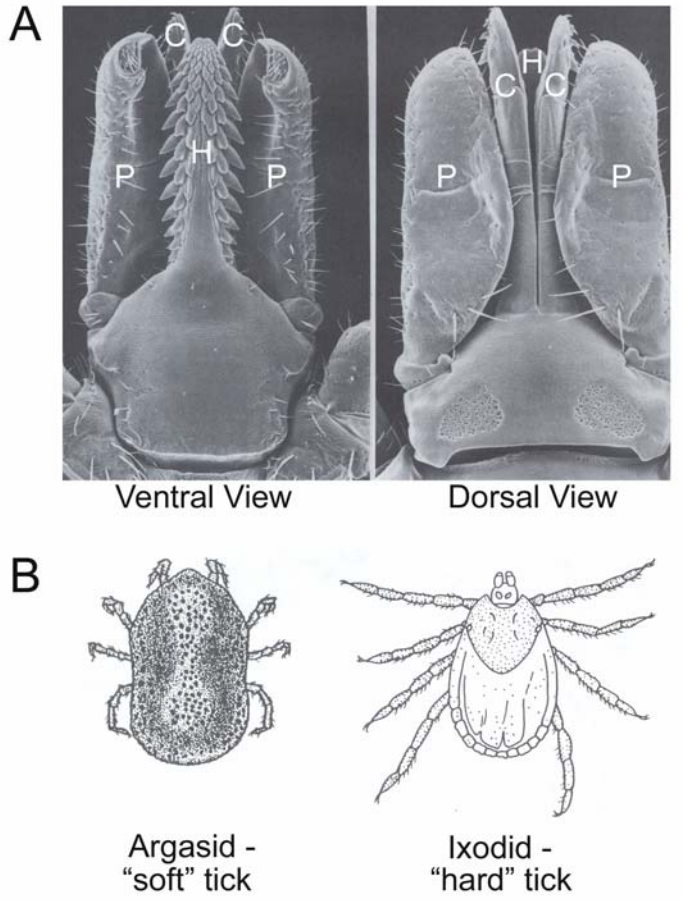


Figure 1.1 Mouthparts and depictions of ticks. ***A***. Ventral and dorsal views of the mouthparts of *Ixodes scapularis*, containing the palps (P), chelicerae (C), and hypostome (H), are pictured. ***B***. Illustrations of representative “soft” and “hard” ticks are depicted. Figure adapted from (137) and (42).

	Activity	Protein	Found in:	Ref.
Platelet Aggregation Inhibitors	hydrolyzes ATP and ADP into AMP	apyrase	*Argas monolakensis*	88
			Ixodes scapularis	121
			Ornithodoros moubata	118
			Ornithodoros savignyi	89
	bind integrin GPIIbIIIa	MG1	*Argas monolakensis*	88
		MG2		88
		variabilin	*Dermacentor variablis*	155
		disagregin	*Ornithodoros moubata*	66
		savignygrin	*Ornithodoros savignyi*	91, 92
	proteolysis of fibrinogen		*Ixodes scapularis*	30
	inhibits collagen induced platelet aggregation	TAI	*Ornithodoros moubata*	67
		moubatin		70, 156
Vasodilators	relax smooth muscles	prostaglandin E_2	*Amblyomma americanum*	3, 13, 119
			Boophilus microplus	23, 57
			Haemaphysalis longicornis	57
			Ixodes holocyclus	57
			Ixodes scapularis	130
		prostaglandin I_2		122
		prostaglandin F_2	*Amblyomma americanum*	3, 13, 119
	bind Ca^{2+}	calreticulin	*Amblyomma americanum*	59, 128
			Boophilus microplus	29
			Dermacentor variablis	163
			Haemaphysalis longicornis	163
			Haemaphysalis qinghaiensis	37
			Ixodes scapularis	163
			Rhipicephalus sanguineus	163
Anticoagulants	inhibit FXa activity	NTI-1	*Hyalomma dromedarii*	55
		NTI-2		55
			Hyalomma truncatum	64
			Ixodes holocyclus	5
		Salp14	*Ixodes scapularis*	100
		TAP	*Ornithodoros moubata*	101, 157
			Ornithodoros savignyi	39
			Rhipicephalus appendiculatus	86
	inhibit thrombin activity	americanin	*Amblyomma americanum*	165, 166
		variegin	*Amblyomma variegatum*	72
		monobin	*Argas monolakensis*	88
		calcaratin	*Boophilus calcaratus*	97
		BmAP	*Boophilus microplus*	50
		microphilin		19
		madanin 1	*Haemaphysalis longicornis*	58
		madanin 2		58
		NTI-1	*Hyalomma dromedarii*	54
		NTI-2		54
			Ixodes holocyclus	5
		ixin	*Ixodes ricinus*	49
		ornithodorin	*Ornithodoros moubata*	148
		savignin	*Ornithodoros savignyi*	90, 103
	inhibit FVII, FV		*Dermacentor andersoni*	43
	inhibit FVIIa/TF complex	Ixolaris	*Ixodes scapularis*	32
		Penthalaris		31
	inhibit FXII activity	haemaphysalin	*Haemaphysalis longicornis*	69
	inhibit extrinsic pathway	BSAP-1	*Ornithodoros savignyi*	24
		BSAP-2		24

Table 1.1 Anti-hemostatic tick salivary proteins.

	Activity	Protein	Found In:	Ref.
Histamine-binding proteins	bind histamine		*Amblyomma americanum*	11
			Ixodes scapularis	147
		Ra-HBP1	*Rhipicephalus appendiculatus*	107
		Ra-HBP2		107
		Ra-HBP3		107
			Rhipicephalus sanguineus	18
	bind histamine and serotonin	SHBP	*Dermacentor reticulatus*	11, 129
Bradykinin inhibitors	inhibits kallikrein	BmTI-A	*Boophilus microplus*	140
	degrades bradykinin	carboxypeptidase	*Ixodes scapularis*	123
	inhibit cathepsin L	sialostatin L		76
		sialostatin L2		75
Anaphylatoxin Inhibitors	inhibit the alternative complement pathway	IRACI	*Ixodes ricinus*	20
		IRACII		20
		Isac	*Ixodes scapularis*	146
		Salp20		143
	binds C5	OmCI	*Ornithodoros moubata*	127

Table 1.2 Anti-inflammatory tick salivary proteins.

	Activity	Protein	Found In:	Ref
Antagonists of leukocyte adhesion	reduce espression of ICAM-1, LFA-1, VLA-4		*Dermacentor andersoni*	87
	reduce expression of P-selectin, VCAM-1		*Ixodes scapularis*	94
	reduce expression of LFA-1			95
Natural killer cell, neutrophil, and macrophage inhibitors	impair phagocytosis and killing activity		*Amblyomma variegatum*	80
			Dermacentor andersoni	114
			Dermacentor reticulatus	79
			Haemaphysalis inermis	80
			Ixodes ricinus	73, 81
			Ixodes scapularis	95, 125
			Rhipicephalus sanguineus	27
Antioxidants	peroxiredoxin	HlPrx	*Haemaphysalis longicornis*	142
	glutathione peroxidase	Salp25D	*Ixodes scapularis*	21
Cytokine regulators	inhibition of IL-2, IFN-γ, IL-12, IL-1, TNF-α, IL-6 unaffect or enhance IL-4 and IL-10 expression		*Dermacentor andersoni*	113
			Ixodes pacificus	132
		Iris	*Ixodes ricinus*	36, 74, 77, 78, 85, 111
			Ixodes scapularis	40, 98, 131, 132, 144
			Rhipicephalus appendiculatus	44
			Rhipicephalus sanguineus	27, 28
Chemokine inhibitors	inhibit IL-8, MCP-1, MIP-1α, RANTES, exotaxin		*Amblyomma variegatum*	45, 46, 149
			Dermacentor reticulatus	45, 46
			Haemaphysalis inermis	45
			Ixodes ricinus	45, 46
			Rhipicephalus appendiculatus	45
			Rhipicephalus sanguineus	33
Inhibitors of dendritic cell migration	inhibits production of IL-12 and TNF-α by DCs	PGE_2	*Ixodes scapularis*	130
	suppresses DC stimulated CD4+ T cell activation			130
	downregulates CCR5 and reduces migration towards MIP-1α		*Rhipicephalus sanguineus*	15, 106
Inhibitors of T-cell proliferation	binds T-cell CD4 coreceptor	Salp15	*Ixodes scapualris*	8, 38, 65
	suppresses T-cell activation and proliferation	p36	*Dermancentor andersoni*	2, 9, 10
		Iris	*Ixodes ricinus*	53, 85
			Rhipicephalus sanguineus	27
Inhibitors of B-cell proliferation	suppresses B-cell activation and proliferation	BIF	*Hyalomma asiaticum*	164
		BIP	*Ixodes ricinus*	47
Immunoglobulin binding proteins	bind host IgG	IGBP	*Amblyomma variegatum*	154
			Ixodes hexagonus	154
			Rhipicephalus appendiculatus	152, 153,154
Anticomplement proteins	prevent the formation and cause dissociation of C3bBb	Irac-I	*Ixodes ricinus*	20
		Irac-II		20
		Isac	*Ixodes scapularis*	146
		Salp20		143
	binds C5	OmCI	*Ornithodoros moubata*	104, 127

Table 1.3 Immunosuppressive tick salivary proteins.

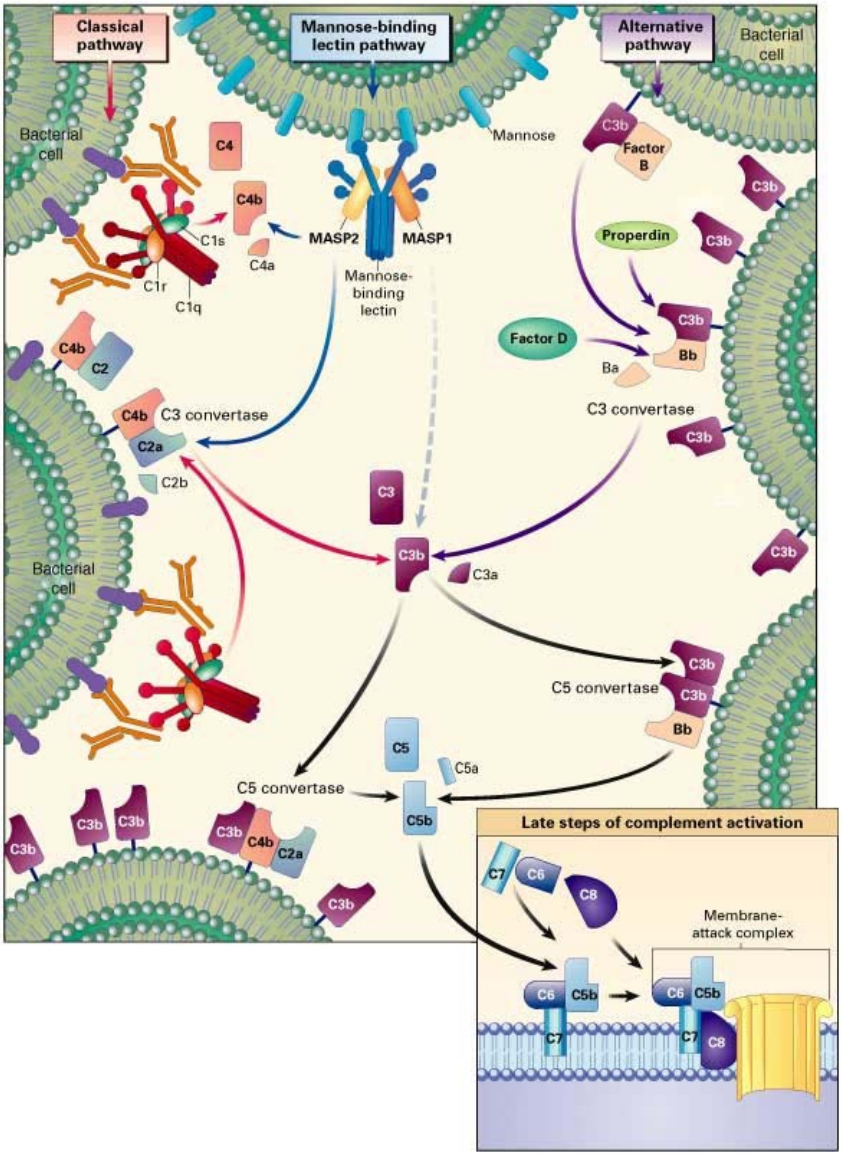


Figure 1.2 The classical, alternative, MBL complement pathways. The classical pathway is initiated when C1q binds surface bound IgG or IgM. C1s and C1r then bind C1q, and C1s cleaves C4 into C4a and C4b. C4b covalently binds an activating surface, allowing C2 to bind. C2 is then cleaved by C1s, resulting in the formation of the C3 convertase, C4b2a. The MBL pathway is initiated when MBL binds carbohydrate residues on activating surfaces. MASP-1 and MASP-2 then bind MBL, and MASP-2 cleaves C4 and C2 to form the C3 convertase, C4b2a. The alternative pathway is initiated when C3b covalently binds activating surfaces. Factor B then binds C3b and is cleaved by fD, resulting in the formation of the C3 convertase, C3bBb, which is stabilized by properdin. The C3 convertase cleaves additional C3 molecules that opsonize surfaces, generate additional convertases, or bind C3 convertases to produce C5 convertases. C5 convertases cleave C5, which initiates activation of the late steps of complement and ultimately leads to the formation of MACs in the cell membrane. Figure taken from (150) with permission from publisher.

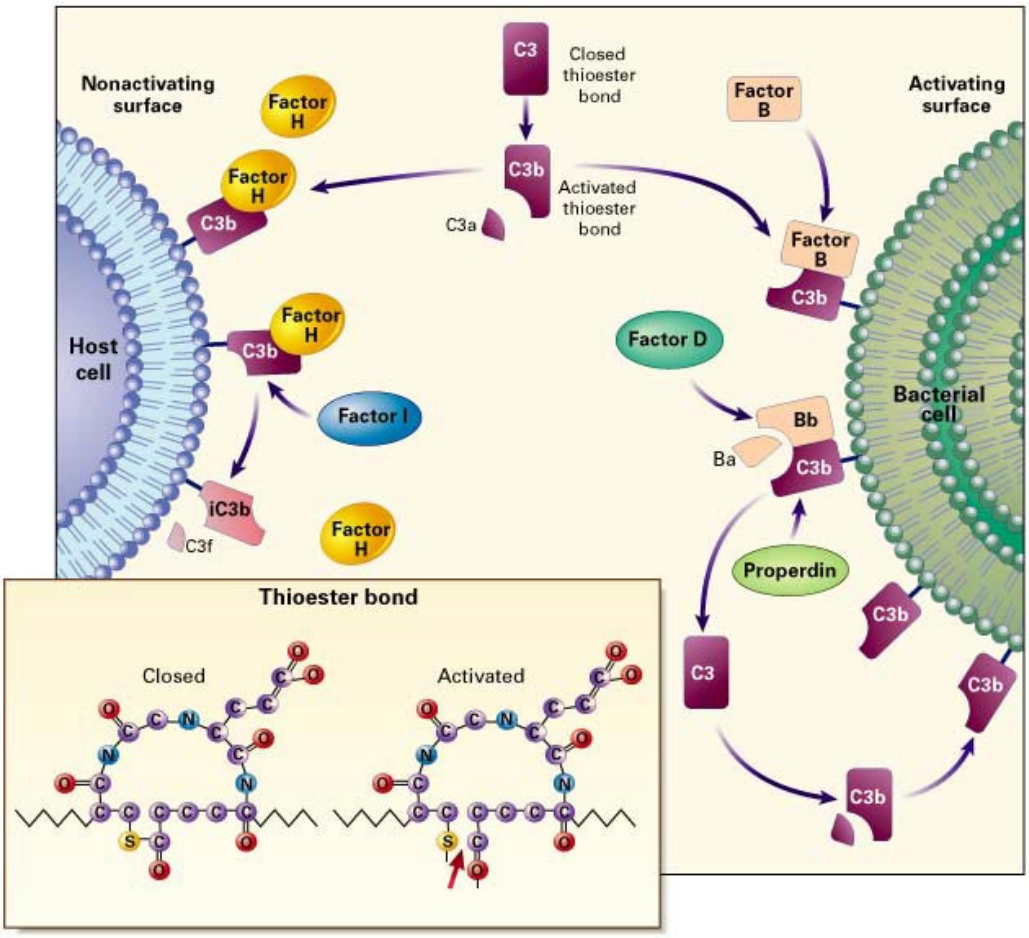


Figure 1.3 Regulatory activities of fH and fI in the presence of bound C3b. C3 is cleaved, generating C3b, which contains an exposed thioester group. This thioester allows C3b to bind covalently to the surfaces of either host cells or invading microbes. If C3b binds to host cell surfaces, which are typically negatively charged due to the presence of sialic acid, fH preferably binds to C3b. fH then acts as a cofactor for fI mediated degradation of C3b into inactive C3b (iC3b) and C3f. If C3b binds to microbial surfaces, which are typically not negatively charged due to a lack of sialic acids, fB preferably binds C3b. fB is subsequently cleaved by fD, releasing Ba and leaving Bb bound to C3b, or the C3 convertase. The C3 convertase is stabilized by properdin and cleaves additional C3 molecules into C3b and C3a. C3b then binds more sites on the activating surface or binds the C3 convertase to form a C5 convertase. Figure taken from (150) with permission from the publisher.

1.10 References

1. Abbas, A. K., Lichtman A. H., Pober J. S., 2000. *Cellular and Molecular Immunology*. 4th ed. W.B. Saunders Company, Philadelphia, PA.

2. Alarcon-Chaidez, F. J., U. U. Muller-Doblies, and S. Wikel. 2003. Characterization of a recombinant immunomodulatory protein from the salivary glands of *Dermacentor andersoni. Parasite Immunol*. 25: 69-77.

3. Aljamali, M., A. S. Bowman, J. W. Dillwith, J. S. Tucker, G. W. Yates, R. C. Essenberg, and J. R. Sauer. 2002. Identity and synthesis of prostaglandins in the lone star tick, *Amblyomma americanum* (L.), as assessed by radio-immunoassay and gas chromatography/mass spectrometry. *Insect Biochem Mol Biol*. 32: 331-41.

4. Aljamali, M. N., A. D. Bior, J. R. Sauer, and R. C. Essenberg. 2003. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum. Insect Mol Biol*. 12: 299-305.

5. Anastopoulos, P., M. J. Thurn, and K. W. Broady. 1991. Anticoagulant in the tick *Ixodes holocyclus. Aust Vet J*. 68: 366-7.

6. Anderson, J. F. 2002. The natural history of ticks. *Med Clin North Am*. 86: 205-18.

7. Andrade, B. B., C. R. Teixeira, A. Barral, and M. Barral-Netto. 2005. Haematophagous arthropod saliva and host defense system: a tale of tear and blood. *An Acad Bras Cienc*. 77: 665-93.

8. Anguita, J., N. Ramamoorthi, J. W. Hovius, S. Das, V. Thomas, R. Persinski, D. Conze, P. W. Askenase, M. Rincon, F. S. Kantor, and E. Fikrig. 2002. Salp15, an *Ixodes scapularis* salivary protein, inhibits CD4(+) T cell activation. *Immunity*. 16: 849-59.

9. Bergman, D. K., M. J. Palmer, M. J. Caimano, J. D. Radolf, and S. K. Wikel. 2000. Isolation and molecular cloning of a secreted immunosuppressant protein from *Dermacentor andersoni* salivary gland. *J Parasitol*. 86: 516-25.

10. Bergman, D. K., R. N. Ramachandra, and S. K. Wikel. 1998. Characterization of an immunosuppressant protein from *Dermacentor andersoni* (Acari: Ixodidae) salivary glands. *J Med Entomol*. 35: 505-9.

11. Bior, A. D., R. C. Essenberg, and J. R. Sauer. 2002. Comparison of differentially expressed genes in the salivary glands of male ticks, *Amblyomma americanum* and *Dermacentor andersoni. Insect Biochem Mol Biol*. 32: 645-55.

12. Bowman, A. S., J. W. Dillwith, and J. R. Sauer. 1996. Tick salivary prostaglandins: Presence, origin and significance. *Parasitol Today*. 12: 388-96.

13. Bowman, A. S., J. R. Sauer, K. Zhu, and J. W. Dillwith. 1995. Biosynthesis of salivary prostaglandins in the lone star tick, *Amblyomma americanum*. *Insect Biochem Mol Biol*. 25: 735-41.

14. Brossard, M. and S. K. Wikel. 2004. Tick immunobiology. *Parasitology*. 129 Suppl: S161-76.

15. Cavassani, K. A., J. C. Aliberti, A. R. Dias, J. S. Silva, and B. R. Ferreira. 2005. Tick saliva inhibits differentiation, maturation and function of murine bone-marrow-derived dendritic cells. *Immunology*. 114: 235-45.

16. Champagne, D. E. 2004. Antihemostatic strategies of blood-feeding arthropods. *Curr Drug Targets Cardiovasc Haematol Disord*. 4: 375-96.

17. Champagne, D. E. 2005. Antihemostatic molecules from saliva of blood-feeding arthropods. *Pathophysiol Haemost Thromb*. 34: 221-7.

18. Chinery, W. A. and E. Ayitey-Smith. 1977. Histamine blocking agent in the salivary gland homogenate of the tick *Rhipicephalus sanguineus sanguineus. Nature*. 265: 366-7.

19. Ciprandi, A., S. K. de Oliveira, A. Masuda, F. Horn, and C. Termignoni. 2006. *Boophilus microplus*: its saliva contains microphilin, a small thrombin inhibitor. *Exp Parasitol*. 114: 40-6.

20. Daix, V., H. Schroeder, N. Praet, J. P. Georgin, I. Chiappino, L. Gillet, K. de Fays, Y. Decrem, G. Leboulle, E. Godfroid, A. Bollen, P. P. Pastoret, L. Gern, P. M. Sharp, and A. Vanderplasschen. 2007. *Ixodes* ticks belonging to the *Ixodes ricinus* complex encode a family of anticomplement proteins. *Insect Mol Biol*. 16: 155-66.

21. Das, S., G. Banerjee, K. DePonte, N. Marcantonio, F. S. Kantor, and E. Fikrig. 2001. Salp25D, an *Ixodes scapularis* antioxidant, is 1 of 14 immunodominant antigens in engorged tick salivary glands. *J Infect Dis*. 184: 1056-64.

22. Dennis, D. T., Piesman, J. F., *Overview of Tick-Borne Infections of Humans*, In *Tick-Borne Diseases of Humans*, D.T.D. Jesse L. Goodman, Daniel E. Sonenshine, Editor. 2005, American Society for Microbiology Press: Washingtion, DC. p. 3-11.

23. Dickinson, R. G., J. E. O'Hagan, M. Schotz, K. C. Binnington, and M. P. Hegarty. 1976. Prostaglandin in the saliva of the cattle tick *Boophilus microplus. Aust J Exp Biol Med Sci*. 54: 475-86.

24. Ehebauer, M. T., B. J. Mans, A. R. Gaspar, and A. W. Neitz. 2002. Identification of extrinsic blood coagulation pathway inhibitors from the tick *Ornithodoros savignyi* (Acari: Argasidae). *Exp Parasitol*. 101: 138-48.

25. Fearon, D. T. 1979. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc Natl Acad Sci U S A*. 76: 5867-71.

26. Fearon, D. T. and K. F. Austen. 1975. Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J Exp Med*. 142: 856-63.

27. Ferreira, B. R. and J. S. Silva. 1998. Saliva of *Rhipicephalus sanguineus* tick impairs T cell proliferation and IFN-gamma-induced macrophage microbicidal activity. *Vet Immunol Immunopathol*. 64: 279-93.

28. Ferreira, B. R. and J. S. Silva. 1999. Successive tick infestations selectively promote a T-helper 2 cytokine profile in mice. *Immunology*. 96: 434-9.

29. Ferreira, C. A., I. Da Silva Vaz, S. S. da Silva, K. L. Haag, J. G. Valenzuela, and A. Masuda. 2002. Cloning and partial characterization of a *Boophilus microplus* (Acari: Ixodidae) calreticulin. *Exp Parasitol*. 101: 25-34.

30. Francischetti, I. M., T. N. Mather, and J. M. Ribeiro. 2003. Cloning of a salivary gland metalloprotease and characterization of gelatinase and fibrin(ogen)lytic activities in the saliva of the Lyme disease tick vector *Ixodes scapularis*. *Biochem Biophys Res Commun*. 305: 869-75.

31. Francischetti, I. M., T. N. Mather, and J. M. Ribeiro. 2004. Penthalaris, a novel recombinant five-Kunitz tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick vector of Lyme disease, *Ixodes scapularis*. *Thromb Haemost*. 91: 886-98.

32. Francischetti, I. M., J. G. Valenzuela, J. F. Andersen, T. N. Mather, and J. M. Ribeiro. 2002. Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, *Ixodes scapularis*: identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. *Blood*. 99: 3602-12.

33. Frauenschuh, A., C. A. Power, M. Deruaz, B. R. Ferreira, J. S. Silva, M. M. Teixeira, J. M. Dias, T. Martin, T. N. Wells, and A. E. Proudfoot. 2007. Molecular cloning and characterization of a highly selective chemokine-binding protein from the tick *Rhipicephalus sanguineus*. *J Biol Chem*. 282: 27250-8.

34. Fujita, T., T. Inoue, K. Ogawa, K. Iida, and N. Tamura. 1987. The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J Exp Med*. 166: 1221-8.

35. Gadjeva, M., S. Thiel, and J. C. Jensenius. 2001. The mannan-binding-lectin pathway of the innate immune response. *Curr Opin Immunol*. 13: 74-8.

36. Ganapamo, F., B. Rutti, and M. Brossard. 1995. *In vitro* production of interleukin-4 and interferon-gamma by lymph node cells from BALB/c mice infested with nymphal *Ixodes ricinus* ticks. *Immunology*. 85: 120-4.

37. Gao, J., J. Luo, R. Fan, V. Fingerle, G. Guan, Z. Liu, Y. Li, H. Zhao, M. Ma, J. Liu, A. Liu, Q. Ren, Z. Dang, C. Sugimoto, and H. Yin. 2007. Cloning and characterization of a cDNA clone encoding calreticulin from *Haemaphysalis qinghaiensis* (Acari: Ixodidae). *Parasitol Res*.

38. Garg, R., I. J. Juncadella, N. Ramamoorthi, Ashish, S. K. Ananthanarayanan, V. Thomas, M. Rincon, J. K. Krueger, E. Fikrig, C. M. Yengo, and J. Anguita. 2006. Cutting edge: CD4 is the receptor for the tick saliva immunosuppressor, Salp15. *J Immunol*. 177: 6579-83.

39. Gaspar, A. R., A. M. Joubert, J. C. Crause, and A. W. Neitz. 1996. Isolation and characterization of an anticoagulant from the salivary glands of the tick, *Ornithodoros savignyi* (Acari: Argasidae). *Exp Appl Acarol*. 20: 583-98.

40. Gillespie, R. D., M. C. Dolan, J. Piesman, and R. G. Titus. 2001. Identification of an IL-2 binding protein in the saliva of the Lyme disease vector tick, *Ixodes scapularis. J Immunol*. 166: 4319-26.

41. Gillespie, R. D., M. L. Mbow, and R. G. Titus. 2000. The immunomodulatory factors of bloodfeeding arthropod saliva. *Parasite Immunol*. 22: 319-31.

42. Goddard, J., 1993. *Physician's Guide to Arthropods of Medical Importance*. CRC Press, Boca Raton, FL.

43. Gordon, J. R. and J. R. Allen. 1991. Factors V and VII anticoagulant activities in the salivary glands of feeding *Dermacentor andersoni* ticks. *J Parasitol*. 77: 167-70.

44. Gwakisa, P., K. Yoshihara, T. Long To, H. Gotoh, F. Amano, and E. Momotani. 2001. Salivary gland extract of *Rhipicephalus appendiculatus* ticks inhibits *in vitro* transcription and secretion of cytokines and production of nitric oxide by LPS-stimulated JA-4 cells. *Vet Parasitol*. 99: 53-61.

45. Hajnicka, V., P. Kocakova, M. Slavikova, M. Slovak, J. Gasperik, N. Fuchsberger, and P. A. Nuttall. 2001. Anti-interleukin-8 activity of tick salivary gland extracts. *Parasite Immunol*. 23: 483-9.

46. Hajnicka, V., I. Vancova, P. Kocakova, M. Slovak, J. Gasperik, M. Slavikova, R. S. Hails, M. Labuda, and P. A. Nuttall. 2005. Manipulation of host cytokine network by ticks: a potential gateway for pathogen transmission. *Parasitology*. 130: 333-42.

47. Hannier, S., J. Liversidge, J. M. Sternberg, and A. S. Bowman. 2004. Characterization of the B-cell inhibitory protein factor in *Ixodes ricinus* tick saliva: a potential role in enhanced *Borrelia burgdorferi* transmission. *Immunology*. 113: 401-8.

48. Harris, C. L., D. M. Pettigrew, S. M. Lea, and B. P. Morgan. 2007. Decay-accelerating factor must bind both components of the complement alternative pathway C3 convertase to mediate efficient decay. *J Immunol*. 178: 352-9.

49. Hoffmann, A., P. Walsmann, G. Riesener, M. Paintz, and F. Markwardt. 1991. Isolation and characterization of a thrombin inhibitor from the tick *Ixodes ricinus*. *Pharmazie*. 46: 209-12.

50. Horn, F., P. C. dos Santos, and C. Termignoni. 2000. *Boophilus microplus* anticoagulant protein: an antithrombin inhibitor isolated from the cattle tick saliva. *Arch Biochem Biophys*. 384: 68-73.

51. Hourcade, D. E. 2006. The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *J Biol Chem*. 281: 2128-32.

52. Hourcade, D. E., L. M. Mitchell, and M. E. Medof. 1999. Decay acceleration of the complement alternative pathway C3 convertase. *Immunopharmacology*. 42: 167-73.

53. Hovius, J. W., N. Ramamoorthi, C. Van't Veer, K. A. de Groot, A. M. Nijhof, F. Jongejan, A. P. van Dam, and E. Fikrig. 2007. Identification of Salp15 homologues in *Ixodes ricinus* ticks. *Vector Borne Zoonotic Dis*. 7: 296-303.

54. Ibrahim, M. A., A. H. Ghazy, T. Maharem, and M. Khalil. 2001. Isolation and properties of two forms of thrombin inhibitor from the nymphs of the camel tick *Hyalomma dromedarii* (Acari: Ixodidae). *Exp Appl Acarol*. 25: 675-98.

55. Ibrahim, M. A., A. H. Ghazy, T. M. Maharem, and M. I. Khalil. 2001. Factor Xa (FXa) inhibitor from the nymphs of the camel tick *Hyalomma dromedarii*. *Comp Biochem Physiol B Biochem Mol Biol*. 130: 501-12.

56. Inokuma, H., T. Aita, K. Tamura, and T. Onishi. 1997. Effect of infestation with *Rhipicephalus sanguineus* on the antibody productivity in dogs. *Med Vet Entomol*. 11: 201-2.

57. Inokuma, H., D. H. Kemp, and P. Willadsen. 1994. Comparison of prostaglandin E2 (PGE2) in salivary gland of *Boophilus microplus*, *Haemaphysalis longicornis* and *Ixodes holocyclus*, and quantification of PGE2 in saliva, hemolymph, ovary and gut of B. microplus. *J Vet Med Sci*. 56: 1217-8.

58. Iwanaga, S., M. Okada, H. Isawa, A. Morita, M. Yuda, and Y. Chinzei. 2003. Identification and characterization of novel salivary thrombin inhibitors from the ixodidae tick, *Haemaphysalis longicornis. Eur J Biochem*. 270: 1926-34.

59. Jaworski, D. C., Simmen, F. A., Lamoreaux, W., Coons, L. B., Muller, M. T., Needham, G. R. 1995. A secreted calreticulin protein in the ixodid tick (*Amblyomma americanum*) saliva. *Journal of Insect Physiology*. 41: 369-75.

60. Jones, L. D., E. Hodgson, T. Williams, S. Higgs, and P. A. Nuttall. 1992. Saliva activated transmission (SAT) of Thogoto virus: relationship with vector potential of different haematophagous arthropods. *Med Vet Entomol*. 6: 261-5.

61. Jones, L. D., W. R. Kaufman, and P. A. Nuttall. 1992. Modification of the skin feeding site by tick saliva mediates virus transmission. *Experientia*. 48: 779-82.

62. Jones, L. D., M. Matthewson, and P. A. Nuttall. 1992. Saliva-activated transmission (SAT) of Thogoto virus: dynamics of SAT factor activity in the salivary glands of *Rhipicephalus appendiculatus*, *Amblyomma variegatum*, and *Boophilus microplus* ticks. *Exp Appl Acarol*. 13: 241-8.

63. Jongejan, F. and G. Uilenberg. 2004. The global importance of ticks. *Parasitology*. 129 Suppl: S3-14.

64. Joubert, A. M., J. C. Crause, A. R. Gaspar, F. C. Clarke, A. M. Spickett, and A. W. Neitz. 1995. Isolation and characterization of an anticoagulant present in the salivary glands of the bont-legged tick, *Hyalomma truncatum. Exp Appl Acarol*. 19: 79-92.

65. Juncadella, I. J., R. Garg, S. K. Ananthnarayanan, C. M. Yengo, and J. Anguita. 2007. T-cell signaling pathways inhibited by the tick saliva immunosuppressor, Salp15. *FEMS Immunol Med Microbiol*. 49: 433-8.

66. Karczewski, J., R. Endris, and T. M. Connolly. 1994. Disagregin is a fibrinogen receptor antagonist lacking the Arg-Gly-Asp sequence from the tick, *Ornithodoros moubata. J Biol Chem*. 269: 6702-8.

67. Karczewski, J., L. Waxman, R. G. Endris, and T. M. Connolly. 1995. An inhibitor from the argasid tick *Ornithodoros moubata* of cell adhesion to collagen. *Biochem Biophys Res Commun*. 208: 532-41.

68. Kashino, S. S., J. Resende, A. M. Sacco, C. Rocha, L. Proenca, W. A. Carvalho, A. A. Firmino, R. Queiroz, M. Benavides, L. J. Gershwin, and I. K. De Miranda Santos. 2005. *Boophilus microplus*: the pattern of bovine immunoglobulin isotype responses to high and low tick infestations. *Exp Parasitol*. 110: 12-21.

69. Kato, N., S. Iwanaga, T. Okayama, H. Isawa, M. Yuda, and Y. Chinzei. 2005. Identification and characterization of the plasma kallikrein-kinin system inhibitor, haemaphysalin, from hard tick, *Haemaphysalis longicornis. Thromb Haemost*. 93: 359-67.

70. Keller, P. M., L. Waxman, B. A. Arnold, L. D. Schultz, C. Condra, and T. M. Connolly. 1993. Cloning of the cDNA and expression of moubatin, an inhibitor of platelet aggregation. *J Biol Chem*. 268: 5450-6.

71. Kimura, Y., T. Miwa, L. Zhou, and W. C. Song. 2008. Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood*. 111: 732-40.

72. Koh, C. Y., M. Kazimirova, A. Trimnell, P. Takac, M. Labuda, P. A. Nuttall, and R. M. Kini. 2007. Variegin, a novel fast and tight binding thrombin inhibitor from the tropical bont tick. *J Biol Chem*. 282: 29101-13.

73. Kopecky, J. and M. Kuthejlova. 1998. Suppressive effect of *Ixodes ricinus* salivary gland extract on mechanisms of natural immunity in vitro. *Parasite Immunol*. 20: 169-74.

74. Kopecky, J., M. Kuthejlova, and J. Pechova. 1999. Salivary gland extract from *Ixodes ricinus* ticks inhibits production of interferon-gamma by the upregulation of interleukin-10. *Parasite Immunol*. 21: 351-6.

75. Kotsyfakis, M., S. Karim, J. F. Andersen, T. N. Mather, and J. M. Ribeiro. 2007. Selective cysteine protease inhibition contributes to blood-feeding success of the tick *Ixodes scapularis. J Biol Chem*. 282: 29256-63.

76. Kotsyfakis, M., A. Sa-Nunes, I. M. Francischetti, T. N. Mather, J. F. Andersen, and J. M. Ribeiro. 2006. Antiinflammatory and immunosuppressive activity of sialostatin L, a salivary cystatin from the tick *Ixodes scapularis. J Biol Chem*. 281: 26298-307.

77. Kovar, L., J. Kopecky, and B. Rihova. 2001. Salivary gland extract from *Ixodes ricinus* tick polarizes the cytokine profile toward Th2 and suppresses proliferation of T lymphocytes in human PBMC culture. *J Parasitol*. 87: 1342-8.

78. Kovar, L., J. Kopecky, and B. Rihova. 2002. Salivary gland extract from *Ixodes ricinus* tick modulates the host immune response towards the Th2 cytokine profile. *Parasitol Res*. 88: 1066-72.

79. Kubes, M., N. Fuchsberger, M. Labuda, E. Zuffova, and P. A. Nuttall. 1994. Salivary gland extracts of partially fed *Dermacentor reticulatus* ticks decrease natural killer cell activity *in vitro. Immunology*. 82: 113-6.

80. Kubes, M., P. Kocakova, M. Slovak, M. Slavikova, N. Fuchsberger, and P. A. Nuttall. 2002. Heterogeneity in the effect of different ixodid tick species on human natural killer cell activity. *Parasite Immunol*. 24: 23-8.

81. Kuthejlova, M., J. Kopecky, G. Stepanova, and A. Macela. 2001. Tick salivary gland extract inhibits killing of *Borrelia afzelii* spirochetes by mouse macrophages. *Infect Immun*. 69: 575-8.

82. Labuda, M., L. D. Jones, T. Williams, and P. A. Nuttall. 1993. Enhancement of tick-borne encephalitis virus transmission by tick salivary gland extracts. *Med Vet Entomol*. 7: 193-6.

83. Lawrie, C. H., S. E. Randolph, and P. A. Nuttall. 1999. *Ixodes* ticks: serum species sensitivity of anticomplement activity. *Exp Parasitol*. 93: 207-14.

84. Lawrie, C. H., R. B. Sim, and P. A. Nuttall. 2005. Investigation of the mechanisms of anti-complement activity in *Ixodes ricinus* ticks. *Mol Immunol*. 42: 31-8.

85. Leboulle, G., M. Crippa, Y. Decrem, N. Mejri, M. Brossard, A. Bollen, and E. Godfroid. 2002. Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. *J Biol Chem*. 277: 10083-9.

86. Limo, M. K., W. P. Voigt, A. G. Tumbo-Oeri, R. M. Njogu, and O. K. ole-MoiYoi. 1991. Purification and characterization of an anticoagulant from the salivary glands of the ixodid tick *Rhipicephalus appendiculatus. Exp Parasitol*. 72: 418-29.

87. Macaluso, K. R. and S. K. Wikel. 2001. *Dermacentor andersoni*: effects of repeated infestations on lymphocyte proliferation, cytokine production, and adhesion-molecule expression by BALB/c mice. *Ann Trop Med Parasitol*. 95: 413-27.

88. Mans, B. J., J. F. Andersen, T. G. Schwan, and J. M. Ribeiro. 2008. Characterization of anti-hemostatic factors in the argasid, *Argas monolakensis*: Implications for the evolution of blood-feeding in the soft tick family. *Insect Biochem Mol Biol*. 38: 22-41.

89. Mans, B. J., A. R. Gaspar, A. I. Louw, and A. W. Neitz. 1998. Apyrase activity and platelet aggregation inhibitors in the tick *Ornithodoros savignyi* (Acari: Argasidae). *Exp Appl Acarol*. 22: 353-66.

90. Mans, B. J., A. I. Louw, and A. W. Neitz. 2002. Amino acid sequence and structure modeling of savignin, a thrombin inhibitor from the tick, *Ornithodoros savignyi. Insect Biochem Mol Biol*. 32: 821-8.

91. Mans, B. J., A. I. Louw, and A. W. Neitz. 2002. Disaggregation of aggregated platelets by savignygrin, a alphaIIbeta3 antagonist from *Ornithodoros savignyi. Exp Appl Acarol*. 27: 231-9.

92. Mans, B. J., A. I. Louw, and A. W. Neitz. 2002. Savignygrin, a platelet aggregation inhibitor from the soft tick *Ornithodoros savignyi*, presents the RGD integrin recognition motif on the Kunitz-BPTI fold. *J Biol Chem*. 277: 21371-8.

93. Mans, B. J. and A. W. Neitz. 2004. Adaptation of ticks to a blood-feeding environment: evolution from a functional perspective. *Insect Biochem Mol Biol*. 34: 1-17.

94. Maxwell, S. S., T. A. Stoklasek, Y. Dash, K. R. Macaluso, and S. K. Wikel. 2005. Tick modulation of the *in-vitro* expression of adhesion molecules by skin-derived endothelial cells. *Ann Trop Med Parasitol*. 99: 661-72.

95. Montgomery, R. R., D. Lusitani, A. De Boisfleury Chevance, and S. E. Malawista. 2004. Tick saliva reduces adherence and area of human neutrophils. *Infect Immun*. 72: 2989-94.

96. Morgan, B. P., Harris, C. L., 1999. *Complement regulatory proteins*. Academic Press, San Diego, CA.

97. Motoyashiki, T., A. T. Tu, D. A. Azimov, and K. Ibragim. 2003. Isolation of anticoagulant from the venom of tick, *Boophilus calcaratus*, from Uzbekistan. *Thromb Res*. 110: 235-41.

98. Muller-Doblies, U. U., S. S. Maxwell, V. D. Boppana, M. A. Mihalyo, S. J. McSorley, A. T. Vella, A. J. Adler, and S. K. Wikel. 2007. Feeding by the tick, *Ixodes scapularis*, causes CD4(+) T cells responding to cognate antigen to develop the capacity to express IL-4. *Parasite Immunol*. 29: 485-99.

99. Muller-Doblies, U. U. and S. K. Wikel, *The Human Reaction to Ticks*, In *Tick-Borne Diseases of Humans*, D.T.D. Jesse L. Goodman, Daniel E. Sonenshine, Editor. 2005, American Society for Microbiology: Washington D.C. p. 102-122.

100. Narasimhan, S., R. A. Koski, B. Beaulieu, J. F. Anderson, N. Ramamoorthi, F. Kantor, M. Cappello, and E. Fikrig. 2002. A novel family of anticoagulants from the saliva of *Ixodes scapularis. Insect Mol Biol*. 11: 641-50.

101. Neeper, M. P., L. Waxman, D. E. Smith, C. A. Schulman, M. Sardana, R. W. Ellis, L. W. Schaffer, P. K. Siegl, and G. P. Vlasuk. 1990. Characterization of recombinant tick anticoagulant peptide. A highly selective inhibitor of blood coagulation factor Xa. *J Biol Chem*. 265: 17746-52.

102. Nicholson-Weller, A., J. Burge, D. T. Fearon, P. F. Weller, and K. F. Austen. 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J Immunol*. 129: 184-9.

103. Nienaber, J., A. R. Gaspar, and A. W. Neitz. 1999. Savignin, a potent thrombin inhibitor isolated from the salivary glands of the tick *Ornithodoros savignyi* (Acari: Argasidae). *Exp Parasitol*. 93: 82-91.

104. Nunn, M. A., A. Sharma, G. C. Paesen, S. Adamson, O. Lissina, A. C. Willis, and P. A. Nuttall. 2005. Complement inhibitor of C5 activation from the soft tick *Ornithodoros moubata*. *J Immunol*. 174: 2084-91.

105. Nuttall, P. A. and M. Labuda. 2004. Tick-host interactions: saliva-activated transmission. *Parasitology*. 129 Suppl: S177-89.

106. Oliveira, C. J., K. A. Cavassani, D. D. More, G. P. Garlet, J. C. Aliberti, J. S. Silva, and B. R. Ferreira. 2007. Tick saliva inhibits the chemotactic function of MIP-1alpha and selectively impairs chemotaxis of immature dendritic cells by down-regulating cell-surface CCR5. *Int J Parasitol*.

107. Paesen, G. C., P. L. Adams, K. Harlos, P. A. Nuttall, and D. I. Stuart. 1999. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. *Mol Cell*. 3: 661-71.

108. Pangburn, M. K. 1986. Differences between the binding sites of the complement regulatory proteins DAF, CR1, and factor H on C3 convertases. *J Immunol*. 136: 2216-21.

109. Pangburn, M. K. and H. J. Muller-Eberhard. 1986. The C3 convertase of the alternative pathway of human complement. Enzymic properties of the bimolecular proteinase. *Biochem J*. 235: 723-30.

110. Pangburn, M. K., R. D. Schreiber, and H. J. Muller-Eberhard. 1981. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J Exp Med*. 154: 856-67.

111. Pechova, J., J. Kopecky, and J. Salat. 2004. Effect of tick salivary gland extract on the cytokine production by mouse epidermal cells. *Folia Parasitol (Praha)*. 51: 367-72.

112. Pechova, J., G. Stepanova, L. Kovar, and J. Kopecky. 2002. Tick salivary gland extract-activated transmission of *Borrelia afzelii* spirochaetes. *Folia Parasitol (Praha)*. 49: 153-9.

113. Ramachandra, R. N. and S. K. Wikel. 1992. Modulation of host-immune responses by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production. *J Med Entomol*. 29: 818-26.

114. Ramachandra, R. N. and S. K. Wikel. 1995. Effects of *Dermacentor andersoni* (Acari: Ixodidae) salivary gland extracts on *Bos indicus* and *B. taurus* lymphocytes and macrophages: in vitro cytokine elaboration and lymphocyte blastogenesis. *J Med Entomol*. 32: 338-45.

115. Ramamoorthi, N., S. Narasimhan, U. Pal, F. Bao, X. F. Yang, D. Fish, J. Anguita, M. V. Norgard, F. S. Kantor, J. F. Anderson, R. A. Koski, and E. Fikrig. 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature*. 436: 573-7.

116. Ribeiro, J. M. 1987. *Ixodes dammini*: salivary anti-complement activity. *Exp Parasitol*. 64: 347-53.

117. Ribeiro, J. M., F. Alarcon-Chaidez, I. M. Francischetti, B. J. Mans, T. N. Mather, J. G. Valenzuela, and S. K. Wikel. 2006. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol*. 36: 111-29.

118. Ribeiro, J. M., T. M. Endris, and R. Endris. 1991. Saliva of the soft tick, *Ornithodoros moubata*, contains anti-platelet and apyrase activities. *Comp Biochem Physiol A*. 100: 109-12.

119. Ribeiro, J. M., P. M. Evans, J. L. MacSwain, and J. Sauer. 1992. *Amblyomma americanum*: characterization of salivary prostaglandins E2 and F2 alpha by RP-HPLC/bioassay and gas chromatography-mass spectrometry. *Exp Parasitol*. 74: 112-6.

120. Ribeiro, J. M. and I. M. Francischetti. 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol*. 48: 73-88.

121. Ribeiro, J. M., G. T. Makoul, J. Levine, D. R. Robinson, and A. Spielman. 1985. Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, *Ixodes dammini*. *J Exp Med*. 161: 332-44.

122. Ribeiro, J. M., G. T. Makoul, and D. R. Robinson. 1988. *Ixodes dammini*: evidence for salivary prostacyclin secretion. *J Parasitol*. 74: 1068-9.

123. Ribeiro, J. M. and T. N. Mather. 1998. *Ixodes scapularis*: salivary kininase activity is a metallo dipeptidyl carboxypeptidase. *Exp Parasitol*. 89: 213-21.

124. Ribeiro, J. M. and A. Spielman. 1986. *Ixodes dammini*: salivary anaphylatoxin inactivating activity. *Exp Parasitol*. 62: 292-7.

125. Ribeiro, J. M., J. J. Weis, and S. R. Telford, 3rd. 1990. Saliva of the tick *Ixodes dammini* inhibits neutrophil function. *Exp Parasitol*. 70: 382-8.

126. Ross, G. D., J. D. Lambris, J. A. Cain, and S. L. Newman. 1982. Generation of three different fragments of bound C3 with purified factor I or serum. I. Requirements for factor H vs CR1 cofactor activity. *J Immunol*. 129: 2051-60.

127. Roversi, P., O. Lissina, S. Johnson, N. Ahmat, G. C. Paesen, K. Ploss, W. Boland, M. A. Nunn, and S. M. Lea. 2007. The structure of OMCI, a novel lipocalin inhibitor of the complement system. *J Mol Biol*. 369: 784-93.

128. Sanders, M. L., D. C. Jaworski, J. L. Sanchez, R. F. DeFraites, G. E. Glass, A. L. Scott, S. Raha, B. C. Ritchie, G. R. Needham, and B. S. Schwartz. 1998. Antibody to a cDNA-derived calreticulin protein from *Amblyomma americanum* as a biomarker of tick exposure in humans. *Am J Trop Med Hyg*. 59: 279-85.

129. Sangamnatdej, S., G. C. Paesen, M. Slovak, and P. A. Nuttall. 2002. A high affinity serotonin- and histamine-binding lipocalin from tick saliva. *Insect Mol Biol*. 11: 79-86.

130. Sa-Nunes, A., A. Bafica, D. A. Lucas, T. P. Conrads, T. D. Veenstra, J. F. Andersen, T. N. Mather, J. M. Ribeiro, and I. M. Francischetti. 2007. Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in *Ixodes scapularis* saliva. *J Immunol*. 179: 1497-505.

131. Schoeler, G. B., S. A. Manweiler, and S. K. Wikel. 1999. *Ixodes scapularis*: effects of repeated infestations with pathogen-free nymphs on macrophage and T lymphocyte cytokine responses of BALB/c and C3H/HeN mice. *Exp Parasitol*. 92: 239-48.

132. Schoeler, G. B., S. A. Manweiler, and S. K. Wikel. 2000. Cytokine responses of C3H/HeN mice infested with *Ixodes scapularis* or *Ixodes pacificus* nymphs. *Parasite Immunol*. 22: 31-40.

133. Schoeler, G. B. and S. K. Wikel. 2001. Modulation of host immunity by haematophagous arthropods. *Ann Trop Med Parasitol*. 95: 755-71.

134. Seya, T. and J. P. Atkinson. 1989. Functional properties of membrane cofactor protein of complement. *Biochem J*. 264: 581-8.

135. Seya, T., J. R. Turner, and J. P. Atkinson. 1986. Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. *J Exp Med*. 163: 837-55.

136. Soares, C. A., C. M. Lima, M. C. Dolan, J. Piesman, C. B. Beard, and N. S. Zeidner. 2005. Capillary feeding of specific dsRNA induces silencing of the *isac* gene in nymphal *Ixodes scapularis* ticks. *Insect Mol Biol*. 14: 443-52.

137. Sonenshine, D. E., 1991. *Biology of Ticks*. Oxford University Press, New York, NY.

138. Sonenshine, D. E., *The Biology of Tick Vectors of Human Disease*, In *Tick-Borne Diseases of Human*, D.T.D. Jesse L. Goodman, Daniel E. Sonenshine, Editor. 2005, American Society for Microbiology: Washington D.C. p. 12-35.

139. Spitzer, D., L. M. Mitchell, J. P. Atkinson, and D. E. Hourcade. 2007. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J Immunol*. 179: 2600-8.

140. Tanaka, A. S., R. Andreotti, A. Gomes, R. J. Torquato, M. U. Sampaio, and C. A. Sampaio. 1999. A double headed serine proteinase inhibitor--human plasma kallikrein and elastase inhibitor--from *Boophilus microplus* larvae. *Immunopharmacology*. 45: 171-7.

141. Titus, R. G., J. V. Bishop, and J. S. Mejia. 2006. The immunomodulatory factors of arthropod saliva and the potential for these factors to serve as vaccine targets to prevent pathogen transmission. *Parasite Immunol*. 28: 131-41.

142. Tsuji, N., T. Kamio, T. Isobe, and K. Fujisaki. 2001. Molecular characterization of a peroxiredoxin from the hard tick *Haemaphysalis longicornis. Insect Mol Biol*. 10: 121-9.

143. Tyson, K., C. Elkins, H. Patterson, E. Fikrig, and A. de Silva. 2007. Biochemical and functional characterization of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Mol Biol*. 16: 469-79.

144. Urioste, S., L. R. Hall, S. R. Telford, 3rd, and R. G. Titus. 1994. Saliva of the Lyme disease vector, *Ixodes dammini*, blocks cell activation by a nonprostaglandin E2-dependent mechanism. *J Exp Med*. 180: 1077-85.

145. Valenzuela, J. G. 2004. Exploring tick saliva: from biochemistry to 'sialomes' and functional genomics. *Parasitology*. 129 Suppl: S83-94.

146. Valenzuela, J. G., R. Charlab, T. N. Mather, and J. M. Ribeiro. 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis. J Biol Chem*. 275: 18717-23.

147. Valenzuela, J. G., I. M. Francischetti, V. M. Pham, M. K. Garfield, T. N. Mather, and J. M. Ribeiro. 2002. Exploring the sialome of the tick *Ixodes scapularis. J Exp Biol*. 205: 2843-64.

148. van de Locht, A., M. T. Stubbs, W. Bode, T. Friedrich, C. Bollschweiler, W. Hoffken, and R. Huber. 1996. The ornithodorin-thrombin crystal structure, a key to the TAP enigma? *Embo J*. 15: 6011-7.

149. Vancova, I., M. Slovak, V. Hajnicka, M. Labuda, L. Simo, K. Peterkova, R. S. Hails, and P. A. Nuttall. 2007. Differential anti-chemokine activity of *Amblyomma variegatum* adult ticks during blood-feeding. *Parasite Immunol*. 29: 169-77.

150. Walport, M. J. 2001. Complement. First of two parts. *N Engl J Med*. 344: 1058-66.

151. Walport, M. J. 2001. Complement. Second of two parts. *N Engl J Med*. 344: 1140-4.

152. Wang, H. and P. A. Nuttall. 1994. Excretion of host immunoglobulin in tick saliva and detection of IgG-binding proteins in tick haemolymph and salivary glands. *Parasitology*. 109 (Pt 4): 525-30.

153. Wang, H. and P. A. Nuttall. 1995. Immunoglobulin G binding proteins in male *Rhipicephalus appendiculatus* ticks. *Parasite Immunol*. 17: 517-24.

154. Wang, H. and P. A. Nuttall. 1995. Immunoglobulin-G binding proteins in the ixodid ticks, *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Ixodes hexagonus*. *Parasitology*. 111 (Pt 2): 161-5.

155. Wang, X., L. B. Coons, D. B. Taylor, S. E. Stevens, Jr., and T. K. Gartner. 1996. Variabilin, a novel RGD-containing antagonist of glycoprotein IIb-IIIa and platelet aggregation inhibitor from the hard tick *Dermacentor variabilis*. *J Biol Chem*. 271: 17785-90.

156. Waxman, L. and T. M. Connolly. 1993. Isolation of an inhibitor selective for collagen-stimulated platelet aggregation from the soft tick *Ornithodoros moubata*. *J Biol Chem*. 268: 5445-9.

157. Waxman, L., D. E. Smith, K. E. Arcuri, and G. P. Vlasuk. 1990. Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. *Science*. 248: 593-6.

158. Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein beta1H. *Proc Natl Acad Sci U S A*. 73: 3268-72.

159. Whaley, K. and S. Ruddy. 1976. Modulation of the alternative complement pathways by beta 1 H globulin. *J Exp Med*. 144: 1147-63.

160. Wikel, S. K. 1979. Acquired resistance to ticks: expression of resistance by C4-deficient guinea pigs. *Am J Trop Med Hyg*. 28: 586-90.

161. Wikel, S. K. 1985. Effects of tick infestation on the plaque-forming cell response to a thymic dependent antigen. *Ann Trop Med Parasitol*. 79: 195-8.

162. Wikel, S. K. 1996. Host immunity to ticks. *Annu Rev Entomol*. 41: 1-22.

163. Xu, G., Q. Q. Fang, J. E. Keirans, and L. A. Durden. 2004. Cloning and sequencing of putative calreticulin complementary DNAs from four hard tick species. *J Parasitol*. 90: 73-8.

164. Yu, D., J. Liang, H. Yu, H. Wu, C. Xu, J. Liu, and R. Lai. 2006. A tick B-cell inhibitory protein from salivary glands of the hard tick, *Hyalomma asiaticum asiaticum*. *Biochem Biophys Res Commun*. 343: 585-90.

165. Zhu, K., A. S. Bowman, D. L. Brigham, R. C. Essenberg, J. W. Dillwith, and J. R. Sauer. 1997. Isolation and characterization of americanin, a specific inhibitor of thrombin, from the salivary glands of the lone star tick *Amblyomma americanum* (L.). *Exp Parasitol*. 87: 30-8.

166. Zhu, K., J. R. Sauer, A. S. Bowman, and J. W. Dillwith. 1997. Identification and characterization of anticoagulant activities in the saliva of the lone star tick, *Amblyomma americanum* (L.). *J Parasitol*. 83: 38-43.

167. Zipfel, P. F., T. S. Jokiranta, J. Hellwage, V. Koistinen, and S. Meri. 1999. The factor H protein family. *Immunopharmacology*. 42: 53-60.

CHAPTER 2

Biochemical and functional characterization of Salp20, and *Ixodes scapularis* salivary protein that inhibits the complement pathway

Katharine Tyson[1], Chris Elkins[2], Holly Patterson[1], Erol Fikrig[3] and Aravinda de Silva[1]

[1]Department of Microbiology and Immunology and [2]Department of Medicine, University of North Carolina at Chapel Hill, NC 27599 and [3]Section of Rheumatology, Department of Medicine, Yale University School of Medicine, New Haven, CT 06510

2.1 Abstract

Ixodes ticks are vectors of several pathogens including *B. burgdorferi*. Tick saliva contains numerous molecules that facilitate blood feeding without host immune recognition and rejection. We have expressed, purified, and characterized Salp20, a potential inhibitor of the alternative complement pathway that shares homology with the Isac protein family. When analyzed by SDS-PAGE and size exclusion chromatography, Salp20 was approximately 48 kDa, more than double its predicted mass, primarily due N- and O-linked glycosylations. Recombinant Salp20 inhibited the alternative complement pathway by dissociating the C3 convertase, and partially protected a serum sensitive species of *Borrelia* from lysis by NHS. We propose that Salp20 facilitates tick feeding and possibly protects tick-borne pathogens from complement components.

2.2 Introduction

The deer tick, *I. scapularis*, transmits several pathogens including *Anaplasma phagocytophilum*, *Babesia microti*, and *B. burgdorferi*, the causative agent of Lyme disease and most prevalent vector-borne pathogen in the United States and Europe (3, 36). *I. scapularis* ticks typically feed on their natural hosts for several days without rejection by the host immune system due to several anti-hemostatic, vasodilatory, and immunosuppressive components present in their saliva (4, 7, 14, 35, 36). Salivary proteins capable of eliminating host pain and irritation at the feeding site include histamine binding proteins, apryases, prostaglandins, and kininases (16, 19, 22, 29). In addition, tick saliva also impairs natural killer cell activity, reduces circulating and localized antibodies, suppresses T-cell proliferation, and decreases the production of Th1 cytokines (6, 7, 9, 21, 28, 36). *Ixodes sp.* salivary gland extracts and saliva inhibit the alternative pathway of complement and prevent the production of anaphylatoxins (7, 11, 12, 19, 23, 36).

An *I. scapularis* salivary protein, Isac, with a predicted mass of 18 kDa, inhibits the alternative pathway of complement by dissociating the components of the C3 convertase and preventing the deposition of C3b onto surfaces, similar to fH and factor H-like protein 1 (8, 13, 28, 33). The alternative pathway of complement is activated when C3b binds covalently through its reactive thioester to activating surfaces (5, 13, 24, 31, 32). Surface bound C3b binds fB, which is then cleaved by fD, producing the cleavage products Bb and Ba. Bb remains bound to C3b, while Ba is released. The surface bound C3bBb complex, or C3 convertase, cleaves additional C3 components producing more C3b that either binds to activating surfaces or to the C3 convertase, forming the C5 convertase. The C5 convertase then initializes the formation of the MAC.

The alternative complement pathway can be initiated by metastable C3(H_2O), a naturally occurring hydrolyzed C3 molecule. C3(H_2O) resembles C3b and binds fB in solution, allowing fB to then be cleaved by fD. The resulting fluid-phase convertase, C3(H_2O)Bb, then cleaves C3, releasing C3b that deposits onto surfaces activating the complement cascade (13, 17, 24).

Invading microbes coated with C3b are either eventually lysed by the development of the MAC or phagocytosed by macrophages and neutrophils. In addition to mediating lysis and opsonization of invading pathogens, the alternative complement cascade also leads to the production of anaphylatoxins, which are proinflammatory mediators that recruit neutrophils and monocytes to the site of complement activation (5, 13, 31, 32).

Inhibition of the alternative complement pathway by *I. scapularis* is important for preventing host inflammation and immune recognition at the feeding site, allowing the tick to feed successfully to repletion. In addition, inhibition of the alternative complement pathway by tick saliva during feeding potentially allows the successful transmission of pathogens throughout the feeding period of 5 days.

Two other *I. scapularis* salivary proteins, Salp9 and Salp20, sharing homology with Isac were identified from a nymphal *I. scapularis* salivary gland cDNA library (4). Salp9 is a protein with 50% similarity to Isac and a predicted mass of 8.8 kDa. Salp20 is a protein with 83% similarity to Isac and a predicted mass of 20.4 kDa. These three salivary proteins, Salp9, Salp20, and Isac, are included in a large family of related *I. scapularis* salivary anticomplement proteins recently identified by Soares et al. (25) and Ribeiro et al. (20). Interestingly, when RNAi was used to silence *isac* in *I. scapularis* nymphs, tick feeding was

delayed and the average weight of engorged ticks was reduced, indicating the importance of anticomplement proteins for successful tick feeding (25).

The goal of the current study was to identify the properties and characterize the functions of Salp20, which shares homology with the *I. scapularis* salivary anti-complement protein Isac. We identified additional members of the Isac protein family that share homology with both Salp20 and Isac. We demonstrated that recombinant Salp20 purified from the media of insect cells inhibits the alternative complement pathway by dissociating the components of the C3 convertase and preventing the cleavage of C3 and the deposition of C3b onto cell surfaces. Additionally, we established that Salp20 protects serum sensitive strains of *Borrelia* from complement mediated lysis indicating that tick transmitted pathogens potentially benefit from the activity of Salp20 and related protein family members.

2.3 Materials and Methods

Ticks and tick saliva

Ixodes scapularis ticks were raised as previously described by Sonenshine (26). Tick saliva was produced following a modified protocol from Tatchell et al. (27). Briefly, adult ticks were allowed to feed on New Zealand White rabbits for 5 days. The ticks were removed and attached to glass slides with adhesive tape. Capillaries were placed over the mouthparts, and ~1-2 μL of pilocarpine (25 mg/ml) and dopamine (25 mg/ml) in 95% ethanol were applied on the dorsum of the ticks. The ticks were allowed to salivate into the capillaries ~2 hrs. at 27^{o}C in humidity chambers.

Cell lines and media

Adherent cultures of High Five cells (Invitrogen, Carlsbad, CA), derived from the cabbage looper, *Trichoplusia ni*, were seeded and maintained according to the instructions of the manufacturer. The cells were grown in Express Five Serum free media (SFM) (Gibco, Carlsbad, CA) supplemented with L-glutamine (18 mM) (Gibco, Carlsbad, CA), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) (Gibco, Carlsbad, CA) at 28^{o}C.

Borrelia burgdorferi B31C1 and *Borrelia garinii* (ATCC, Manassas, VA) were grown and maintained in complete BSK-II media at 33^{o}C as described by Ohnishi et al. (15).

PCR from *I. scapularis* bacteriophage libraries

In order to construct the cDNA library from 48 hr fed nymphs, total RNA was first extracted from ~300 fed *I. scapularis* nymphs using the ToTALLY RNA extraction kit (Ambion, Austin, TX) according to the instructions of the manufacturer. Isolation and purification of mRNA from total RNA was performed using the poly(A)Pure mRNA Purification Kit (Ambion, Austin, TX). The cDNA library from 48 hr fed nymphal ticks was subsequently constructed in the phagemid vector, pBK-CMV, using the ZAP Express cDNA Synthesis and ZAP Express cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA). The average size of an insert in the phagemid vector was approximately 1.8 kilobases (kb). The titer of the resulting phage library was 2.0 x10^{9} plaque forming units (pfu)/ml with a complexity of 1.0 x 10^{6} clones. The method used to generate the cDNA library generated from fed nymph salivary glands has been described previously (4).

Products were PCR amplified from each of the bacteriophage libraries directly using the following primer sets: KS20F – 5` CCAGCCATGAGGACTGCGCT 3` and S20R – 5`

TCAGGAAATTGCCTCGAAT TGAGT 3`, and IsacF – 5` CACTGAGGTTC AGAGCAAG 3` and IsacR – 5` GTATCAGAACTGTGCTTGCAC 3`. The Salp20 primers, KS20F and S20R, anneal to the 5` and 3` ends of the *salp20* open reading frame (ORF), while the Isac primers, IsacF and IsacR, anneal upstream and downstream of the *isac* ORF, respectively. After amplification, the PCR products were cloned into pCR2.1 TOPO following the instructions of the manufacturer (Invitrogen, Carlsbad, CA). Plasmids containing the PCR products were purified using the QIAprep Mini-Prep Kit (Qiagen, Valencia, CA) and then transformed into chemically competent *E. coli* TOP 10 cells. Transformants were selected and screened by restriction digests of plasmid DNA and PCR analysis using M13F and M13R primers (Invitrogen, Carlsbad, CA), which anneal outside of the multiple cloning region of pCR2.1 TOPO. To determine the identity of the PCR products, plasmids containing inserts of the correct size were sequenced using the M13 primers at the UNC-Chapel Hill Genome Analysis Facility.

Expression and purification of recombinant Salp20 in High Five cells

To express Salp20 in High Five cells, we used an approach similar to Alarcon-Chaidez et al. (1). Salp20 was first PCR amplified from the pBlue vector using the following primers: KS20F – 5` CCAGCCATGAGGACTGCGCT 3`, and S20RNS – 5` GGAAATTGCCTCGAATGAGTCTC 3`. The 5` primer contained a Kozak sequence to allow for efficient protein expression from the cloning vector, while the 3` primer lacked a stop codon to allow the fusion of a V5-epitope and 6X-histidine (His) tag to the C-terminus of the protein. Once amplified, the PCR product was cloned into the expression pIB/V5-His-TOPO (Invitrogen), which contains both ampicillin and blasticidin resistance genes allowing for selection in *E. coli* and High Five cells, generating the construct pIB-S20NS.

Constitutive expression of Salp20-V5-His (S20NS) was controlled by the *Orgyia pseudotsugata* baculovirus promoter, *OpIE2*. The resulting construct, pIB-S20NS, was purified using the QIAfilter Plasmid Midi Kit (Qiagen, Valencia, CA) and transfected into High Five cells following the manufacturer's protocol. As a positive control for protein expression and purification, High Five cells were also transfected with pIB-CAT (Invitrogen, Carlsbad, CA), the pIB/V5-His vector containing chloramphenicol acetyltransferase (CAT). Stably transfected cells were selected and maintained by the addition of blasticidin (10 μg/ml) to the cell culture media.

For protein purification, adherent cultures of stably transfected cells were grown to 90% confluency in T-175 flasks. Cells were then collected and seeded into 500 ml of culture media in 3L Fernbach flasks (Corning, Corning, NY) at a density of 5×10^5 cells/ml. Cultures were grown at 28°C with gentle agitation until reaching a density of $2\text{-}3 \times 10^6$ cells/ml in 1L. The media was then collected and centrifuged to remove all cells and debris. After centrifugation, the media was dialyzed 24 hrs. at 4°C against Ni-NTA Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Throughout dialysis, the buffer was changed three times. After dialysis, the media was loaded onto a 500 μL column of Ni2+-Nitriloacetic acid (Ni-NTA, Qiagen, Valencia, CA) agarose. After all media passed through the column, the column was washed two times with 4 column volumes of Ni-NTA Wash Buffer, and eluted four times with ½ the column volume of Ni-NTA Elution Buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, pH 8.0). All elutions were pooled and dialyzed 24 hrs. at 4°C against phosphate buffered saline (PBS) with two buffer changes. After dialysis, the elution sample was concentrated using Amicon Ultra-15 YM-10 centrifugal filter devices (10 kDa MWCO, Millipore, Billerica, MA). Protein concentrations and purity were determined by

Bradford analysis and SDS-PAGE, respectively. The final concentration of protein was 50-100 μg/ml. Purified protein was stored at -20^{o}C until needed.

SDS-PAGE and Immunoblot or Lectin Blot Analysis

For SDS-PAGE, all samples were electrophoresed under reducing conditions by either 10% SDS-PAGE or 4-12% SDS-PAGE. Gels were then stained using SimplyBlue SafeStain (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. For immunoblots, following separation by SDS-PAGE under reducing conditions, samples were transferred to nitrocellulose membranes by semi-dry transfer at 380 mA for 60 min. Membranes were then blocked with 1X Tris-buffered saline (TBS), 2% milk for 12-16 hrs at 4^{o}C. Mouse anti-V5 monoclonal antibody (Invitrogen, Carlsbad, CA) was diluted 1:5000 in 1X TBS, 2% milk and incubated with membranes for 1 hr at room temperature. Membranes were next incubated with alkaline phosphatase (AP) conjugated goat anti-mouse antibody (Sigma, St. Louis, MO) diluted 1:1000 in 1X TBS, 2% milk for 45 min at room temperature. For C3a blots, rabbit anti-human C3a antibody (CompTech, Tyler, TX) was diluted 1:1000, and secondary AP-conjugated goat anti-rabbit IgG (Sigma) was diluted 1:1000 in 1X TBS, 2% milk. For C3 blots, goat anti-human C3 antibody (CompTech, Tyler, TX) was diluted 1:1000, and AP-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO) was diluted 1:25000 following the same procedures as described. Incubation times were the same as described previously. Bound antibody was detected directly on the membranes using the phosphatase substrate, 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) (KPL). For lectin blots, blots were subjected to the DIG Glycan Differentiation Kit (Roche, Indianapolis, IN) following the instructions of the manufacturer. Briefly, blots were blocked and incubated with digoxigenin (DIG)-labeled lectins at various dilutions. Blots were then

washed and incubated with AP-conjugated sheep anti-DIG Fab fragments. Bands were then visualized with diluted NBT/X-phosphate staining solution.

Deglycosylations with Peptide N-Glycosidase F (PNGase F) and trifluoromethanesulfonic acid (TFMS)

To remove N-linked glycans from S20NS, approximately 0.5 μg of purified S20NS from insect cells was digested with 30 U/μL of PNGase F (New England Biolabs, Ipswich, MA) following the instructions provided by the manufacturer. Briefly, protein was denatured in 1X Glycoprotein Denaturing Buffer (0.5% SDS, 1% β-mercaptoethanol) (New England Biolabs, Ipswich, MA) for 10 min at 100^{o}C. The denatured protein was then incubated with PNGase F in 1X G7 Buffer (50 mM sodium phosphate, pH 7.5, New England Biolabs, Ipswich, MA) and 1% NP-40 for 24 hr at 37^{o}C. The deglycosylated protein was then analyzed by SDS-PAGE and immunoblots.

To remove N- and O-linked glycans from S20NS, 2 μg of S20NS were treated with the Glycoprofile IV Chemical Deglycosylation Kit containing TFMS according to the manufacturer's instructions (Sigma, St. Louis, MO).

Rabbit Erythrocyte Lysis Assays

RbEs (CompTech, Tyler, TX) were washed three times with 5 volumes of alternative pathway (AP) Buffer (5 mM $MgCl_2$, 5 mM EGTA in gelatin veronal buffer (GVB), CompTech, Tyler, TX), and resuspended to a final concentration of 2.0 x 10^8 cells/ml in AP buffer. Prior to performing the alternative pathway assays, the dilution of NHS (CompTech, Tyler, TX) resulting in 80-90% lysis of the RbEs was determined and used for experimental assays. Experimental and control proteins were serially diluted in PBS and added to NHS in AP buffer. For some experiments, equal amounts of C5 depleted ($C5^{-/-}$) human serum

(CompTech, Tyler, TX) were used instead of NHS. The volume of protein sample used for each reaction remained constant, keeping concentrations of divalent cations and chelators invariable. The final volume of the mixture was 25 μl. This mixture was then incubated with 25 μl of washed, resuspended RbEs (2×10^8 cells/ml) in disposable borosilicate glass culture tubes for 30 min at 37°C. Control reactions contained RbEs (2×10^8 cells/ml) incubated with only NHS in AP buffer or AP buffer alone. After incubation, 75 μl of N-saline (0.15 M NaCl) was added to each tube. The tubes were then centrifuged at 1000 g for 5 min to pellet all remaining intact RbEs. Supernatants were collected and loaded into 96-well plates. The O.D. of the supernatants was measured at a λ of 405 nm.

Human C3 or fB deposition ELISAs

To determine if Salp20 inhibited the deposition of human C3 or fB onto surfaces, we performed deposition ELISAs using agarose coated plates following a modified protocol of Ribeiro and Valenzuela et al. (19, 28). In the presence of polysaccharides, C3 is activated and covalently attaches to the sugars (30). Factor B then noncovalently binds bound C3b. Since agarose is made of primarily galactose, C3 is activated in the presence of agarose and attaches covalently to agarose surfaces (19, 28). Briefly, 96-well plates were coated with 100 μl of 0.1% agarose in water. The agarose in the plates was dried for 48 hrs at 37°C. Once dry, 50 μl of AP buffer containing a 1:4 dilution of NHS, and either S20NS or CAT at various concentrations was added to the wells and incubated at 37°C for 30 min. In some reactions, S20NS or CAT was added 30 min after the addition of NHS and the wells were incubated an additional 20 min at 37°C. Control wells lacked NHS or contained 25 mM EDTA and NHS. After incubation with NHS, the plates were washed 3 times, 5 min each on an orbital shaker, with 200 μl/well of 1X TBS containing 10 mg/ml bovine serum albumin

(BSA) and 2 mM $MgCl^{2+}$. After washing, 100 μl of a 1:1000 dilution of goat α-human C3 antibody (CompTech, Tyler, TX) or goat α-human fB antibody (CompTech, Tyler, TX) in 1X TBS, 10mg/ml BSA, 2mM $MgCl^{2+}$ was added to each well and the plate was incubated 1 hr at 37°C. The plate was then washed 3 times as previously described, and 100 μl of either a 1:5000 or 1:1000 dilution of an AP-conjugated rabbit α-goat IgG (Sigma, St. Louis, MO) was added to each well. The plate was incubated 45 min at 37°C. Following the incubation, the plate was washed 2 times, 5 min each, with 200 μl/well of 1X TBS, 10 mg/ml BSA. The plate was next washed once with 1X TBS, 10 mg/ml BSA, 0.1% Tween followed by an additional wash with N-saline. After washing, 100 μl of Sigma Fast p-Nitrophenyl phosphate (PNPP, Sigma, St. Louis, MO) was added to each well and the plate was incubated at room temperature for 15 min. After incubation, the O.D. was measured at a λ of 405 nm.

Bactericidal Assays

Bactericidal assays were performed following a modified protocol of Brooks et al (2). Briefly, *B. burgdorferi* (serum resistant) and *B. garinii* (serum sensitive) were grown in BSK-II media at 33°C to a density of 1-5 x 10^7 cells/ml. Cells were collected and washed 2 times with serum free BSK-II. Cells were then resuspended at a density of 1 x 10^7 cells/ml in serum-free BSK-II containing either 20% NHS, 20% heat-inactivated NHS (HI NHS), S20NS (10 μg/ml), CAT (10 μg/ml), or 1X PBS. The bacteria were then incubated at 33°C for 1 hour in the presence of NHS, HI NHS, or NHS and S20NS or various controls. After incubation, live bacteria were counted by dark field microscopy. Spirochetes were considered live if they were moving and maintained their morphology and refractiveness. The lower detection limit of the assay was 5 x 10^4 cells/ml.

2.4 Results

Isac and Salp20 are members of a large family of proteins

Soares et al. (25) and Ribeiro et al. (20) have identified multiple cDNA clones from adult and nymphal cDNA libraries that share homology with Isac and Salp20. In this study, we have discovered an additional 15 unique clones related to Isac and Salp20, increasing the size of the Isac protein family. In order to identify members of this family, we PCR amplified products using two primer sets, S20F & S20R and IsacF & Isac R, from two different cDNA libraries, one generated from the salivary glands of fed *I. scapularis* nymphs and the other from whole fed *I. scapularis* nymphs. We sequenced the PCR products and identified 15 unique clones sharing homology with Isac and Salp20 (Fig 2.1). The translated amino acid sequences of each of the isolated clones ranged from 69-95% sequence similarity to Isac and Salp20. Twelve of the 15 unique clones contained a 5-10 amino acid deletion at positions 134 through 146 (Fig 2.1). Additionally, S20Lclone 5 contained a frameshift mutation at position 171 altering the location of the stop codon by 3 amino acids. In all of the clones identified, a putative secretion signal was present, four cysteines in the mature protein were conserved, and four of the seven N-linked glycosylation sites found in Isac and Salp20 were maintained (Fig 2.1).

Production and purification of Salp20 from *E. coli* and High Five cells

In order to determine if other members of the Isac protein family shared the complement inhibitory properties of Isac, we expressed recombinant Salp20 in insect cells for use in functional assays. For insect cell expression, a V5 epitope and 6X-histidine tag were fused to the C-terminus of Salp20, which was expressed with its native putative N-terminal secretion signal. Insect cells were stably transfected with pIB-S20NS vector to

produce recombinant Salp20-V5-His (S20NS). To evaluate expression of S20NS, cell lysates and media were collected and analyzed by Western blot analysis using a monoclonal antibody directed against the V5-epitope tag (Fig 2.2). As seen in the blot, S20NS is present as a smear in the media of the transfected cells due to its native secretion signal. Some S20NS is also found in the cell lysates.

To purify S20NS from cell media, we subjected media from stably transfected cells to immobilized-metal affinity chromatography. All fractions were collected and analyzed by SDS-PAGE (Fig 2.3). Roughly 50-100 μg of S20NS was purified from 1 liter of cell media. Unexpectedly, S20NS was approximately 48 kDa, much larger than the expected size of 24 kDa, which is the mass of Salp20 containing the C-terminal V5-epitope and 6X-histidine tags (4 kDa) (Fig 2.3). Size exclusion chromatography also indicated that the mass of the protein was approximately 48 kDa (data not shown). In order to confirm the identity of S20NS, the purified protein sample was analyzed by mass spectroscopy at the UNC-Duke Michael Hooker Proteomics Center. By mass spectroscopy, we attained 30% peptide coverage of S20NS, with the majority of the peptides corresponding to only S20NS (data not shown).

Glycosylation Status of S20NS

We believed S20NS was approximately 48 kDa rather than 24 kDa due to post-translational modifications, including N-linked and O-linked glycosylations. Sequence analysis of S20NS reveals 7 potential N-linked glycosylation sites based on the consensus sequence Asn-X-Ser/Thr (Fig 2.1) and 8-14 O-linked glycosylation sites within the C-terminus (20) as predicted by NetOGlyc 3.1 server, indicating the protein could display a large mass due to presence of carbohydrates. In addition, when analyzed by Western blots, S20NS appears as a smear possibly representing differentially glycosylated forms of the

protein (Fig 2.2). To determine if N-linked sugars were responsible for the size increase of S20NS, we digested S20NS with PNGase F, a glycosidase that removes N-linked carbohydrates. After digestion with PNGase F, S20NS shifted from 48 kDa to approximately 38 kDa (Fig 2.4A), indicating S20NS contained N-linked glycosylations. However, the protein did not shift to the expected size of 24 kDa. In addition, mass spectroscopy of S20NS treated with PNGase F indicated the addition of N-linked carbohydrates at 2 potential sites.

In order to determine if S20NS contained O-linked glycosylations in addition to N-linked glycosylations, we treated S20NS with trifluoromethanesulfonic acid (TFMS), which removes N- and O-linked glycans without destroying the peptide backbone. After TFMS treatment, S20NS shifted from 48 kDa to approximately 30 kDa (Fig 2.4A), indicating both N- and O-linked glycosylations were primarily responsible for the size shift of S20NS. The smear below 30 kDa after S20NS was treated with TFMS is most likely the result of protein degradation (Fig 2.4A).

To confirm S20NS contained both N- and O-linked glycans, we subjected S20NS and S20NS digested with PNGase F or treated with TFMS to lectin blotting using the lectin Glanthus nivalis agglutinin (GNA), which recognizes terminal mannose residues. GNA recognized S20NS and S20NS treated with PNGase F (Fig 2.4B), indicating PNGase F partially removed N-linked carbohydrates, leaving O- and some N-linked sugars on S20NS. After TFMS treatment, S20NS was no longer recognized by GNA (Fig 2.4B), suggesting all carbohydrates, both N- and O-linked had been removed.

Alternative Complement Pathway Assays with S20NS

Small quantities of *I. scapularis* saliva are capable of inhibiting the alternative pathway of complement (Fig 2.5A). Valenzuela et al. (28) demonstrated that Isac, a tick salivary protein with 83% amino acid sequence similarity to Salp20, inhibited the alternative complement pathway. To determine if S20NS inhibited the alternative complement pathway, we incubated RbEs with NHS and S20NS in the presence of Mg^{2+}. After incubation, we removed any whole cells or cellular debris by centrifugation. We then calculated the percent lysis of the RbEs by measuring the optical density of the supernatants. As little as 0.625 μg/ml of S20NS inhibited the alternative complement pathway by approximately 70% (Fig 2.5B), indicating S20NS functions similarly to Isac. Interestingly, the percent inhibition by S20NS did not increase beyond 70% with increasing concentrations of S20NS. Cell culture media from chloramphenicol acetyltransferase-V5-His (CAT) expressing High Five cells that was subjected to the same purification procedures as S20NS media and *E. coli* expressed Salp20 did not inhibit RbE lysis at any concentration (Fig 2.5B and data not shown, respectively), indicating that only S20NS produced in insect cells had anti-complement activity. S20NS did not inhibit the classical complement pathway (data not shown), consistent with Isac, tick saliva, and salivary gland extracts (11, 34).

Effect of S20NS on the cleavage and activation of C3

Since S20NS inhibited the alternative complement pathway, we next wanted to determine the step of the alternative pathway that was blocked by S20NS. Activation of the alternative pathway begins with the covalent attachment of C3b to activating surfaces (13, 24, 31, 32). fB then binds surface bound C3b, allowing cleavage of fB into Bb and Ba by fD. The surface bound C3bBb complex, C3 convertase, then cleaves additional C3, allowing

amplification of the pathway by the deposition of more C3b onto surfaces. In order to examine the cleavage state of C3 in the presence of S20NS, we analyzed supernatants and erythrocyte cell pellets from RbE lysis assays by immunoblots. We performed the RbE lysis assays with C5 ($C5^{-/-}$) depleted human serum to allow deposition of C3b on cell surfaces but prevent complete lysis of the erythrocytes by the membrane attack complex. Supernatants and cell pellets were subjected to Western blot analysis to determine if C3 was cleaved into C3a and C3b, and if C3b deposition occurred on RbE surfaces. At a concentration of 10 μg/ml, S20NS significantly reduced the production of C3a from the cleavage of C3 when compared to reactions containing only PBS as indicated by the lower band (Fig 2.6A). When present in lower concentrations, S20NS had little effect on C3 cleavage. In the blots, the upper band represents the α-chain of C3 and the middle bands represent the α-chain of iC3(H_2O) and an α-chain degradation product of C3 (~ 45 kDa).

C3 and C3b are composed of two chains, the α- and β-chains (13). When activated, the α-chain of C3 covalently attaches to a surface through its reactive thioester. The β-chain of C3 remains attached to the covalently bound α-chain by a disulfide bond. In the blot shown, S20NS (10 μg/ml) prevented the covalent attachment of the C3b to RbE surfaces when compared to pellets from reactions containing only PBS, as indicated by the upper arrow representing covalently attached C3b α-chains (C3 α`-chains) and the middle arrow representing C3b β-chains. (Fig 2.6B). Under the reducing conditions of SDS-PAGE, the β-chain dissociates from the α-chain of C3b. The lower arrow indicates C3b α-chain degradation products. Purified CAT protein containing the C-terminal V5 epitope and 6X-His tag (CAT) had no effect on the cleavage of C3 or the deposition of C3b at equivalent concentrations. No protein was detected in the reaction containing NHS and PBS because no

cell pellet formed due to presence of all complement components, including C5, in NHS causing complete lysis of the erythrocytes.

To further determine how S20NS was acting to prevent C3a production and C3b deposition, we performed C3 and fB deposition ELISAs following the protocol of Valenzuela et al. (28). In this system, C3 is activated in the presence of Mg^{2+} and agarose and covalently deposits to agarose coated wells of a 96-well plate (28, 30). Factor B then noncovalently attaches to agarose-bound C3b, forming the C3 convertase. S20NS (10 μg/ml) inhibited the deposition of both C3b and fB when added immediately with NHS to the agarose coated wells (Fig 2.7A and 2.7B, respectively). However, when added 30 min after the addition of NHS, S20NS (10 μg/ml) did not displace covalently bound C3b from the agarose (Fig 2.7A). Interestingly, S20NS (10 μg/ml) displaced fB from covalently bound C3b when added 30 min after the addition of NHS (Fig 2.7B). These data indicate S20NS works similarly to Isac, dissociating fB from covalently bound C3b and disrupting the activity of the C3 convertase.

Borreliacidal Assays with S20NS

Recently, Kyckova et al. have demonstrated *I. ricinus* salivary gland extracts protect *B. afzelii* from complement mediated lysis *in vitro* (10). Since S20NS is an *I. scapularis* salivary protein secreted into a host during feeding and pathogen transmission, the anticomplement activity of S20NS could potentially protect vector transmitted pathogens from complement mediated killing. To address this hypothesis, we performed bactericidal assays with NHS, S20NS (10 μg/ml), and a serum sensitive species of *Borrelia*. In the presence of 20% NHS, S20NS protected *Borrelia garinii*, a serum sensitive species of *Borrelia* causing Lyme disease in Europe, from complement mediated killing by

approximately 70% (Fig 2.8). *B. burgdorferi*, a serum resistant species of *Borrelia*, was unaffected by NHS in presence or absence of S20NS.

2.5 Discussion

In the current study, we identified additional members of the ILP family previously characterized by Soares et al. (25) and Ribeiro et al.(20). All family members identified except Salp9 possess putative secretion signals, contain 4 conserved cysteines in the mature protein, and retain at least four of the seven N-linked glycosylation sites. An individual tick may express many or all of these genes, with expression patterns that change over time or in different tissues. Alternatively, proteins encoded by genes of this family with potentially similar functions within an individual tick may display antigenic variation, which may be needed to escape host immune responses during prolonged feeding periods. However, since the family members were isolated from cDNA libraries generated from hundreds of nymphal ticks, variation in the sequences may be a result of genetic variation between individual ticks, rather than each tick possessing several family members.

In this work, we also successfully produced and purified Salp20 from *E. coli* and High Five insect cells. Only the protein produced in insect cells had functional activity. Furthermore, the insect expressed protein had an approximate molecular weight of 48 kDa, unlike the expected molecular weight of 24 kDa. N- and O-linked glycosylations were primarily responsible for the increased molecular mass. S20NS may contain other post-translational modifications since removal of all carbohydrates shifted the protein to approximately 30 kDa, rather than 24 kDa. Interestingly, other members of this salivary protein family also appear to have increased masses as Isac was approximately 45 kDa, even

though the predicted size was only 18.5 kDa (29) and additional family members identified in this study have larger masses than predicted when expressed in High Five cells (data not shown).

We found S20NS inhibited the alternative pathway of complement but not the classical pathway. The protein inhibited the alternative pathway by preventing the cleavage of C3 into C3a and C3b, thereby preventing the deposition of C3b onto RbE surfaces. Further investigation demonstrated S20NS dissociated fB from covalently bound C3b, disrupting the C3 convertase. This mechanism of inhibition is similar to fH and the mechanism previously demonstrated for Isac (28).

Inhibition of the alternative pathway by a family of *I. scapularis* complement inhibitors including both Isac and Salp20 potentially allows successful feeding of the tick by limiting the production of an inflammatory response at the site of tick feeding. In fact, Soares et al. (25) reported that RNAi mediated silencing of Salp20 in ticks interfered with successful tick feeding. In addition to allowing for successful tick feeding, inhibition of the alternative complement cascade by *I. scapularis* salivary proteins may also allow successful pathogen transmission from an infected tick. Ramamoorthi et al. (18) have demonstrated Salp15, an *I. scapularis* salivary protein, binds *B. burgdorferi* surfaces and significantly enhances its transmission to a host during tick feeding. In this work, we have demonstrated that serum sensitive species of *Borrelia* survive killing by human complement components in the presence of recombinant Salp20, indicating this family of proteins potentially facilitates efficient pathogen transmission during tick feeding.

At this time, we are interested in determining the importance and necessity of the large family of salivary proteins that possibly inhibit host complement. We are currently

investigating whether other family members block the alternative complement pathway in a manner similar to both Isac and Salp20. Furthermore, we are interested in determining if any of these family members display antigenic variation or posses other activities that may be essential for successful tick feeding and/or pathogen transmission.

2.6 Acknowledgements

We thank Dan Sonenshine and Anne Broadwater for their assistance with tick salivations. We also thank Carol Parker of the UNC-Duke Michael Hooker Proteomics Center for her assistance with mass spectroscopy, Ruth Silversmith for her assistance with size exclusion chromatography, and David Klapper for his assistance with purification of deglycosylated proteins. This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (5U01AI058263-04), USA.

2.7 Contributions

In this work, I designed and performed the experiments. Holly Patterson, a lab technician under my direction, assisted with the production of purified recombinant insect proteins. Erol Fikrig provided the original *salp20* cDNA construct, and Chris Elkins and Aravinda de Silva assisted with the design of experiments. This work is published in Insect Molecular Biology, Volume 16(4), pages 469-479 and permission for reprint in this dissertation is not required by the publisher.

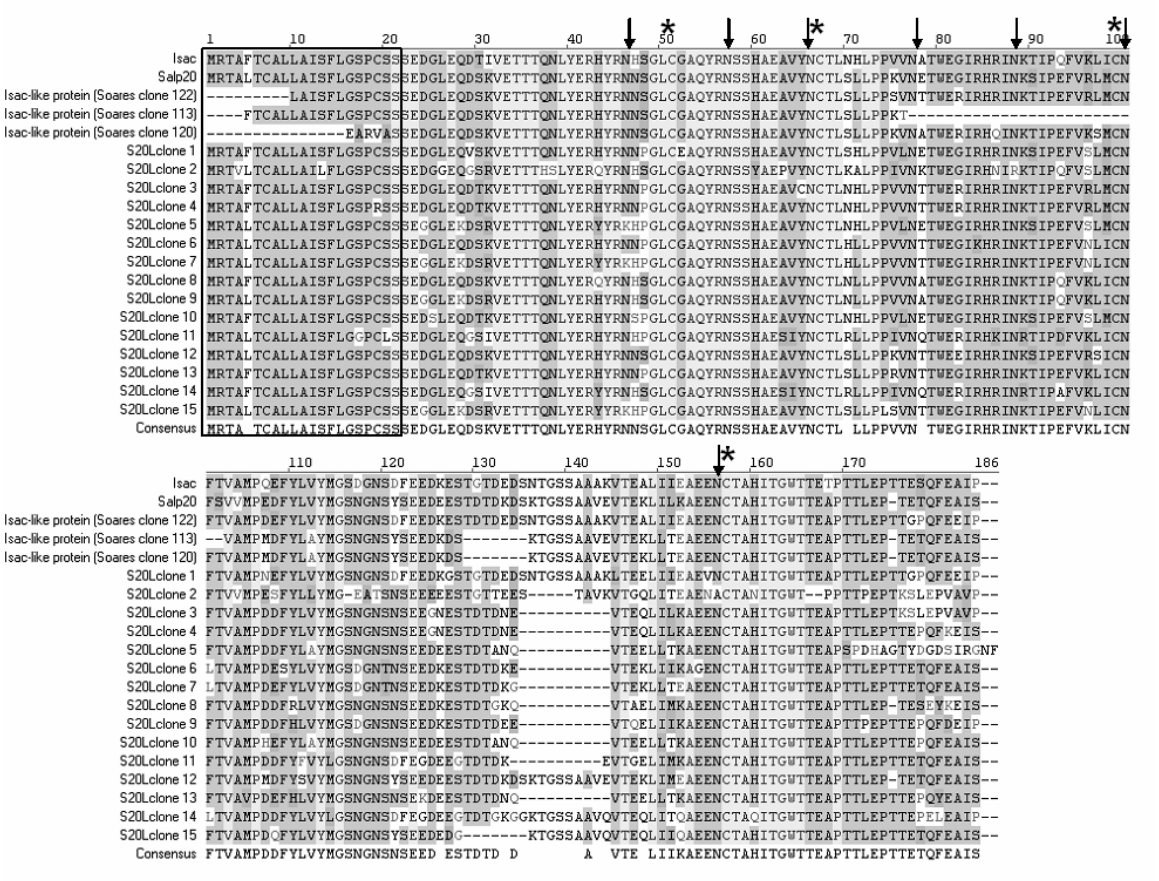


Figure 2.1 Amino acid alignment of Salp20, Isac, and cDNA clones related to both Salp20 and Isac. Clones identified by PCR analysis from whole tick and salivary gland cDNA libraries were aligned with Salp20, Isac, and cDNA clones previously identified by Soares et al. (25). Boxed light gray residues indicate conserved amino acids in all clones, and dark gray boxed residues indicate conservation among some of the clones. The putative secretion signals of Salp20, Isac, and all cDNA clones are boxed. Potential N-linked glycosylation sites are marked by arrows and cysteines conserved in the mature proteins of all clones are indicated asterisks.

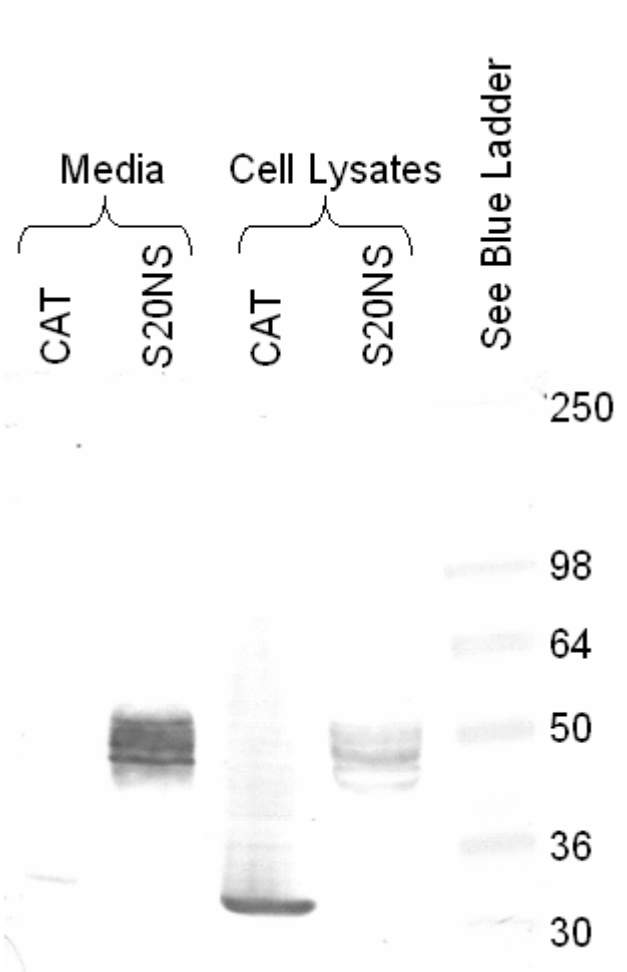


Figure 2.2 Expression of Salp20 in High Five (*Trichoplusia ni*) cells. Western blot analysis of either media or cell lysates from cultures of High Five cells stably transfected with pIB-CAT (CAT) (~ 34 kDa), pIB-S20NS (S20NS) (~ 48 kDa). Blots were probed with mouse α-V5 IgG (1:5000), washed, and subsequently probed with AP-conjugated goat α-mouse IgG (1:1000).

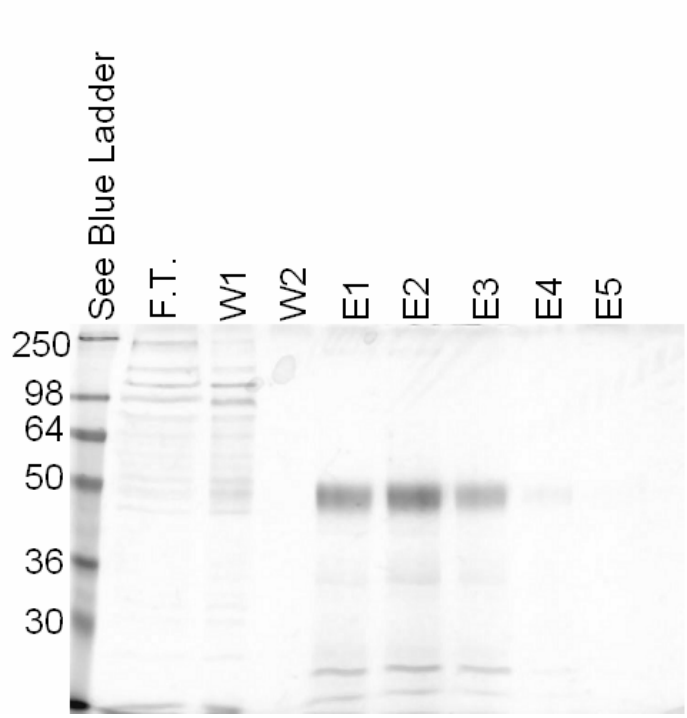


Figure 2.3 Purification of S20NS from transfected High Five cell media. Media from transfected High five cells was dialyzed and then subjected to immobilized metal affinity chromatography. Fractions were collected and subjected to SDS-PAGE analysis followed by Simply Blue Staining. F.T. – flow through, W1 – wash 1, E1 – elution 1.

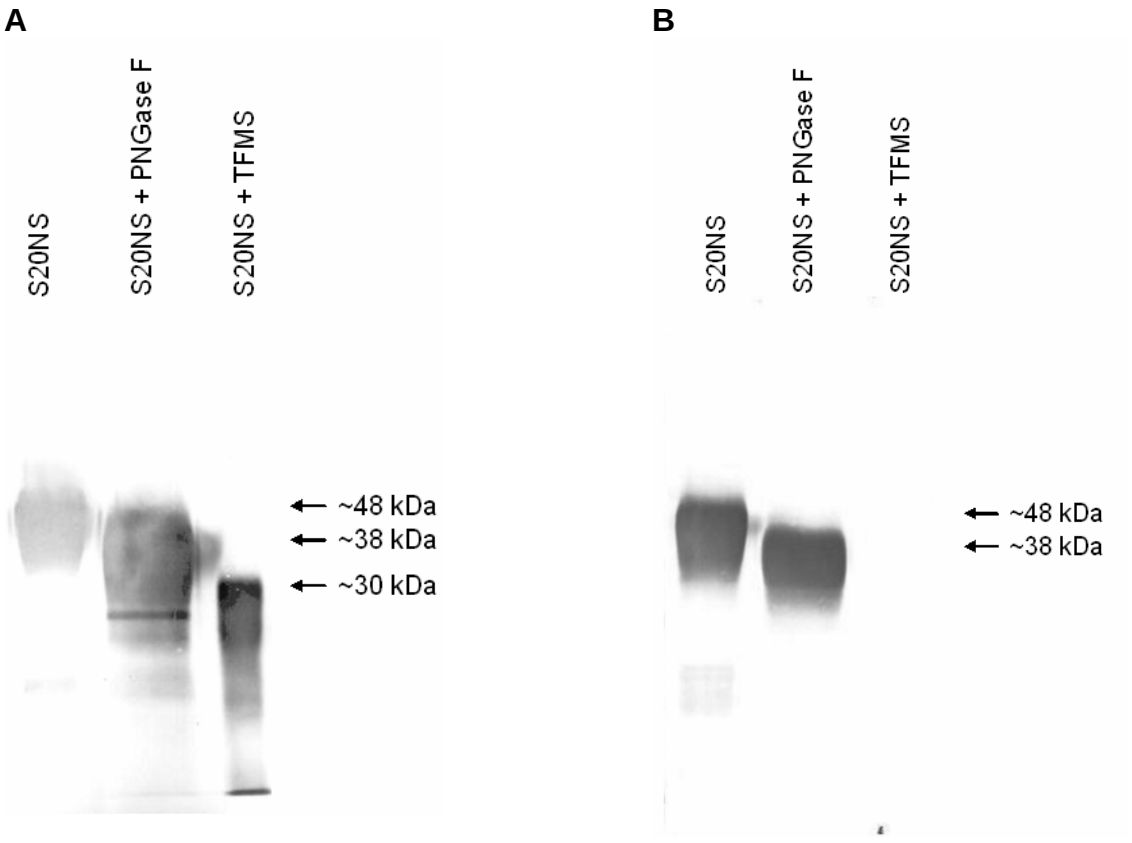
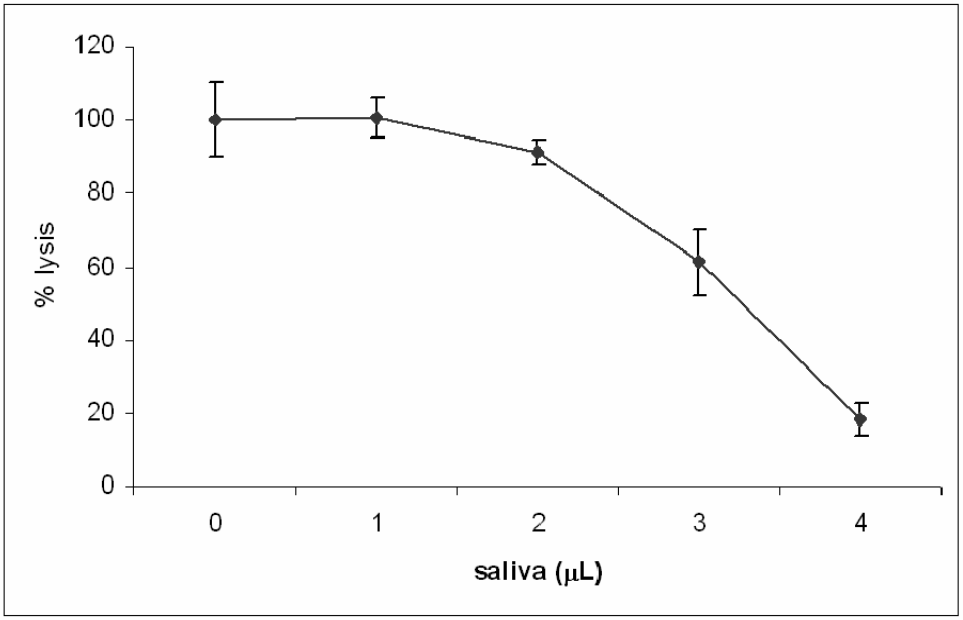


Figure 2.4 Deglycosylations and lectin blotting of S20NS. For deglycosylations, S20NS was either digested with PNGase F overnight at 37^{o}C or treated with TFMS for 3 hrs at 4^{o}C. The glycosylated and deglycosylated proteins were subjected to 10% SDS-PAGE and Western blot analysis with a monoclonal mouse anti-V5 antibody (1:5000) (***A***) or lectin blot analysis with DIG-labeled GNA (1:1000) and AP-conjugated anti-DIG sheep Fab fragments (1:1000) (***B***).

A



B

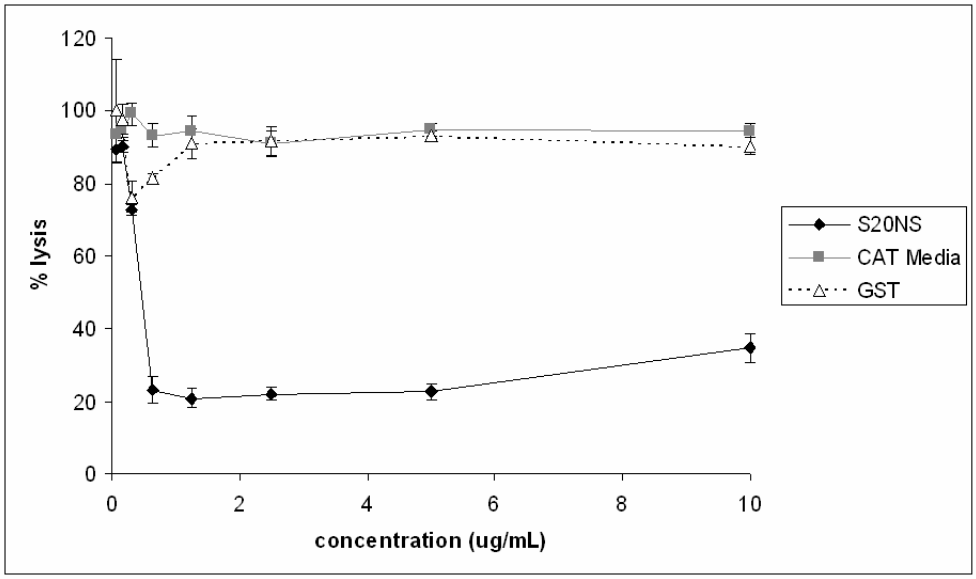


Figure 2.5 RbE Lysis Assays with *I. scapularis* saliva (***A***) or S20NS (***B***). Various amounts of tick saliva (***A***) or various concentrations of S20NS or a negative control protein, glutathione-S-transferase (GST), (***B***) were incubated with RbEs and NHS (1:4) in the presence of Mg^{2+} for 30 min at 37°C. The O.D. of supernatants from pelleted cells after lysis had occurred was measured and the percent lysis was calculated. The RbE lysis assay with S20NS (***B***) is a representative experiment of 10 different experiments giving similar results. The error bars for each assay depict the standard deviation of the mean O.D. value of triplicates.

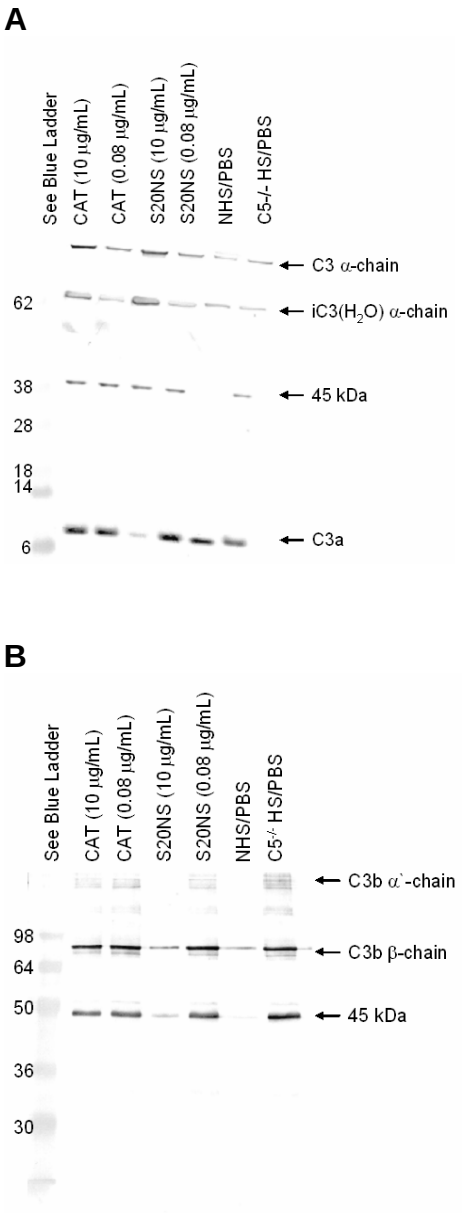
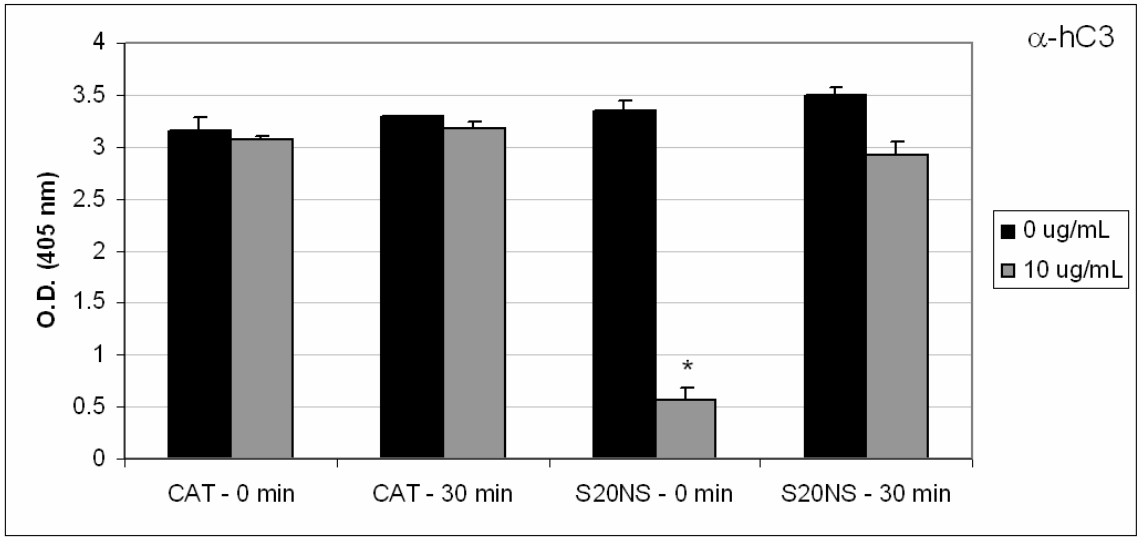


Figure 2.6 Activation of C3 and deposition of C3b on RbE surfaces during RbE lysis assays using C5 depleted human serum. (***A***) Supernatants from RbE lysis assays using $C5^{-/-}$ human serum were collected and subjected to Western blot analysis with rabbit α-human C3a antibody (1:1000) and AP-conjugated goat α-rabbit IgG (1:1000). C3a (10 kDa), C3 α-chains (~ 110 kDa), iC3(H2O) α-chains (~ 77 kDa), and C3 α-chain degradation products (~ 45 kDa) are all indicated by the arrows. (***B***) Pellets from the lysis assays were collected, washed, resuspended, and subjected to Western blot analysis using goat α-human C3 antibody (1:1000) and AP-conjugated rabbit α-goat IgG (1:25000). Covalently attached C3 α-chains (α`), C3 β-chains (~ 76 kDa), and α-chain degradation products (~ 45 kDa) are indicated by the arrows. As a positive control for activation of the alternative pathway and lysis of the RbEs, NHS was used instead of $C5^{-/-}$ human serum in both (***A***) and (***B***).

A



B

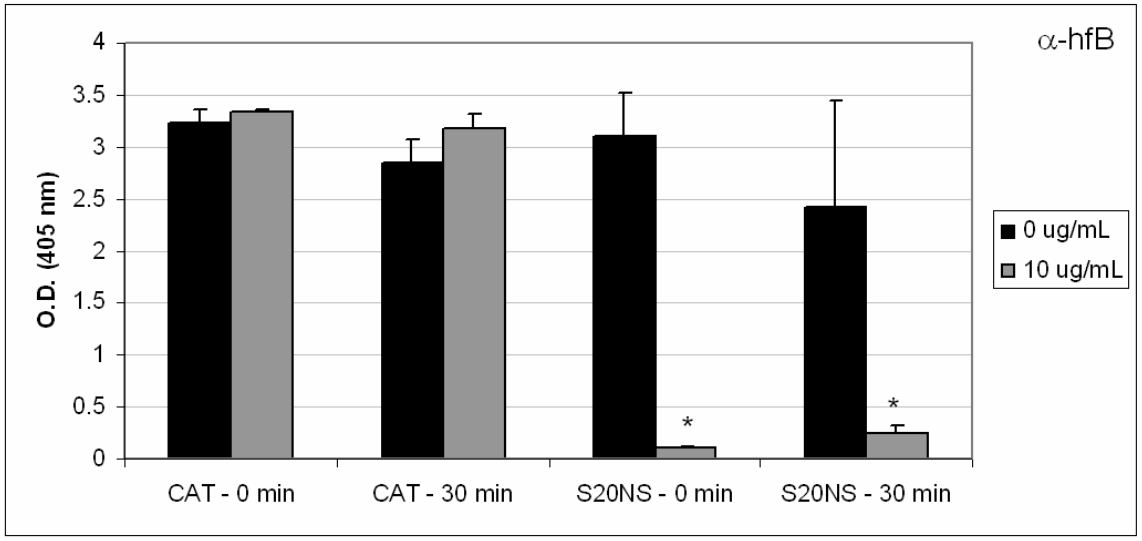


Figure 2.7 Deposition of human C3 (***A***) or Factor B (***B***) in the presence of recombinant S20NS. NHS (1:4) in AP Buffer was added to agarose coated wells and either S20NS or CAT (10 μg/ml) were added to the wells of a 96-well plate either immediately with NHS (0 min) or 30 min after NHS (30 min). (***A***) Plates were washed and incubated with a primary goat α-human C3 antibody (1:1000) and a secondary rabbit α-goat AP conjugated antibody (1:1000). (***B***) Plates were washed and incubated with a primary goat α-human fB antibody (1:5000) and a secondary rabbit α-goat IgG AP conjugated antibody (1:1000). Each graph depicts a single experiment performed in triplicate that is representative of 4 experiments with similar results. The error bars represent the standard deviation of the mean O.D value. The asterisks represent statistically significant differences between negative controls (0 μg/ml) and S20NS samples (10 μg/ml) at each timepoint where $p<0.05$.

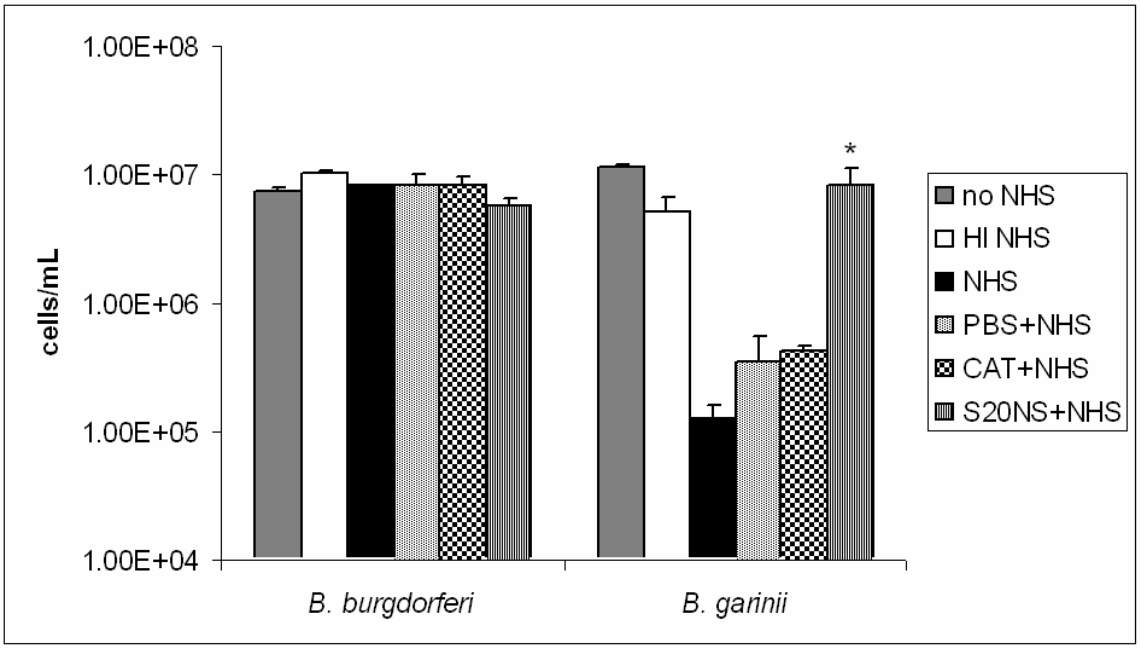


Figure 2.8 *Borreliacidal* assays in the presence of S20NS. *B. burgdorferi* (serum resistant) or *B. garinii* (serum sensitive) at a density of 1 x 10^7 cells/ml were incubated with 20% NHS for 1 hour at 33^{o}C in the absence or presence of 10 μg/ml of S20NS or CAT. As a negative control for killing, cells were also incubated with 20% heat inactivated NHS (HI NHS). After incubation, live cells were counted by dark field microscopy and cell densities were determined. The lower detection limit of the assay was 5 x 10^4 cells/ml. The results depict a single experiment performed in duplicate that represents 3 experiments with similar results. The error bars represent the standard deviation of the mean cell density. The asterisk represents statistically significant difference between the NHS and S20NS+NHS groups where $p<0.06$.

2.8 References

1. Alarcon-Chaidez, F. J., U. U. Muller-Doblies, and S. Wikel. 2003. Characterization of a recombinant immunomodulatory protein from the salivary glands of *Dermacentor andersoni*. *Parasite Immunol*. 25: 69-77.

2. Brooks, C. S., S. R. Vuppala, A. M. Jett, A. Alitalo, S. Meri, and D. R. Akins. 2005. Complement regulator-acquiring surface protein 1 imparts resistance to human serum in *Borrelia burgdorferi*. *J Immunol*. 175: 3299-308.

3. Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis. 1982. Lyme disease-a tick-borne spirochetosis? *Science*. 216: 1317-9.

4. Das, S., G. Banerjee, K. DePonte, N. Marcantonio, F. S. Kantor, and E. Fikrig. 2001. Salp25D, an *Ixodes scapularis* antioxidant, is 1 of 14 immunodominant antigens in engorged tick salivary glands. *J Infect Dis*. 184: 1056-64.

5. Gasque, P. 2004. Complement: a unique innate immune sensor for danger signals. *Mol Immunol*. 41: 1089-98.

6. Gillespie, R. D., M. C. Dolan, J. Piesman, and R. G. Titus. 2001. Identification of an IL-2 binding protein in the saliva of the Lyme disease vector tick, *Ixodes scapularis*. *J Immunol*. 166: 4319-26.

7. Gillespie, R. D., M. L. Mbow, and R. G. Titus. 2000. The immunomodulatory factors of bloodfeeding arthropod saliva. *Parasite Immunol*. 22: 319-31.

8. Kirkitadze, M. D. and P. N. Barlow. 2001. Structure and flexibility of the multiple domain proteins that regulate complement activation. *Immunol Rev*. 180: 146-61.

9. Kovar, L., J. Kopecky, and B. Rihova. 2002. Salivary gland extract from *Ixodes ricinus* tick modulates the host immune response towards the Th2 cytokine profile. *Parasitol Res*. 88: 1066-72.

10. Kyckova, K. and J. Kopecky. 2006. Effect of tick saliva on mechanisms of innate immune response against *Borrelia afzelii*. *J Med Entomol*. 43: 1208-14.

11. Lawrie, C. H., S. E. Randolph, and P. A. Nuttall. 1999. *Ixodes* ticks: serum species sensitivity of anticomplement activity. *Exp Parasitol*. 93: 207-14.

12. Lawrie, C. H., R. B. Sim, and P. A. Nuttall. 2005. Investigation of the mechanisms of anti-complement activity in *Ixodes ricinus* ticks. *Mol Immunol*. 42: 31-8.

13. Morgan, B. P., Harris, C. L., 1999. *Complement regulatory proteins*. Academic Press, San Diego, CA.

14. Nuttall, P. A., G. C. Paesen, C. H. Lawrie, and H. Wang. 2000. Vector-host interactions in disease transmission. *J Mol Microbiol Biotechnol*. 2: 381-6.

15. Ohnishi, J., J. Piesman, and A. M. de Silva. 2001. Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. *Proc Natl Acad Sci U S A*. 98: 670-5.

16. Paesen, G. C., P. L. Adams, K. Harlos, P. A. Nuttall, and D. I. Stuart. 1999. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. *Mol Cell*. 3: 661-71.

17. Pangburn, M. K., R. D. Schreiber, and H. J. Muller-Eberhard. 1981. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J Exp Med*. 154: 856-67.

18. Ramamoorthi, N., S. Narasimhan, U. Pal, F. Bao, X. F. Yang, D. Fish, J. Anguita, M. V. Norgard, F. S. Kantor, J. F. Anderson, R. A. Koski, and E. Fikrig. 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature*. 436: 573-7.

19. Ribeiro, J. M. 1987. *Ixodes dammini*: salivary anti-complement activity. *Exp Parasitol*. 64: 347-53.

20. Ribeiro, J. M., F. Alarcon-Chaidez, I. M. Francischetti, B. J. Mans, T. N. Mather, J. G. Valenzuela, and S. K. Wikel. 2006. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol*. 36: 111-29.

21. Ribeiro, J. M. and I. M. Francischetti. 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol*. 48: 73-88.

22. Ribeiro, J. M., G. T. Makoul, J. Levine, D. R. Robinson, and A. Spielman. 1985. Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, *Ixodes dammini*. *J Exp Med*. 161: 332-44.

23. Ribeiro, J. M. and A. Spielman. 1986. *Ixodes dammini*: salivary anaphylatoxin inactivating activity. *Exp Parasitol*. 62: 292-7.

24. Schreiber, R. D., M. K. Pangburn, P. H. Lesavre, and H. J. Muller-Eberhard. 1978. Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. *Proc Natl Acad Sci U S A*. 75: 3948-52.

25. Soares, C. A., C. M. Lima, M. C. Dolan, J. Piesman, C. B. Beard, and N. S. Zeidner. 2005. Capillary feeding of specific dsRNA induces silencing of the *isac* gene in nymphal *Ixodes scapularis* ticks. *Insect Mol Biol*. 14: 443-52.

26. Sonenshine, D. E., 1993. *Biology of ticks.* Oxford University Press, New York, NY.

27. Tatchell, R. J. 1967. A modified method for obtaining tick oral secretion. *J Parasitol*. 53: 1106-7.

28. Valenzuela, J. G., R. Charlab, T. N. Mather, and J. M. Ribeiro. 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis. J Biol Chem*. 275: 18717-23.

29. Valenzuela, J. G., I. M. Francischetti, V. M. Pham, M. K. Garfield, T. N. Mather, and J. M. Ribeiro. 2002. Exploring the sialome of the tick *Ixodes scapularis. J Exp Biol*. 205: 2843-64.

30. Vogt, W. 1974. Activation, activities and pharmacologically active products of complement. *Pharmacol Rev*. 26: 125-69.

31. Walport, M. J. 2001. Complement. First of two parts. *N Engl J Med*. 344: 1058-66.

32. Walport, M. J. 2001. Complement. Second of two parts. *N Engl J Med*. 344: 1140-4.

33. Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein beta1H. *Proc Natl Acad Sci U S A*. 73: 3268-72.

34. Wikel, S. K. 1979. Acquired resistance to ticks: expression of resistance by C4-deficient guinea pigs. *Am J Trop Med Hyg*. 28: 586-90.

35. Wikel, S. K. 1996. Host immunity to ticks. *Annu Rev Entomol*. 41: 1-22.

36. Wikel, S. K. 1999. Tick modulation of host immunity: an important factor in pathogen transmission. *Int J Parasitol*. 29: 851-9.

CHAPTER 3

A novel mechanism of complement inhibition unmasked by a tick salivary protein that binds properdin

Katharine R. Tyson[1], Christopher Elkins[2], and Aravinda M. de Silva[1]

[1]Department of Microbiology and Immunology and [2]Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, NC 27599.

3.1 Abstract

Ixodes scapularis Salp20 is a member of the ILP family that inhibits the alternative complement pathway. In this study, we demonstrate that the target of Salp20 is properdin. Properdin is a natural, positive regulator of the alternative pathway that binds to the C3 convertase, stabilizing the molecule. Salp20 directly bound to and displaced properdin from the C3 convertase. Displacement of properdin accelerated the decay of the C3 convertase, leading to inhibition of the alternative pathway. S20NS is distinct from known decay accelerating factors, such as DAF, CR1, and fH, which directly interact with either C3b or Bb.

3.2 Introduction

Ixodes ticks are efficient vectors of various pathogens, including *B. burgdorferi*, the causative agent of Lyme disease (1, 2). Tick saliva contains multiple proteins that modulate host immunity and hemostasis, which facilitate blood feeding and pathogen transmission (3). One property of tick saliva is inhibition of the alternative complement pathway (4-6). Isac, Salp20, Irac-1, and Irac-2 are members of a large family of homologous proteins, referred to as the ILP family, that specifically inhibit the alternative pathway by dissociating the components of the C3 convertase, C3b and Bb (7-11). While preventing the activation of the alternative pathway, Salp20 also protects *B. garinii* from complement mediated killing by NHS (10), suggesting these anti-complement proteins facilitate tick feeding as well as pathogen transmission and survival.

In this work, we have characterized the specific mechanism by which Salp20 inhibits the alternative pathway. We demonstrate that Salp20 directly binds to properdin, a positive regulator of the alternative pathway. Salp20 displaces properdin from the C3 convertase, thus accelerating the decay of the alternative pathway C3 convertase.

3.3 Materials and Methods

Recombinant proteins, purified proteins, and antibodies

S20NS and CAT were expressed and purified from stably transfected High Five cells as described previously (10). Recombinant protein purity was determined by SDS-PAGE, while purified protein concentrations were determined by Bradford analysis. Purified human complement components – C3b (cat no. A114), fB (cat no. A135), fD (cat no. A136), and properdin (cat no. A139) – and antibodies directed against the complement components –

goat α-human C3 (cat no. A213), goat α-human fB (cat no. A235), and goat α-human properdin (cat no. A239) were obtained from CompTech (Tyler, TX). Mouse α-His IgG was obtained from Qiagen and mouse α-V5 IgG was obtained from Invitrogen (Carlsbad, CA).

Assays to measure the decay of C3 convertases

To measure the decay of C3 convertases formed from complement components in NHS in the presence of S20NS, ELISAs were performed as described previously (9, 10). Briefly, microtiter plates were coated with 0.1% agarose for 48 h at 37°C. To form C3 convertases in the wells, the agarose coated wells were then incubated with NHS in AP Buffer (gelatin veronal buffer with Mg^{2+} and Ca^{2+} [GVB^{++}, CompTech, Tyler, TX], 5 mM EGTA, 5 mM $MgCl_2$) for 1 h at 37°C. The plate bound convertases were subsequently washed and incubated with various concentrations of S20NS for 30 min at 37°C. After incubation, the wells were washed with Wash buffer (TBS, 10 mg/ml BSA, 2 mM $MgCl_2$), and any remaining plate-bound Bb or properdin were detected by standard ELISA methods using either a primary goat α-human fB Ab or a goat α-human properdin Ab, followed by a secondary AP-conjugated rabbit α-goat IgG. OD_{405} values were determined and percent deposition was calculated using the following equation: ((OD_{405} sample – OD_{405} NHS with 25mM EDTA)/(OD_{405} sample without S20NS or CAT – OD_{405} NHS with 25mM EDTA)) x 100.

To measure the decay of C3 convertases formed from purified components in the presence of S20NS, we performed an ELISA adapted from Hourcade et al. (12). Microtiter plate wells were coated with 250 ng/well of C3b in PBS for 12 h at 4°C. After coating, the wells were washed with PBS and then blocked for 15 min at 23°C with Binding buffer (PBS, 75 mM NaCl, 5mM $NiCl_2$, 4% BSA, 0.05% Tween-20). To form the C3 convertase, fB (400

ng/well) and fD (25 ng/well) in Binding buffer were added to the wells and incubated at 37°C for 2 h. The wells were subsequently washed with PBS and then incubated with various concentrations of S20NS, CAT, or fH in Binding buffer for 30 min at 37°C. The wells were washed with TBST (TBS, 0.2% Tween-20), and the OD_{405} was determined for any remaining Bb by ELISA using specific antibodies. Percent deposition was calculated using the following equation: ((OD_{405} sample – OD_{405} C3b coated wells)/(OD_{405} sample without S20NS or CAT – OD_{405} C3b coated wells)) x 100.

In some assays, properdin was included in the formation of the C3 convertase from purified complement components. After coating the wells with C3b, fB (50 ng/well), fD (25 ng/well), and properdin (50 ng/well) in Mg^{2+} Binding buffer (PBS, 75 mM NaCl, 10 mM $MgCl_2$, 4% BSA, 0.05% Tween) were incubated in the wells for 2 h at 37°C. Plate bound Bb and properdin were detected by standard ELISAs. In these assays, the concentration of fB was lower than in the assays lacking properdin because properdin stabilized the C3 convertase more efficiently than the substitution of Mg^{2+} with Ni^{2+} in the assays lacking properdin. Since the convertase was stabilized more efficiently, less fB was needed to achieve equivalent OD_{405} readings for fB deposition between the two assays. Percent deposition was calculated as described above. To form C3b-properdin (C3bP) complexes, plates were coated with C3b as described above and properdin (50 ng/well) was subsequently added. Bound properdin was detected as described.

Cofactor activity assays

To investigate the cofactor activity of S20NS during fI mediated degradation of C3b, cofactor activity assays were performed following a modified protocol of McRae et al. (13). Briefly, 200 ng of C3b was incubated with various concentrations of S20NS, fH, or CAT and

400 ng of fI in reaction buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl) for 30 min at 37°C. After incubation, C3b degradation products were analyzed by immunoblots using a primary goat α-C3 Ab and a secondary AP-conjugated rabbit α-goat IgG.

To determine if S20NS degraded C3b in the presence of fH, 200 ng of C3b were incubated with 400 ng of either S20NS or fI and 1 μg of fH in reaction buffer for 30 min at 37°C. C3b degradation products were then detected by immunoblotting.

Assays to detect Salp20 binding to properdin

To detect direct binding of S20NS to properdin, we performed immunoprecipitations and analyzed the precipitates by immunoblot. S20NS (150 ng) was incubated with properdin (450 ng) at 37°C for 30 min in Binding buffer (PBS, 75 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween) and then added to blocked Protein-A sephadex beads (Sigma, St. Louis, MO) coated with 1 μg of mouse α-V5 IgG for 1 hour at 37°C. The sephadex beads were washed and resuspended in non-reducing SDS-PAGE loading dye. Samples were subjected to SDS-PAGE and immunoblotting with antibodies specific for either S20NS or properdin.

As an alternative method to detect S20NS binding to properdin, microtiter plate wells were first coated with 100 ng/well of S20NS, CAT, or C3b for 12 h at 4°C. The wells were then blocked and incubated with 100 ng/well properdin for 1 h at 37°C. After incubation, the wells were washed. To detect plate bound properdin, the wells were incubated with a primary goat α-properdin Ab and a secondary AP-conjugated rabbit α-goat IgG.

Saturation Binding Assays

To determine the relative binding affinity of properdin for either S20NS or C3b, we performed a solid-phase binding assay. Microtiter plates were coated with a saturating amount of either S20NS (10 ng/well) or C3b (10 ng/well) for 12 hrs at 4°C in 0.1M

Carbonate Binding Buffer, pH 9.2. After coating, the wells were blocked with Binding buffer for 1 hr at 37^{o}C and then incubated with increasing concentrations of properdin in Binding buffer (PBS, 75 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween) at 37^{o}C for 1 hr The wells were then washed with TBST, and bound properdin was detected by an ELISA using a primary goat α-human properdin Ab and a secondary AP-conjugated rabbit α-goat Ab. Development of the substrate was stopped after 3 min by the addition of 3M NaOH. The OD_{405} was determined and plotted, and relative K_d values were calculated using GraphPad Prism 4 (GraphPad Software).

3.4 Results

S20NS specifically inhibits the alternative complement pathway by dissociating the C3 convertase

Isac and related tick salivary proteins, including S20NS, specifically inhibit the alternative complement pathway by dissociating the components of the C3 convertase (5, 9-11). In the current study, the mechanism of inhibition of the alternative pathway by S20NS was confirmed by performing an agarose based ELISA as described previously (9). In this assay, C3 present in NHS is activated by agarose coated microtiter plates. C3 activation leads to the formation of an active convertase on the agarose consisting of covalently bound C3b and Bb (9). When increasing concentrations of S20NS were incubated with preformed covalently bound C3 convertases, the amount of bound Bb was reduced (IC_{50} of S20NS = 0.8 μg/ml) (Fig 3.1). Equal concentrations of purified recombinant CAT protein, a negative control protein expressed from the same expression vector as S20NS in High Five cells, did not disrupt the C3 convertase. Previous studies have demonstrated that covalently attached

C3b is unaffected in the presence of S20NS (10). Together, these results indicate that S20NS inhibits the alternative complement pathway by specifically dissociating Bb from the C3 convertase, similar to the activity of Isac and related family members (9-11). Since the IC_{50} of S20NS = 0.8 μg/ml, we chose to use concentrations of either 1 or 2 μg/ml of S20NS for subsequent experiments.

S20NS is a unique regulator of the alternative pathway

Since S20NS and Isac inhibit the alternative pathway by dissociating Bb from the C3 convertase, it has been hypothesized that Salp20 and Isac act in a manner similar to fH, a natural negative regulator of the alternative pathway (9). Human fH is a serum glycoprotein that directly binds C3b, displacing Bb and causing decay acceleration of the C3 convertase (14, 15). In addition, fH also acts as a cofactor for fI mediated degradation of C3b (14, 16, 17). To determine if S20NS acted by the same mechanism as fH, we performed ELISAs to measure the decay of C3 convertases in the presence of S20NS or fH. In these assays, we formed C3 convertases in the wells of microtiter plates from purified complement components (C3b, fB, and fD) and then incubated S20NS or various control proteins with the convertases. After the incubation, we detected any remaining bound Bb in the convertases by ELISA. The C3 convertases formed from purified components were disrupted by fH as indicated by the reduction in the amount of deposited Bb (Fig 3.2A). Surprisingly, however, S20NS displayed no effect (Fig 3.2A). These results indicate that in this assay S20NS does not share similar activity to fH. Moreover, these results also demonstrate that S20NS dissociates C3 convertases formed from NHS (Fig 3.1) but not convertases formed from purified complement components (Fig 3.2A).

Experiments were also performed to determine if S20NS acted as a cofactor for fI mediated degradation of C3b, similar to fH. S20NS was mixed with purified fI and the mixture was then added to purified C3b. Degradation products of the C3b α-chain (C3b α`-chain), 67 and 43 kDa fragments, were detected by immunoblots with specific Abs. Various concentrations of either S20NS or CAT were incapable of mediating fI degradation of C3b, unlike fH, which when incubated in the presence of fI, resulted in the degradation of C3b (Fig 3.2B).

Since S20NS did not act as a cofactor for fI mediated C3b degradation like fH, experiments were done to test if S20NS functioned similarly to fI and degraded C3b in the presence of fH. When S20NS was mixed with fH and then incubated with C3b, we observed no degradation of C3b, whereas fI incubated with fH and C3b resulted in C3b degradation (Fig 3.2C). Together, these results demonstrate that S20NS disrupts the C3 convertase by a mechanism that is different from both fH and fI.

S20NS inhibits the alternative pathway by displacing properdin from the C3 convertase

S20NS dissociated the components of the C3 convertase when the convertase was formed from NHS (Fig 3.1) but not from purified complement components (Fig 3.2A). The discrepancy in the activity of S20NS between the two assays is likely due to differences in the composition of the convertases formed from either NHS, which potentially contain C3b, Bb, and properdin, or from purified complement components, which contain only C3b and Bb. Properdin is a positive regulator of the alternative pathway that binds and stabilizes the C3 convertase, significantly increasing its half life (18, 19). To determine if the inhibitory activity of S20NS was potentially mediated through properdin, we formed C3 convertases from purified complement components in the presence of properdin and then incubated

S20NS or control proteins with the convertases. When S20NS was incubated with C3 convertases containing properdin, approximately 90% of Bb was displaced (Fig 3.3), in contrast to its effect on convertases lacking properdin (Fig 3.2A). Factor H displaced Bb from C3 convertases formed in either the presence or absence of properdin (Fig 3.2A and Fig 3.3).

After establishing that S20NS was only active against convertases containing properdin, experiments were done to determine if S20NS displaced properdin from the C3 convertase. S20NS displaced properdin from C3 convertases formed from purified components (Fig 3.4A) as well as from convertases formed from NHS (Fig 3.4B). In addition, S20NS also displaced properdin from complexes containing only C3bP, demonstrating the specificity of S20NS for properdin (Fig 3.4C). Unlike S20NS, fH did not displace properdin from C3 convertases (Fig 3.4A) or from C3bP complexes (Fig 3.4C). Together, these results demonstrate that S20NS uniquely accelerates the decay of C3 convertases by specifically displacing properdin from the convertase.

S20NS binds properdin

To determine if S20NS directly interacted with properdin to dissociate the C3 convertase, S20NS and properdin were incubated together and S20NS was next immunoprecipitated using an antibody that bound to its C-terminal V5-epitope tag. The precipitates were then immunoblotted for either S20NS or properdin with specific Abs. In the immunoblots, we detected S20NS as well as properdin in the precipitates (Figure 3.5A), indicating that S20NS directly bound to properdin.

The interaction between Salp20 and properdin was also confirmed by ELISA. Microtiter plate wells were coated with S20NS and then incubated with properdin. After

incubation, bound properdin was detected with specific Abs. In wells coated with either S20NS or C3b, we detected specific binding of properdin when compared to the negative control, CAT (Fig 3.5B).

In addition to studying the direct interaction between S20NS and properdin, we also calculated the relative binding affinity of properdin for either S20NS or C3b by performing solid-phase saturation binding assays. In these assays, microtiter plates were coated with equal amounts of either S20NS or C3b. Increasing concentrations of properdin were then added to the wells, and bound properdin was detected with specific antibodies. Properdin binding to S20NS saturated at a lower concentration than properdin binding to C3b (Fig 3.5C). The relative K_d of properdin binding to S20NS = 0.669 nM where the relative K_d of properdin binding to C3b > 85 nM. These results indicate properdin binds to S20NS with an affinity that is >100 fold higher than its affinity for C3b.

3.5 Discussion

In this study, we have demonstrated that S20NS is only active against C3 convertases containing properdin. The simplest mechanism consistent with our data is that S20NS directly interacts with properdin, causing its dissociation from the C3 convertase and the subsequent decay acceleration of the convertase. This model is supported by the observations that 1) properdin directly bound to Salp20 with a relative affinity that was at least 100 fold higher than the affinity of properdin for C3b and 2) Salp20 treatment reduced the levels of properdin on preformed C3 convertases and C3bP complexes. We cannot completely rule out alternative models such as properdin facilitating necessary contacts between Salp20 and C3bBb, allowing S20NS to bind the convertase directly and cause decay

acceleration. However, as we have not found any S20NS physically associated with the inactivated convertase (data not shown), we favor the model in which Salp20 acts by directly displacing properdin from the convertase.

All of our studies were performed with insect cell expressed recombinant S20NS, which we believe functions almost identically to native Salp20 expressed in tick saliva. Valenzuela et al. have demonstrated that native Isac, purified directly from tick salivary gland extracts, inhibited the alternative complement pathway, likely by dissociating the C3 convertase (9). This result supports the idea that the activity S20NS closely mimics the activity of native Salp20.

Our proposed model for S20NS mediated displacement of properdin from the C3 convertase is consistent with the previous studies (5). Lawrie et al. determined that *I. ricinus* salivary gland extracts (SGE) inhibited the activity of C3 convertases formed from NHS on erythrocyte surfaces, but had no effect on C3 convertases formed from purified complement components. In addition, when cobra venom factor (CVF) was used as an activator of complement in the presence of NHS, *I. ricinus* SGE displayed no inhibitory activity against the CVFBb convertase. Since properdin was absent in the pure component and CVF assays, these negative results could be explained by our model where the active inhibitory component in tick SGE, in particular, S20NS or related ILP family members, acts through properdin.

The decay accelerating activity of S20NS is unique and distinct from any of the characterized alternative pathway decay accelerating factors, DAF, CR1, and fH, which directly interact with C3bBb or C3b to destabilize the C3 convertase (15, 20-25). S20NS displaced properdin from C3 convertases and C3bP complexes, whereas fH did not displace

properdin in our assays. In a previous study, Hourcade used surface plasmon resonance to demonstrate that fH binding to C3 convertases results in the decay of C3 convertases and the dissociation of properdin (18). We may not have observed properdin dissociation following fH treatment because C3 complexes formed in our ELISAs differ from the convertases formed in the surface plasmon resonance study. Specifically, the C3 convertase complexes formed in our assays are likely to contain both complete C3 convertases and C3bP complexes. The properdin displaced by S20NS in our assays might be mainly derived from C3bP complexes, which are not affected by fH.

Even though properdin is not an active component of the C3 convertase, it is essential for the stabilization and full activity of the convertase (19, 26). Gupta-Bansal et al. and Perdikoulis et al. have demonstrated that Abs directed against properdin are capable of inhibiting the alternative pathway (26, 27). Recent studies have also shown that properdin is capable of binding to cell surfaces and initiating the alternative pathway by providing a platform for the assembly of the C3 convertase (28). Since properdin is vital for effective complement activation, it is an attractive target for inactivation by pathogens or blood feeding organisms. One example of a virulence factor that targets properdin is streptococcal pyrogenic exotoxin B, which acts to degrade properdin, allowing the pathogenic group A streptococci to resist opsonophagocytosis mediated by complement (29).

Salp20 is a member of the ILP family, containing at least 49 members (7, 8, 10, 11). In addition to Salp20, several members of this family, specifically Isac, Irac-1, Irac-2, S20Lclone 12, and S20Lclone 2, inhibit the alternative pathway by decay acceleration of the C3 convertase (9-11) (data not shown). It is likely that these proteins also interact with properdin.

Properdin is composed of short N- and C-terminal regions separated by 6 TSRs (30), which make up the majority of the protein. We propose that Salp20 and other ILP family members specifically bind the TSRs of properdin to cause its displacement from the C3 convertase. The TSRs found in properdin and several other proteins primarily bind sulfated glycoconjugates and glycosaminoglycans (GAGs) (31, 32). Interestingly, S20NS contains multiple N- and O-linked glycans that make up almost half the molecular weight of the mature protein (10). These carbohydrate modifications may potentially be sulfated glycoconjugates and GAGs, allowing S20NS to resemble the sulfated glycoconjugates and bind the TSRs of properdin.

In addition to properdin, TSRs are found in other complement proteins, cell adhesion molecules, and proteases, many of which regulate host hemostasis and innate immunity (33). In addition to their roles in complement inhibition, we speculate different ILP family members may target different TSR containing proteins to alter host hemostasis and innate immunity, facilitating tick feeding. ILP family members may prove to be useful for developing anti-tick vaccines as well as novel therapies for complement mediated diseases.

3.6 Acknowledgements

We thank Anne Broadwater and Holly Patterson (UNC) for technical assistance and Nate Rigel and Miriam Braunstein (UNC) for providing the anti-His antibody. We also thank Bill Messer (UNC) for assistance with statistical analyses. We thank Michael Frank (Duke University School of Medicine), Kari Hacker (UNC), and Vivian Chen (UNC) for helpful discussions.

3.7 Contributions

In this work, I designed and performed all the experiments. Chris Elkins and Aravinda de Silva assisted in the design of experiments. This work is published in the Journal of Immunology, 2008, volume 180(6), pages 3964-3968,

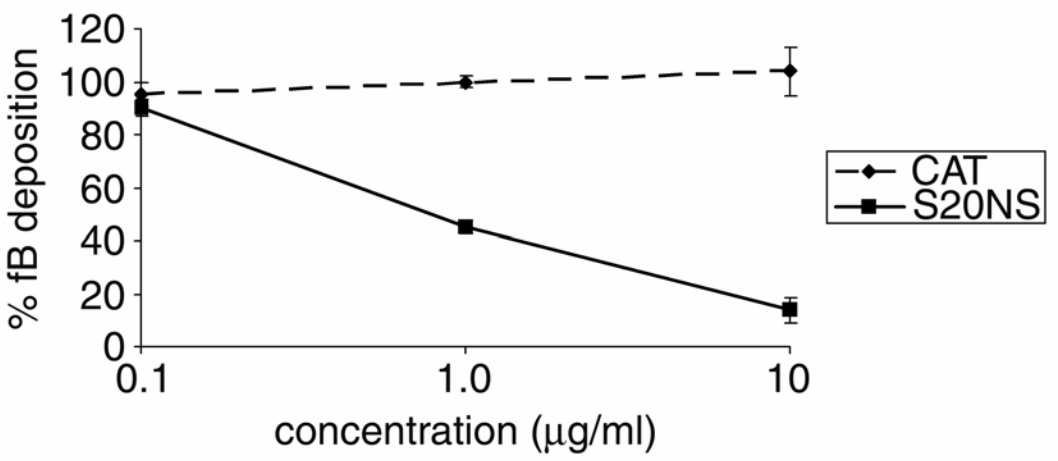


Figure 3.1 S20NS inhibits the alternative complement pathway by dissociating the C3 convertase. C3 convertases were preformed on agarose surfaces from complement components in NHS. Ten-fold dilutions of S20NS or CAT were then added to the preformed convertases and the amount of remaining Bb was determined by ELISA. The error bars represent 2 standard deviations from the mean where N=6.

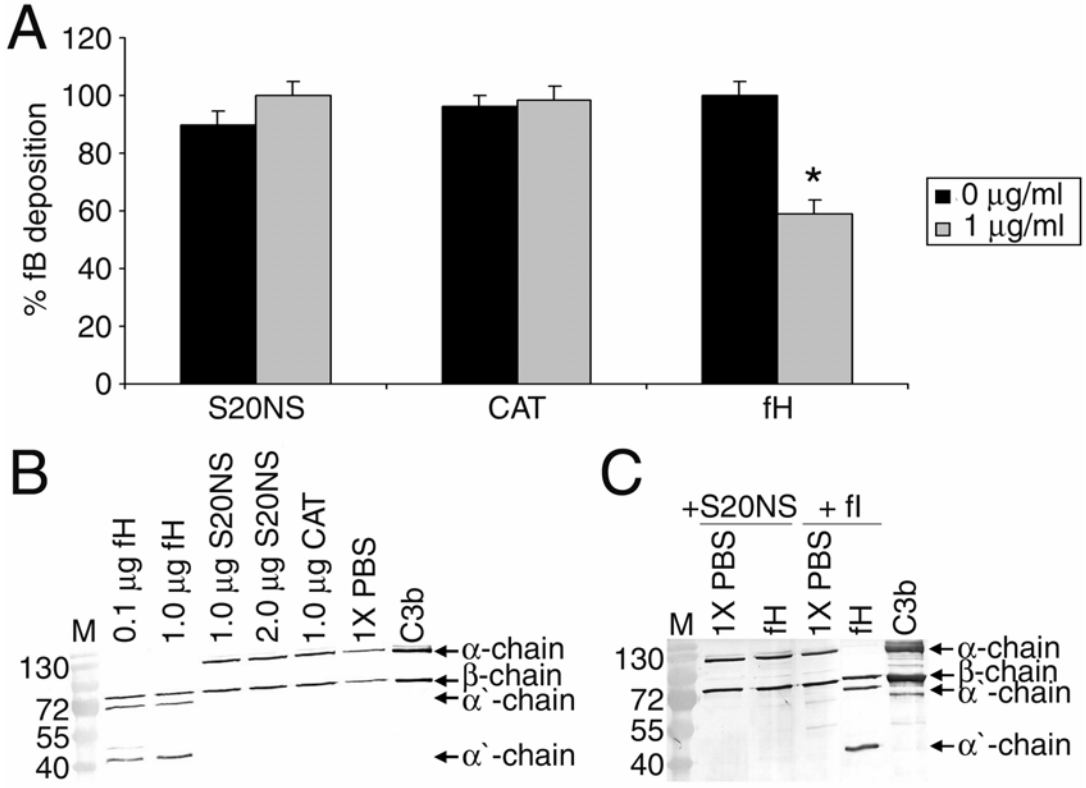


Figure 3.2 S20NS does not dissociate the C3 convertase by a mechanism similar to fH or fI. ***A***, C3 convertases were preformed in microtiter plate wells from purified complement components (C3b, fB, and fD) and washed. S20NS (1 µg/ml), fH (1 µg/ml), CAT (1 µg/ml, negative control), or buffer alone (0 µg/ml) were then added to the preformed convertases and the amount of remaining bound Bb was determined by ELISA. The error bars represent 2 standard deviations from the mean where N=6. The asterisk indicates statistical significance (p=0.008) between the 0 µg/ml and 1 µg/ml samples of fH as measured by a student t-test. ***B,*** Various concentrations of S20NS or fH were incubated with C3b in the presence of fI, and C3b degradation products, represented by the 67 and 43 kDa bands, were visualized by Western blots using a polyclonal goat α-hC3 Ab. ***C,*** S20NS or fI were incubated with C3b in the presence of fH. C3b degradation products were visualized by Western blots as described in Fig 2B. M, marker.

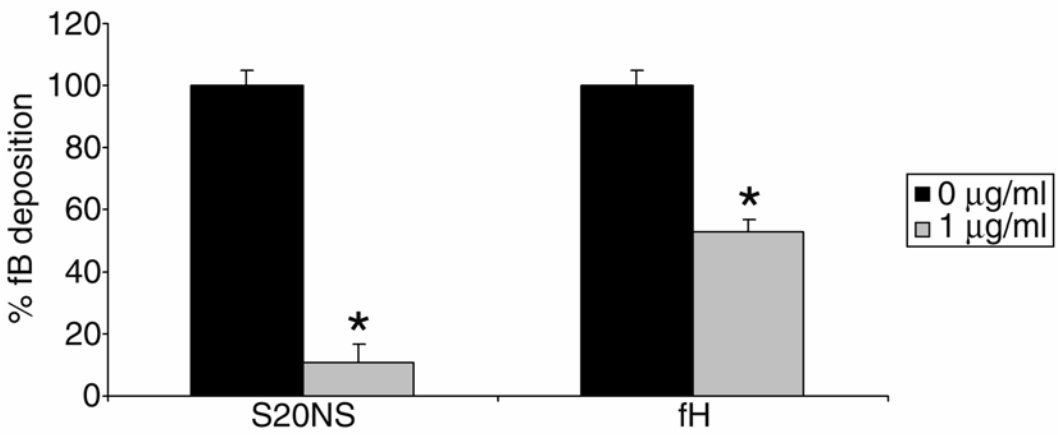


Figure 3.3 S20NS dissociates the C3 convertase only in the presence of properdin. C3 convertases were formed from purified components (C3b, fB, and fD) in the presence of properdin and then washed. S20NS (1 µg/ml), fH (1 µg/ml), or buffer (0 µg/ml) were added to the preformed convertases and the amount of remaining bound Bb was determined by ELISA. The error bars represent 2 standard deviations from the mean where N=6. The asterisks indicate statistical significance between the 0 µg/ml and 1 µg/ml samples as measured by a student's t-test where $p<0.001$.

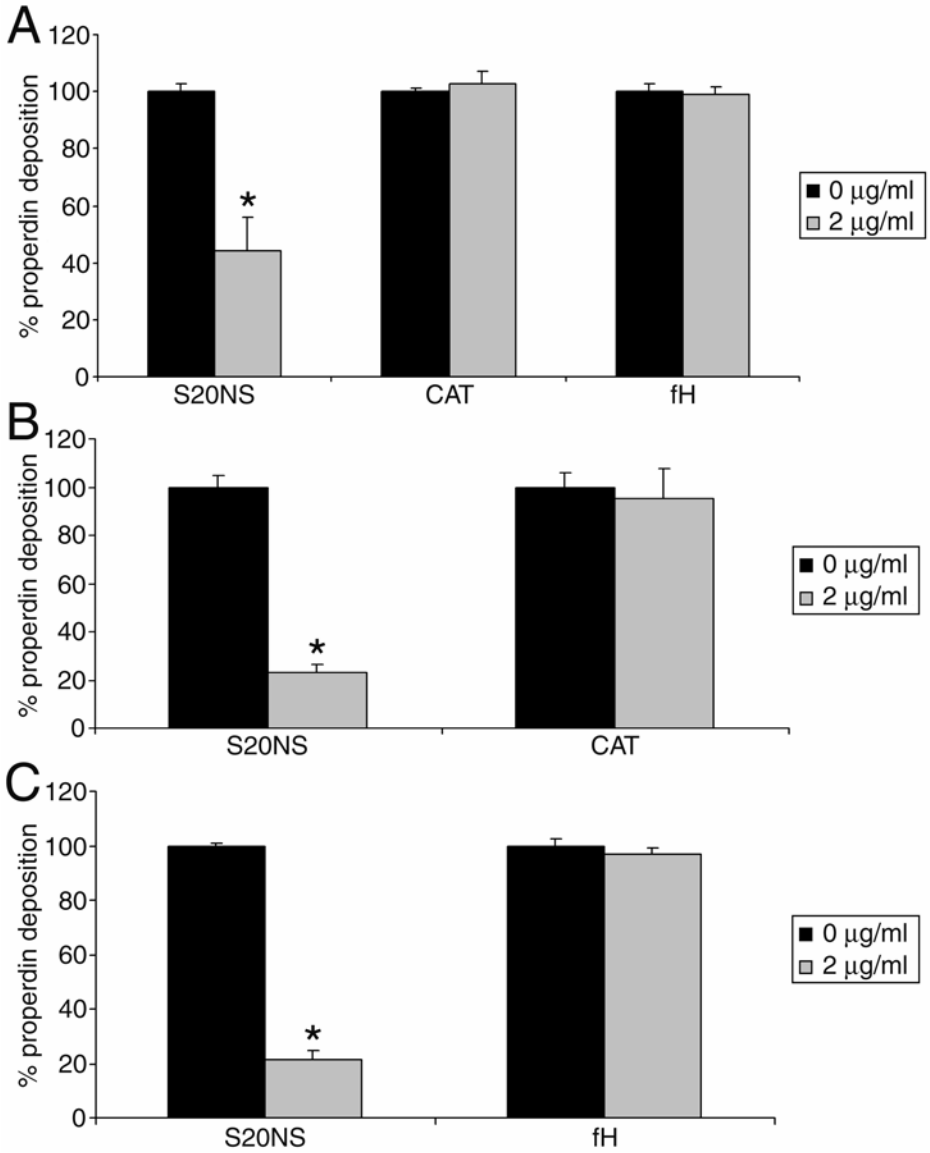


Figure 3.4 S20NS dissociates properdin from the C3 convertase. ***A***, C3 convertases were formed from purified complement components as described in Fig 3. S20NS (2 µg/ml), fH (2 µg/ml) or buffer (0 µg/ml) were then incubated with the preformed convertases, and bound properdin was detected by ELISA. ***B,*** C3 convertases were formed from complement components in NHS as described in Fig 1. S20NS (2 µg/ml), CAT (2 µg/ml, negative control), or buffer (0 µg/ml) were then incubated with the preformed convertases and bound properdin was detected by ELISA. ***C***, C3bP complexes were formed from purified C3b and properdin. S20NS (2 µg/ml), fH (2 µg/ml) or buffer (0 µg/ml) were then incubated with the complexes, and bound properdin was detected by ELISA. The error bars represent 2 standard deviations from the mean where N=6. The asterisks indicate statistical significance between the 0 µg/ml and 2 µg/ml samples of S20NS as measured by a student's t-test where $p<0.001$.

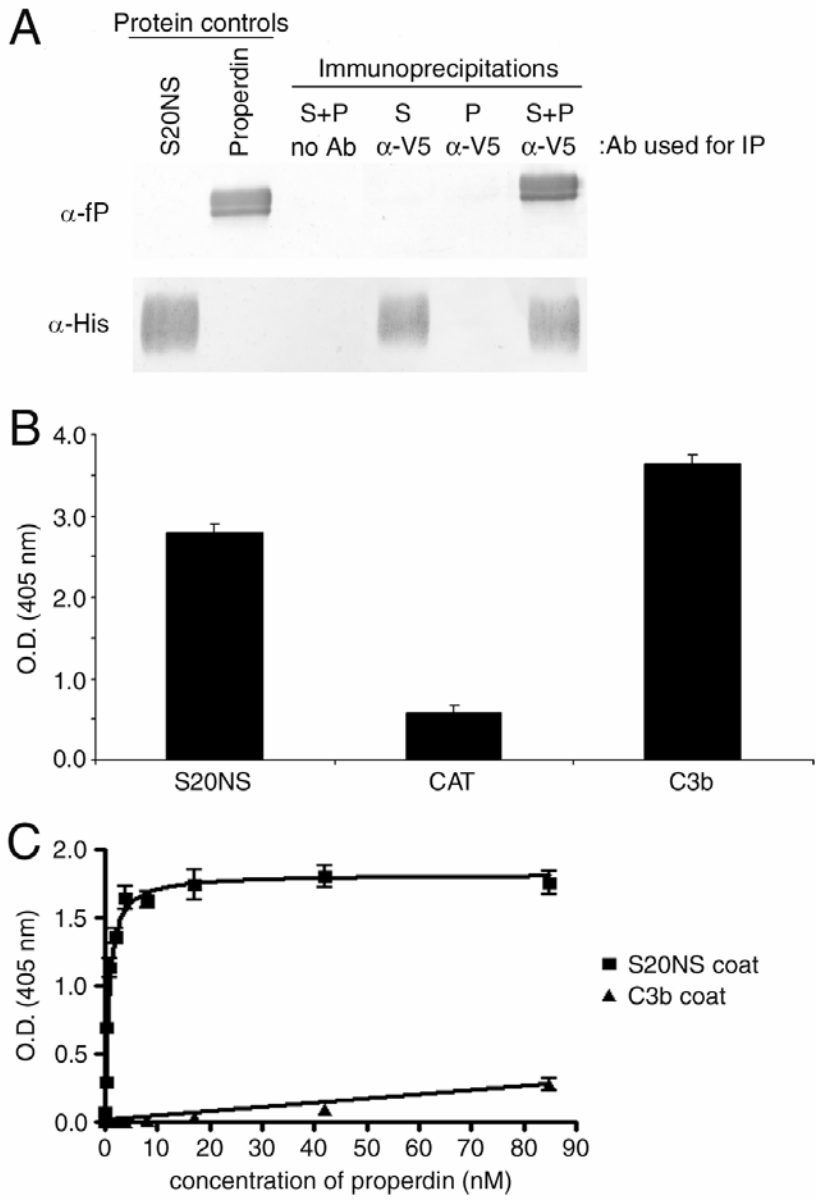


Figure 3.5 S20NS binds properdin. ***A,*** S20NS (S), properdin (P), or S20NS previously incubated with properdin (S+P) were immunoprecipitated (IP) with a monoclonal α-V5 Ab against an epitope tag on S20NS. Immunoprecipitates were then analyzed by Western Blots using specific Abs directed against properdin (α-fP) or S20NS (α-His). ***B,*** Microtiter plate wells were coated with S20NS, CAT (negative control), or C3b (positive control). The wells were washed, blocked, and then incubated with properdin. Bound properdin was detected by ELISA. The error bars represent 2 standard deviations from the mean where N=6. ***C,*** Microtiter plate wells were coated with either S20NS or C3b. Increasing concentrations of properdin were then added to the wells, and bound properdin was detected by ELISA. The data depict a single experiment performed in triplicate that is representative of 3 independent experiments. The error bars represent the standard error from the mean.

3.8 References

1. Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis. 1982. Lyme disease-a tick-borne spirochetosis? *Science*. 216: 1317-19.

2. Sonenshine, D. E., 1993. *Biology of ticks.* Oxford University Press, New York, NY.

3. Wikel, S. K. 1999. Tick modulation of host immunity: an important factor in pathogen transmission. *Int J Parasitol*. 29: 851-9.

4. Lawrie, C. H., S. E. Randolph, and P. A. Nuttall. 1999. *Ixodes* ticks: serum species sensitivity of anticomplement activity. *Exp Parasitol*. 93: 207-14.

5. Lawrie, C. H., R. B. Sim, and P. A. Nuttall. 2005. Investigation of the mechanisms of anti-complement activity in *Ixodes ricinus* ticks. *Mol Immunol*. 42: 31-8.

6. Ribeiro, J. M. 1987. *Ixodes dammini*: salivary anti-complement activity. *Exp Parasitol*. 64: 347-53.

7. Soares, C. A., C. M. Lima, M. C. Dolan, J. Piesman, C. B. Beard, and N. S. Zeidner. 2005. Capillary feeding of specific dsRNA induces silencing of the *isac* gene in nymphal *Ixodes scapularis* ticks. *Insect Mol Biol*. 14: 443-52.

8. Ribeiro, J. M., F. Alarcon-Chaidez, I. M. Francischetti, B. J. Mans, T. N. Mather, J. G. Valenzuela, and S. K. Wikel. 2006. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol*. 36: 111-29.

9. Valenzuela, J. G., R. Charlab, T. N. Mather, and J. M. Ribeiro. 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis. J Biol Chem*. 275: 18717-23.

10. Tyson, K., C. Elkins, H. Patterson, E. Fikrig, and A. de Silva. 2007. Biochemical and functional characterization of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Mol Biol*. 16: 469-79.

11. Daix, V., H. Schroeder, N. Praet, J. P. Georgin, I. Chiappino, L. Gillet, K. de Fays, Y. Decrem, G. Leboulle, E. Godfroid, A. Bollen, P. P. Pastoret, L. Gern, P. M. Sharp, and A. Vanderplasschen. 2007. *Ixodes* ticks belonging to the *Ixodes ricinus* complex encode a family of anticomplement proteins. *Insect Mol Biol*. 16: 155-66.

12. Hourcade, D. E., L. M. Mitchell, and M. E. Medof. 1999. Decay acceleration of the complement alternative pathway C3 convertase. *Immunopharmacology*. 42: 167-73.

13. McRae, J. L., T. G. Duthy, K. M. Griggs, R. J. Ormsby, P. J. Cowan, B. A. Cromer, W. J. McKinstry, M. W. Parker, B. F. Murphy, and D. L. Gordon. 2005. Human factor H-related protein 5 has cofactor activity, inhibits C3 convertase activity, binds heparin and C-reactive protein, and associates with lipoprotein. *J Immunol*. 174: 6250-6.

14. Zipfel, P. F., T. S. Jokiranta, J. Hellwage, V. Koistinen, and S. Meri. 1999. The factor H protein family. *Immunopharmacology*. 42: 53-60.

15. Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein β1H. *Proc Natl Acad Sci U S A*. 73: 3268-72.

16. Pangburn, M. K., R. D. Schreiber, and H. J. Muller-Eberhard. 1977. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein β1H for cleavage of C3b and C4b in solution. *J Exp Med*. 146: 257-70.

17. Ross, G. D., J. D. Lambris, J. A. Cain, and S. L. Newman. 1982. Generation of three different fragments of bound C3 with purified factor I or serum. I. Requirements for factor H vs CR1 cofactor activity. *J Immunol*. 129: 2051-60.

18. Hourcade, D. E. 2006. The role of properdin in the assembly of the alternative pathway C3 convertases of complement. *J Biol Chem*. 281: 2128-32.

19. Fearon, D. T. and K. F. Austen. 1975. Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *J Exp Med*. 142: 856-63.

20. Morgan, B. P., Harris, C. L., 1999. *Complement regulatory proteins*. Academic Press, San Diego, CA.

21. Pangburn, M. K. and H. J. Muller-Eberhard. 1986. The C3 convertase of the alternative pathway of human complement. Enzymic properties of the bimolecular proteinase. *Biochem J*. 235: 723-30.

22. Fujita, T., T. Inoue, K. Ogawa, K. Iida, and N. Tamura. 1987. The mechanism of action of decay-accelerating factor (DAF). DAF inhibits the assembly of C3 convertases by dissociating C2a and Bb. *J Exp Med*. 166: 1221-28.

23. Nicholson-Weller, A., J. Burge, D. T. Fearon, P. F. Weller, and K. F. Austen. 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J Immunol*. 129: 184-9.

24. Fearon, D. T. 1979. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc Natl Acad Sci U S A*. 76: 5867-71.

25. Whaley, K. and S. Ruddy. 1976. Modulation of the alternative complement pathways by β1H globulin. *J Exp Med*. 144: 1147-63.

26. Gupta-Bansal, R., J. B. Parent, and K. R. Brunden. 2000. Inhibition of complement alternative pathway function with anti-properdin monoclonal antibodies. *Mol Immunol*. 37: 191-201.

27. Perdikoulis, M. V., U. Kishore, and K. B. Reid. 2001. Expression and characterisation of the thrombospondin type I repeats of human properdin. *Biochim Biophys Acta*. 1548: 265-77.

28. Spitzer, D., L. M. Mitchell, J. P. Atkinson, and D. E. Hourcade. 2007. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J Immunol*. 179: 2600-8.

29. Tsao, N., W. H. Tsai, Y. S. Lin, W. J. Chuang, C. H. Wang, and C. F. Kuo. 2006. Streptococcal pyrogenic exotoxin B cleaves properdin and inhibits complement-mediated opsonophagocytosis. *Biochem Biophys Res Commun*. 339: 779-84.

30. Goundis, D. and K. B. Reid. 1988. Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. *Nature*. 335: 82-5.

31. Holt, G. D., M. K. Pangburn, and V. Ginsburg. 1990. Properdin binds to sulfatide [Gal(3-SO_4)β1-1 Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J Biol Chem*. 265: 2852-5.

32. Guo, N. H., H. C. Krutzsch, E. Negre, T. Vogel, D. A. Blake, and D. D. Roberts. 1992. Heparin- and sulfatide-binding peptides from the type I repeats of human thrombospondin promote melanoma cell adhesion. *Proc Natl Acad Sci U S A*. 89: 3040-4.

33. Tucker, R. P. 2004. The thrombospondin type 1 repeat superfamily. *Int J Biochem Cell Biol*. 36: 969-74.

CHAPTER 4

Expression and functional characterization of additional *I. scapularis* ILP family members

Katharine Tyson, Holly Patterson, and Aravinda de Silva

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, NC 27599

4.1 Abstract

I. scapularis ticks secrete numerous anti-hemostatic, anti-inflammatory, and immunosuppressive proteins during feeding. Isac and Salp20, two *I. scapularis* salivary anti-complement proteins, are members of a large family of paralogous tick proteins designated the ILP family. Salp20 specifically binds properdin, resulting in C3 convertase destabilization. In this study, we examined the functions of two ILP family members, S20Lclone 12 and S20Lclone 2, which share 95% and 67% amino acid identity with Salp20, respectively. Recombinant S20Lclone 12 (S20L12) and S20Lclone 2 (S20L2) inhibited the alternative complement pathway by specifically binding properdin with similar affinities, identical to Salp20. When mixed together, recombinant Salp20

(S20NS), S20L12 and S20L2 displayed no synergistic inhibitory effects beyond the activity of the individual proteins. Furthermore, S20NS, S20L12, S20L2 similarly inhibited the alternative complement pathway of various host animals, indicating different ILP family members do not display host specificity. Finally, we determined that S20NS, S20L12, and S20L2 specifically interacted with properdin and no other TSR-containing proteins.

4.2 Introduction

I. scapularis, commonly known as the blacklegged tick, is an exclusively blood sucking ectoparasite that feeds on a host for several days to weeks (28). In order to avoid host recognition and rejection, *I. scapularis* ticks secrete numerous pharmacologically active components in their saliva during feeding, which block host hemostasis, inflammation, and immune recognition (3, 4, 16, 24, 33, 38). Recently, multiple groups have determined that many of the anti-hemostatic, anti-inflammatory, and immunosuppressive components in ixodid tick saliva, including *I. scapularis* saliva, are members of large families of related proteins (7, 10, 12, 17, 19, 23, 27, 35). Daix et al. demonstrated that *I. ricinus* anti-complement proteins, Irac-1 and Irac-2, are both expressed within the salivary glands of an individual tick, indicating that multiple members of different tick salivary protein families are likely co-expressed within individual feeding ticks (7).

Salp20 is an anti-complement protein secreted in *I. scapularis* saliva that inhibits the alternative complement pathway by directly binding properdin, which causes decay acceleration of the C3 convertase (32) (Chapter 3). Salp20 and its homologue, Isac, are both members of the ILP family of anti-complement salivary proteins. Currently, the ILP family is comprised of approximately 49 unique members, sharing between 64% and 95% amino

acid identity to either Isac or Salp20 (23, 27, 32). At this time, only Salp20 and Isac have been demonstrated to be complement inhibitors and the functions of other ILP family members are unknown.

In addition to the anti-complement ILP family members identified in *I. scapularis*, Daix et al. and Couvreur et al. have also identified multiple *I. ricinus* proteins that share homology with Isac and Salp20 (6, 7). Recombinant Irac-1 and Irac-2, two *I. ricinus* Isac homologues, were found to inhibit the alternative complement pathway by preventing C3 convertase assembly and by dissociating Bb from C3b in the C3 convertase, similar to Isac and Salp20 (7). This evidence further supports the idea that related ILP family members likely share functional similarities.

Two *I. scapularis* ILP family members that were identified in the studies described in Chapter 1, S20Lclone 12 and S20Lclone 2, share approximately 95% and 67% amino acid identity with Salp20, respectively. In this chapter, we express recombinant S20Lclone 12 and S20Lclone 2 and examine their functions in relation to S20NS. We also attempt to characterize any differences that may exist between S20Lclone 12, S20Lclone 2, and S20NS in an effort to understand why ticks produce so many different proteins that are closely related.

4.3 Materials and Methods

Cell lines and media

Adherent cultures of High Five cells (Invitrogen, Carlsbad, CA), derived from the cabbage looper, *Trichoplusia ni*, were seeded and maintained according to the instructions of the manufacturer. The cells were grown in Express Five Serum free media (SFM) (Gibco,

Carlsbad, CA) supplemented with L-glutamine (18 mM) (Gibco, Carlsbad, CA), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) (Gibco, Carlsbad, CA) at 28^{o}C.

Expression and purification of S20Lclone 12 and S20Lclone 2 in High Five cells

To express recombinant S20Lclone 12 and S20Lclone 2 in High Five cells, we used an approach similar to Alarcon-Chaidez et al. (2). S20Lclone 12 was first PCR amplified from the pCR 2.1 TOPO vector using the following primers: KS20F – 5` CCAGCCATGAGGACTGCGCT 3` and S20RNS – 5` GGAAATTGCCTCGAATGAGTCTC 3`, while S20Lclone 2 was amplified using the following primers: SLC13F – 5` GCTCAGTTATGAGGACTGTGCTG 3` and SLC13RNS – 5` GGGGACGGCCACAGGCTCAA 3`. The 5` primers contained Kozak sequences to allow for efficient protein expression from the cloning vector, while the 3` primers lacked stop codons to allow the fusion of a V5-epitope and 6X-His tag to the C-terminus of the proteins. Once amplified, the PCR products were cloned into the expression vector pIB/V5-His-TOPO (Invitrogen), which contains both ampicillin and blasticidin resistance genes allowing for selection in *E. coli* and High Five cells, generating the constructs pIB-S20Lclone 12 and pIB-S20Lclone 2. Constitutive expression of S20Lclone12-V5-His (S20L12) and S20Lclone 2-V5-His (S20L2) was controlled by the *Orgyia pseudotsugata* baculovirus promoter, *OpIE2*. The resulting constructs, pIB-S20L12 and pIB-S20L2, were purified using the QIAfilter Plasmid Midi Kit (Qiagen, Valencia, CA) and transfected into High Five cells following the manufacturer's protocol. As a positive control for protein expression and purification, High Five cells were also transfected with pIB-CAT (Invitrogen, Carlsbad, CA), the pIB/V5-His vector containing chloramphenicol acetyltransferase (CAT).

Stably transfected cells were selected and maintained by the addition of blasticidin (10 μg/ml) to the cell culture media.

For protein purification, adherent cultures of stably transfected cells were grown to 90% confluency in T-175 flasks. Cells were then collected and seeded into 500 ml of culture media in 3L Fernbach flasks (Corning, Corning, NY) at a density of 5×10^5 cells/ml. Cultures were grown at 28°C with gentle agitation until reaching a density of $2\text{-}3 \times 10^6$ cells/ml in 1L. The media was then collected and centrifuged to remove all cells and debris. After centrifugation, the media was concentrated and buffer exchanged against Ni-NTA Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) using Hydrosart filters (Sartorius, Edgewood, NY) in the Vivaflow 200 (Sartorius, Edgewood, NY). After the concentration and buffer exchange, the media was loaded onto a 500 μL column of Ni^{2+}-Nitriloacetic acid (Ni-NTA, Qiagen, Valencia, CA) agarose. After all media passed through the column, the column was washed two times with 4 column volumes of Ni-NTA Wash Buffer and eluted five times with ½ the column volume of Ni-NTA Elution Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0). All elutions were pooled and dialyzed 24 hrs at 4°C against 1X PBS with two buffer changes. After dialysis, the elution sample was concentrated using Amicon Ultra-15 YM-10 centrifugal filter devices (10 kDa MWCO, Millipore, Billerica, MA). Protein concentrations and purity were determined by Bradford analysis and SDS-PAGE, respectively. The final concentration of protein was 50-100 μg/ml. Purified protein was stored at -20°C until needed.

SDS-PAGE and immunoblot analysis

For SDS-PAGE, all samples were electrophoresed under reducing conditions by either 10% SDS-PAGE or 4-12% SDS-PAGE. Gels were then stained using SimplyBlue

SafeStain (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. For immunoblots, following separation by SDS-PAGE under reducing conditions, samples were transferred to nitrocellulose membranes by semi-dry transfer at 380 mA for 60 min. Membranes were then blocked with 1X Tris-buffered saline (TBS), 2% milk for 12-16 hrs. at 4^{o}C. Mouse anti-V5 monoclonal antibody (Invitrogen, Carlsbad, CA) was diluted 1:5000 in 1X TBS, 2% milk and incubated with membranes for 1 hr at room temperature. Membranes were next incubated with alkaline phosphatase (AP) conjugated goat anti-mouse antibody (Sigma, St. Louis, MO) diluted 1:1000 in 1X TBS, 2% milk for 45 min at room temperature. For C3a blots, rabbit anti-human C3a antibody (CompTech, Tyler, TX) was diluted 1:1000, and secondary AP-conjugated goat anti-rabbit IgG (Sigma) was diluted 1:1000 in 1X TBS, 2% milk. Bound antibody was detected directly on the membranes using the phosphatase substrate, 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) (KPL).

Deglycosylations with PNGase F and TFMS

To remove N-linked glycans from S20L12 and S20L2, approximately 0.5 μg of purified protein from insect cells was digested with 30 U/μL of PNGase F (New England Biolabs, Ipswich, MA) following the instructions provided by the manufacturer. Briefly, protein was denatured in 1X Glycoprotein Denaturing Buffer (0.5% SDS, 1% β-mercaptoethanol) (New England Biolabs, Ipswich, MA) for 10 min at 100^{o}C. The denatured protein was then incubated with PNGase F in 1X G7 Buffer (50 mM sodium phosphate, pH 7.5, New England Biolabs, Ipswich, MA) and 1% NP-40 for 24 hr at 37^{o}C. The deglycosylated protein was then analyzed by SDS-PAGE and immunoblots.

To remove N- and O-linked glycans from S20L12 and S20L2, 2 μg of protein were treated with the Glycoprofile IV Chemical Deglycosylation Kit containing TFMS according to the manufacturer's instructions (Sigma, St. Louis, MO).

Erythrocyte lysis assays

In order to test the alternative pathway, RbEs (CompTech, Tyler, TX) were washed three times with 5 volumes of AP Buffer (5 mM $MgCl_2$, 5 mM EGTA in gelatin veronal buffer (GVB), CompTech, Tyler, TX), and resuspended to a final concentration of 2.0 x 10^8 cells/ml in AP buffer. Prior to performing the alternative pathway assays, the dilution of NHS (CompTech, Tyler, TX), normal mouse serum (NMS) (courtesy of C. Elkins, UNC-Chapel Hill), or normal pig serum (NPS) (courtesy of T. Kawula, UNC-Chapel Hill) resulting in 80-90% lysis of the RbEs was determined and used for experimental assays. Experimental and control proteins were serially diluted in 1X PBS and added to NHS in AP buffer. The volume of protein sample used for each reaction remained constant, keeping concentrations of divalent cations and chelators invariable. The final volume of the mixture was 25 μL. This mixture was then incubated with 25μL of washed, resuspended RbEs (2 x 10^8 cells/ml) in disposable borosilicate glass culture tubes for 30 min at 37^{o}C. Control reactions contained RbEs (2 x 10^8 cells/ml) incubated with only NHS, NMS, or NPS in AP buffer or AP buffer alone. After incubation, 75 μL of N-saline was added to each tube. The tubes were then centrifuged at 1000 g for 5 min to pellet all remaining intact RbEs. Supernatants were collected and loaded into 96-well plates. The O.D. of the supernatants was measured at a λ of 405 nm. In order to test the classical pathway, assays were performed as described for the alternative pathway using antibody-sensitized sheep erythrocytes (SEs)

(CompTech, Tyler, TX) and GVB^{++} (GVB with Ca^{2+} and Mg^{2+}) (CompTech, Tyler, TX) instead of RbEs and AP Buffer.

Human C3 or fB deposition ELISAs

To determine if S20L12 or S20L2 inhibited the deposition of human C3 or fB onto surfaces, we performed deposition ELISAs using agarose coated plates following a modified protocol of Ribeiro and Valenzuela et al. (22, 34). In the presence of polysaccharides, C3 is activated and covalently attaches to the sugars (36). fB then noncovalently binds bound C3b. Since agarose is made of primarily galactose, C3 is activated in the presence of agarose and attaches covalently to agarose surfaces (22, 34). Briefly, 96-well plates were coated with 100 μL of 0.1% agarose in water. The agarose in the plates was dried for 48 hrs at 37°C. Once dry, 50 μL of AP buffer containing a 1:4 dilution of NHS and S20NS, S20L12, S20L2, or CAT at various concentrations were added to the wells and incubated at 37°C for 30 min. In some reactions, S20NS, S20L12, S20L2, or CAT were added 30 min after the addition of NHS and the wells were incubated an additional 20 min at 37°C. Control wells lacked NHS or contained 25 mM EDTA and NHS. After incubation with NHS, the plates were washed 3 times, 5 min each on an orbital shaker, with 200 μL/well of 1X TBS containing 10 mg/ml bovine BSA and 2 mM $MgCl_2$. After washing, 100 μL of a 1:1000 dilution of goat α-human C3 antibody (CompTech, Tyler, TX) or goat α-human fB antibody (CompTech, Tyler, TX) in 1X TBS, 10 mg/ml BSA, 2mM $MgCl^{2+}$ was added to each well and the plate was incubated 1 hr at 37°C. The plate was then washed 3 times as previously described, and 100 μL of either a 1:5000 or 1:1000 dilution of an AP-conjugated rabbit α-goat IgG (Sigma, St. Louis, MO) was added to each well. The plate was incubated 45 min at 37°C. Following the incubation, the plate was washed 2 times, 5 min each, with 200 μL/well of 1X TBS, 10

mg/ml BSA. The plate was next washed once with 1X TBS, 10 mg/ml BSA, 0.1% Tween followed by an additional wash with N-saline. After washing, 100 μL of Sigma Fast PNPP (Sigma, St. Louis, MO) was added to each well and the plate was incubated at room temperature for 15 min. After incubation, the O.D. was measured at a λ of 405 nm.

Assays to detect S20L12 and S20L2 binding to properdin, TSP-1, or ADAMTS-13

To detect direct binding of S20L12 and S20L2 to properdin, we performed immunoprecipitations and analyzed the precipitates by immunoblot. S20L12 or S20L2 (150 ng) were incubated with properdin (450 ng) at 37^{o}C for 30 min in Binding buffer (PBS, 75 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween) and then added to blocked Protein-A sephadex beads (Sigma) coated with 1 μg of mouse α-V5 IgG for 1 hour at 37^{o}C. The sephadex beads were washed and resuspended in non-reducing SDS-PAGE loading dye. Samples were subjected to SDS-PAGE and immunoblotting with antibodies specific for either S20NS or properdin.

As an alternative method to detect S20NS, S20L12, or S20L2 binding to properdin, microtiter plate wells were first coated with 100 ng/well of S20NS, S20L12, S20L2, CAT, or C3b for 12 h at 4^{o}C. The wells were then blocked and incubated with 100 ng/well properdin for 1 h at 37^{o}C. After incubation, the wells were washed. To detect plate bound properdin, the wells were incubated with a primary goat α-properdin Ab and a secondary AP-conjugated rabbit α-goat IgG.

To detect S20NS, S20L12, or S20L2 binding to thrombospondin-1 (TSP-1), a disintegrin and metalloproteinase with a thrombospondin type I motif member 13 (ADAMTS-13), or properdin, microtiter plates were first coated with 100 ng/well of TSP-1, ADAMTS-13, or properdin in 0.1 M Carbonate Buffer for 12 h at 4^{o}C. Wells were then

blocked with Binding buffer for 15 min at 23°C. After blocking, 1 μg/ml of S20NS, S20L12, or S20L2 in Binding buffer were incubated in the wells for 1 hr at 37°C. After the incubation, the wells were washed with TBST and bound protein was detected by standard ELISAs using a primary monoclonal mouse α-V5 IgG and a secondary AP-conjugated goat α-mouse IgG antibody.

Saturation Binding Assays

To determine the relative binding affinity of properdin for S20NS, S20L12, S20L2, or C3b we performed a solid-phase binding assay. Microtiter plates were coated with a saturating amount of S20NS, S20L12, S20L2, or C3b (10 ng/well) for 12 hrs at 4°C in 0.1M Carbonate Binding Buffer, pH 9.2. After coating, the wells were blocked with Binding buffer (PBS, 75 mM NaCl, 10 mM $MgCl_2$, 4% BSA, 0.05% Tween) for 1 hr at 37°C and then incubated with increasing concentrations of properdin in Binding buffer (PBS, 75 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween) at 37°C for 1 hr The wells were then washed with TBST, and bound properdin was detected by an ELISA using a primary goat α-human properdin Ab and a secondary AP-conjugated rabbit α-goat Ab. Development of the substrate was stopped after 3 min by the addition of 3M NaOH. The OD_{405} was determined and plotted, and relative K_d and B_{max} values were calculated using GraphPad Prism 4 (GraphPad Software).

4.4 Results

Expression and characterization of S20L12 and S20L2

To examine the functional activities of additional ILP family members, we expressed two ILP members, S20Lclone 12 and S20Lclone 2, in an insect cell expression system. S20Lclone 12 shares 95% amino acid identity with Salp20 and possesses a putative secretion

signal, 7 N-linked glycosylation sites, several potential C-terminal O-linked glycosylation sites, and 4 conserved cysteines in the mature protein (Fig 4.1A). S20Lclone 2 shares only 65% amino acid identity with Salp20 but also possesses a putative secretion signal and 4 conserved cysteines in the mature protein. However, unlike Salp20 and S20Lclone 12, S20Lclone 2 only contains 5 potential N-linked glycosylation sites and displays multiple amino acid deletions at positions 116, 129-134, and 167-168, possibly resulting in fewer C-terminal O-linked glycosylation sites (Fig 4.1A).

Recombinant S20L12 (S20Lclone 12 containing C-terminal V5 epitope and 6X-histidine tag) and S20L2 (S20Lclone 2 containing C-terminal V5 epitope and 6X-histidine tag) were produced in insect cells similarly to S20NS as described in Chapter 2. S20L12 and S20L2 were both efficiently expressed and secreted from stably transfected insect cells, as both proteins were found primarily in the culture media by Western blot (Fig 4.2B). Interestingly, similar to S20NS, S20L12 and S20L2 appeared to be ~ 50 kDa and 48 kDa, respectively, much larger than their predicted sizes of 24 kDa and 23 kDa, respectively. In addition, S20L12 and S20L2 migrated as smears by Western blot, indicating the presence of differentially post-translationally modified forms of the proteins. Since S20NS appeared as a smear by Western blot and displayed a size discrepancy between predicted and observed sizes primarily because of the addition of N- and O-linked glycosylations (32), we expected S20L12 and S20L2 likely contained carbohydrate modifications.

To determine if S20L12 and S20L2 contained N- and O-linked glycosylations, we digested the proteins with PNGase F, a glycosidase that removes N-linked carbohydrates, or treated them with TFMS, an acid that removes N- and O-linked carbohydrates without destroying the peptide backbone. After incubations with PNGase F, S20L12 shifted from ~

50 kDa to ~ 45 kDa, while S20L2 shifted from ~ 48 kDa to ~ 40 kDa, indicating the presence of N-linked glycans on both proteins (Fig 4.1C). After TFMS treatment, S20L12 shifted from ~ 50 kDa to ~ 34 kDa, while S20L2 shifted from ~ 48 kDa to ~ 34 kDa, indicating the presence of N- and O-linked glycans on both proteins (Fig 4.1C). Together, these results suggest that S20L12 and S20L2 both contain N- and O-linked glycosylations, similar to S20NS.

Inhibition of the alternative complement pathway by S20L12 and S20L2

To determine if S20L12 and S20L2 inhibited the alternative complement pathway, we performed RbE lysis assays as described in Chapter 2. S20L12 and S20L2 (1 μg/ml) inhibited approximately 50% RbE lysis by NHS, similar to the activity of S20NS (Fig 4.2A). Comparable to S20NS, S20L12 and S20L2 were also incapable of completely blocking RbE lysis by NHS (Fig 4.2A) (32). If S20NS, S20L12, and S20L2 are secreted in tick saliva simultaneously during feeding, then they may display synergistic effects, completing blocking host complement components. To determine if ILP family members acted synergistically, S20L12, S20L2, and S20NS were mixed and incubated with NHS in the presence of RbEs. When mixed together, the ILPs displayed an IC_{50} of 0.687 μg/ml, similar to the IC_{50} of individual ILPs. These results suggest that ILP family members likely do not exhibit synergistic effects when secreted into a host during tick feeding.

Experiments were done to test if S20L12 and S20L2 inhibited the classical complement pathway. To test inhibition in the classical pathway, antibody sensitized erythrocytes (SEs) were incubated with NHS in the presence of S20L12 or S20L2 and Mg^{2+} and Ca^{2+}. After incubation, whole cells and cellular debris were pelleted by centrifugation. We then calculated the percent lysis of the SEs by measuring the optical density of the

supernatants. S20L12 and S20L2 did not inhibit the classical pathway, consistent with the activity of tick saliva, Isac, and S20NS (15, 22, 34, 37) (Fig 4.2B).

Dissociation of the C3 convertase by S20L12 and S20L2

Since S20NS inhibited the alternative complement pathway by preventing assembly and causing dissociation of the C3 convertase (32), we wanted to determine if S20L12 and S20L2 also inhibited the alternative pathway by a similar mechanism. To examine the effects of S20L12 and S20L2 on the C3 convertase, we formed C3 convertases using the agarose-based ELISA described in Chapter 2. In this assay, C3 is activated in the presence of agarose and Mg^{2+}, covalently attaching to the agarose. fB then binds to covalently bound C3b and is cleaved by fD, resulting in the formation of surface-bound C3 convertases (C3bBb). Bound complement components are then detected by standard ELISAs with specific antibodies. S20L12 and S20L2 prevented the deposition of C3b and fB when added simultaneously with NHS to agarose coated microtiter wells (Fig 4.3A & B). When S20L12 and S20L2 were added 30 min after NHS, they did not displace covalently bound C3b (Fig 4.3A & B). However, when added 30 min after NHS, S20L12 and S20L2 dissociated fB from C3b (Fig 4.3A & B). These activities are indistinguishable from the activities of both Isac and S20NS (32, 34), indicating that multiple ILP family members inhibit the alternative complement pathway by destabilizing the C3 convertase.

S20L12 and S20L2 bind properdin

S20NS inhibits the alternative complement pathway by specifically binding properdin, a positive regulator of the C3 convertase (Chapter 3). S20NS displaces properdin from the C3 convertase, promoting decay acceleration of the convertase. Since S20L12 and S20L2 dissociate the C3 convertase similar to S20NS, we wanted to further establish if

S20L12 and S20L2 directly interacted with properdin. In order to detect a direct interaction between S20L12 and S20L2 and properdin, we incubated S20L12 and S20L2 with properdin, immunoprecipitated S20L12 or S20L2 with an antibody directed against the C-terminal V5-epitope tag, and analyzed the precipitates for the presence of properdin by Western blots. Both S20L12 and S20L2 directly interacted with properdin, similar to S20NS, as properdin was detected in precipitates only in the presence of S20L12 or S20L2 (Fig 4.4A). To confirm this interaction, we also preformed binding ELISAs. Microtiter plate wells were coated with S20NS, S20L12, S20L2, CAT (negative control), and C3b (positive control) and then incubated with properdin. Bound properdin was detected by standard ELISAs with specific antibodies. Properdin directly bound S20L12 and S20L2, similar to S20NS and C3b (Fig 4.4B). These results confirm a direct interaction between properdin and multiple ILP family members, specifically S20NS, S20L12, and S20L2.

Relative binding affinities of properdin for different ILP family members

Based on our studies, S20NS, S20L12, and S20L2 inhibited the alternative complement pathway by directly binding properdin and accelerating decay of the C3 convertase. As these ILP family members are likely expressed simultaneously during tick feeding, we wanted to determine any differences between them, justifying the necessity for their co-expression. In order to identify differences between ILP family members, we first examined the possibility that different ILP family members exhibit different binding affinities for human properdin. As S20L2 contains several C-terminal amino acid deletions, it may display a lower binding affinity for properdin (Fig 4.1A). In order to determine relative binding affinities of ILP family members, we performed solid-phase saturation binding assays. Microtiter wells were coated with properdin and then incubated with

dilutions of S20NS, S20L12, and S20L2. After incubation, the wells were washed and bound ILP family members were detected with an antibody directed against the C-terminal V5-epitope tag. The binding affinities of properdin for S20NS, S20L12, or S20L2 were similar, as the relative calculated dissociation constants (K_d) were 1.129, 0.8566, and 1.027 nM, respectively (Fig 4.5). Furthermore, the maximum numbers of binding sites (B_{max}), as indicated by the OD_{405} value where saturation occurs, were also comparable between S20NS, S20L12, and S20L2 (Fig 4.5). These results indicate that multiple ILP family members bind properdin with similar affinities. Therefore, ILP family members may display other functional differences.

Inhibition of the alternative complement pathway in various hosts by ILP family members

I. scapularis feeds on a wide range of hosts including various small and large mammals, birds, and even reptiles (9, 29). To feed successfully without host recognition and rejection, the tick must suppress hemostatic, inflammatory, and immune responses, including complement activation. In order to block the alternative complement pathway in numerous hosts by binding properdin, which contains amino acid variations between different hosts (18), the tick may produce multiple ILP family members that could preferentially bind properdin from specific hosts. To test this possibility, we performed erythrocyte lysis assays using serum collected from three animal hosts (mice, pigs, and humans) and evaluated the complement inhibitory activity of S20NS, S20L12, and S20L2. In the presence of NHS and NPS, 1 μg/ml of S20NS, S20L12, and S20L2 displayed maximum inhibition of RbE lysis (Fig 4.6A & C). In the presence of NMS, 1 μg/ml of S20NS and S20L12 and 5 μg/ml S20L2 displayed maximum inhibition of RbE lysis (Fig 4.6B), suggesting S20NS and S20L12 may

preferentially inhibit complement components in NMS over S20L2. However, when tested repeatedly, these variations in inhibition of RbE lysis were not consistent. These results demonstrate that different ILP family members display no significant differences in their inhibitory activities against the alternative complement pathways from various hosts.

Binding of ILP family members to TSR containing proteins

The majority of properdin, ~ 80%, is composed of 6 TSRs, which are flanked by short N- and C-terminal sequences (18). TSRs are approximately 60 amino acids and are found in a variety of proteins where they facilitate protein-protein interactions (1, 31). In properdin, the TSRs are necessary for binding C3b or the C3 convertase (11, 21). TSP-1, a protein involved in platelet aggregation, inflammation, and angiogenesis during wound healing, and ADAMTS-13, a metalloproteinase that cleaves von Willebrand's factor, both contain multiple TSRs (5, 14, 25). As several ILP family members are likely expressed during tick feeding, they may serve additional roles besides complement inhibition. By binding the TSRs of properdin and other TSR-containing proteins, such as TSP-1 and ADAMTS-13, ILP family members may display multiple functions, potentially inhibiting the alternative complement pathway, platelet aggregation, and inflammation. In order to test this possibility, we performed binding ELISAs with ILP family members and various TSR-containing proteins. Microtiter wells were coated TSP-1, ADAMTS-13, or properdin and then incubated with S20NS, S20L12, S20L2, and CAT (negative control). Bound ILP family members were detected by ELISA with specific antibodies. S20NS, S20L12, and S20L2 specifically interacted with only properdin (Fig 4.7), indicating ILP family members likely only interact with properdin and not other TSR-containing proteins.

4.5 Discussion

In this study, we addressed the functions of additional ILP family members, S20Lclone 12 and S20Lclone 2. We successfully expressed and purified recombinant S20L12 and S20L2 from insect cells. Like S20NS, S20L12 and S20L2 possessed both N- and O-linked glycosylations, resulting in much larger sizes than predicted. Additionally, S20L12 and S20L2 inhibited the alternative complement pathway by directly interacting with properdin. Properdin bound S20NS, S20L12, and S20L2 with similar affinities, suggesting that multiple ILP family members share similar complement inhibitory activities.

A family of salivary anti-complement proteins has also been identified in *I. ricinus* ticks, which are closely related to *I. scapularis*. Daix et al. identified and expressed Irac-1 and Irac-2, which both inhibit the alternative complement pathway by destabilizing the C3 convertase. In addition, Daix et al. determined that Irac-1 and Irac-2 are co-expressed in a single tick during feeding by IFA of individual salivary gland pairs from adult engorged females with monoclonal antibodies directed against either Irac-1 or Irac-2 (7). Couvreur et al. identified additional *I. ricinus* anti-complement proteins related to Irac-1 and Irac-2 and determined that they were also co-expressed in unfed and fed larval, nymphal, and adult ticks (6). Based on these observations, it is likely that Salp20, Isac, S20L12, and S20L2 are co-expressed in *I. scapularis* ticks during feeding. We next wanted to determine the reason for ticks to co-express multiple ILP family members with identical functions during feeding.

Several possibilities exist to explain why the tick would need to co-express a large family of anti-complement proteins with the same functions. One possibility is that ILP family members potentially display synergistic inhibitory effects when co-expressed during feeding, completely blocking host complement activation. In this study, we examined this

possibility by performing erythrocyte lysis assays in the presence of a mixture of S20NS, S20L12, and S20L2. This mixture of ILP proteins displayed no synergistic inhibitory effects when compared to the inhibitory effects of individual ILP family members.

Another reason to co-express multiple ILP family members is that different family members may display host specificity, preferentially binding properdin from specific animal hosts. This is a plausible possibility as ixodid ticks, especially *I. scapularis,* feed on a wide variety of hosts including mammals, birds, and reptiles (9, 29). In this study, we tested this possibility by performing lysis assays in the presence of S20NS, S20L12, and S20L2 with NHS, NMS, or NPS. We detected no significant differences in the inhibitory activities of the ILP family members between the different animal serum samples. Since we only tested three different serum sources, it is possible that different ILPs display host specificity between other animal sources. However, Couvreur et al. and Schroeder et al. have demonstrated that *I. ricinus* anti-complement proteins display similar inhibitory activities against sera from several animals, birds, and reptiles, suggesting *I. ricinus* and potentially *I. scapularis* ILPs do not display host specificity (6, 26).

A third possibility justifying the co-expression of multiple ILP family members during tick feeding is that the ILP family members are potentially multifunctional. Since the ILP family members directly interact with properdin, and properdin is primarily composed of TSRs, then the ILP family members may potentially interact with other TSR-containing proteins and inhibit additional host hemostatic, inflammatory and immune responses. In order to test this possibility, we incubated S20NS, S20L12, and S20L2 with various TSR-containing proteins including TSP-1, ADAMTS-13, and properdin. The ILP family members specifically interacted with only properdin, indicating ILP family members most likely do

not bind other TSR-containing proteins. However, numerous proteins involved in various cellular responses including platelet aggregation, angiogenesis, cell matrix remodeling, and neuronal development contain TSRs. Since we only examined the interactions of S20NS, S20L12, and S20L2 with TSP-1 and ADAMTS-13, the possibility still exists that ILP family members may directly interact with other TSR-containing proteins. Furthermore, the ILP family members may serve additional functions important for tick physiology. Daix et al. and Couvreur et al. determined that *I. ricinus* anti-complement proteins are constituitively co-expressed in unfed ticks, possibly suggesting they may serve important roles in the tick when it is not feeding (6, 7).

In this study, we determined that ILP family members do not display synergy or host specificity, and they do not bind additional TSR-containing proteins. Other possibilities still exist to explain the co-expression of multiple ILPs in the tick, including: 1) differential expression of ILP family members during different life stages of the tick 2) differential target recognition between ILP family members and 3) antigenic variation between different ILP family members. It is not likely that specific ILPs are expressed during specific life stages since the ILP family members examined in this study, Salp20, S20Lclone 12, and S20Lclone 2, are co-expressed during the nymphal life stage of the tick (8, 32). Furthermore, Couvreur at al. have demonstrated that multiple *I. ricinus* anti-complement proteins are co-expressed during all life stages of the tick (6).

Various ILP family members may be co-expressed during tick feeding because different family members potentially bind different domains within the target protein, properdin. Properdin is primarily composed of 6 TSRs, which are flanked by short N- and C-terminal regions (18). In plasma, properdin typically exists as dimers, trimers, or tetramers,

with tetramers and trimers being the most active forms of the protein (20). TSR-6 of properdin facilitates polymerization, while TSR-5 mediates C3b interactions (11, 21). Different ILP family members may bind different domains of properdin, blocking its polymerization or its interaction with C3b, which would ultimately result in C3 convertase decay acceleration. Spitzer et al. have also demonstrated that properdin binds surfaces independently of C3b, acting as a platform to initiate convertase formation and complement activation (30). The domains mediating the interaction of properdin with activating surfaces are currently unknown, but various ILP family members may also bind these domains, blocking the interaction of properdin with activating surfaces and preventing activation of the alternative complement pathway. We are currently examining the domains of properdin that are necessary for binding S20NS, S20L12, and S20L2.

There is a high probability that various ILP family members display antigenic variation. Since *I. scapularis* ticks feed for several days on a host and repeatedly feed on the same host, the host has ample time to mount immune responses, including the generation of neutralizing antibodies, against various tick salivary proteins. Therefore, the tick may need to produce multiple proteins with identical functions that are antigenically variable in order evade host immune responses and prevent complement activation. In support of this theory, Couvreur et al. have recently established that antibodies generated against a specific *I. ricinus* anti-complement protein, IxAC-B1, only detect and neutralize the activity of IxAC-B1 and none of the other *I. ricinus* anticomplement proteins (6). Additionally, Kotsyfakis et al. have demonstrated that two *I. scapularis* salivary sialostatins, which bind and inhibit host cathepsins, also display antigenic variation (13). Based on these findings, Isac, Salp20, S20Lclone 12, and S20Lclone 2 also likely display antigenic variation. At this time, we are

investigating this possibility. Characterization of the functions, properties, and differences of various ILP family members will provide further insight into the mechanisms of salivary proteins important for tick feeding and aid in the development of anti-tick vaccines.

4.6 Contributions

In this work, I designed and performed the experiments. Holly Patterson, a lab technician under my direction, produced and purified of recombinant insect proteins used in this study, including S20NS, S20L12, and S20L2. Aravinda de Silva assisted with the design of experiments.

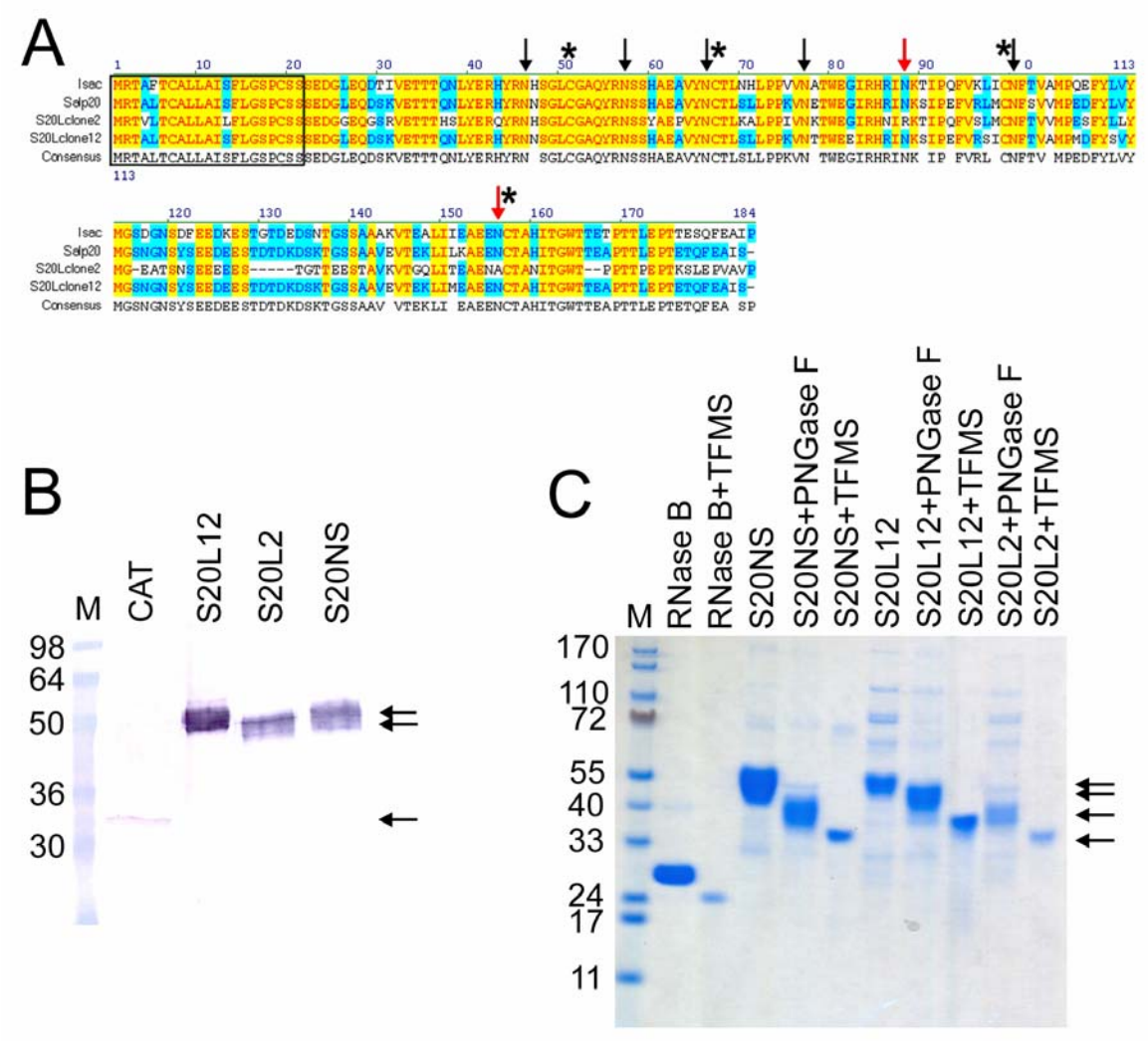


Figure 4.1 Alignment, expression, and deglycosylation of S20L12 and S20L2. ***A.*** Amino acid alignment of Isac, Salp20, S20Lclone 2, and S20Lclone 12. Putative secretion signals are boxed, potential N-linked glycosylation sites are marked with arrows, and conserved cysteines are marked with asterisks. Yellow highlighted amino acids indicate conservation among all 4 sequences while blue highlighted amino acids indicated conservation among 2 or 3 sequences. ***B.*** Western blot using a mouse monoclonal α-V5 antibody of culture media from stably transfected insect cells expressing CAT (~34 kDa), S20L12 (~ 50 kDa), S20L2 (~ 48 kDa), or S20NS (~ 50 kDa). ***C.*** SDS-PAGE of RNaseB (positive control for deglycosylation with TFMS), S20NS, S20L12, and S20L2 treated with either PNGaseF or TFMS. Arrows indicate native and deglycosylated proteins.

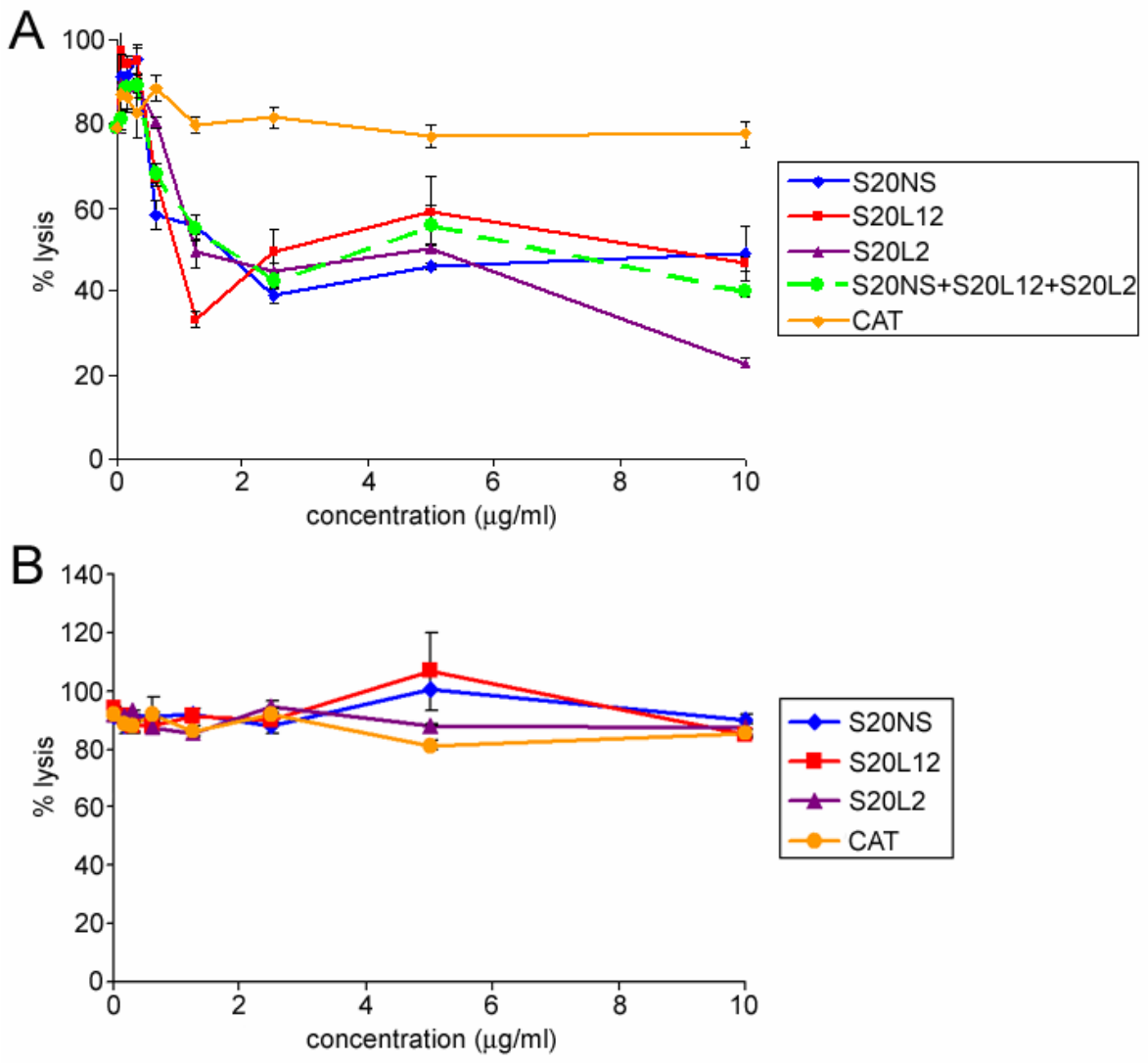


Figure 4.2 Inhibition of the alternative complement pathway by S20L12 and S20L2. RbEs (***A***) or SEs (***B***) were incubated with increasing concentrations S20NS, S20L12, S20L2, CAT, or a mixture of ILP family members in the presence of NHS. After lysis occurred, cells and cell debris were pelleted, and the $O.D._{405}$ of the supernatants was determined and the percent lysis was calculated. The error bars represent the standard error of the mean % lysis where N=3.

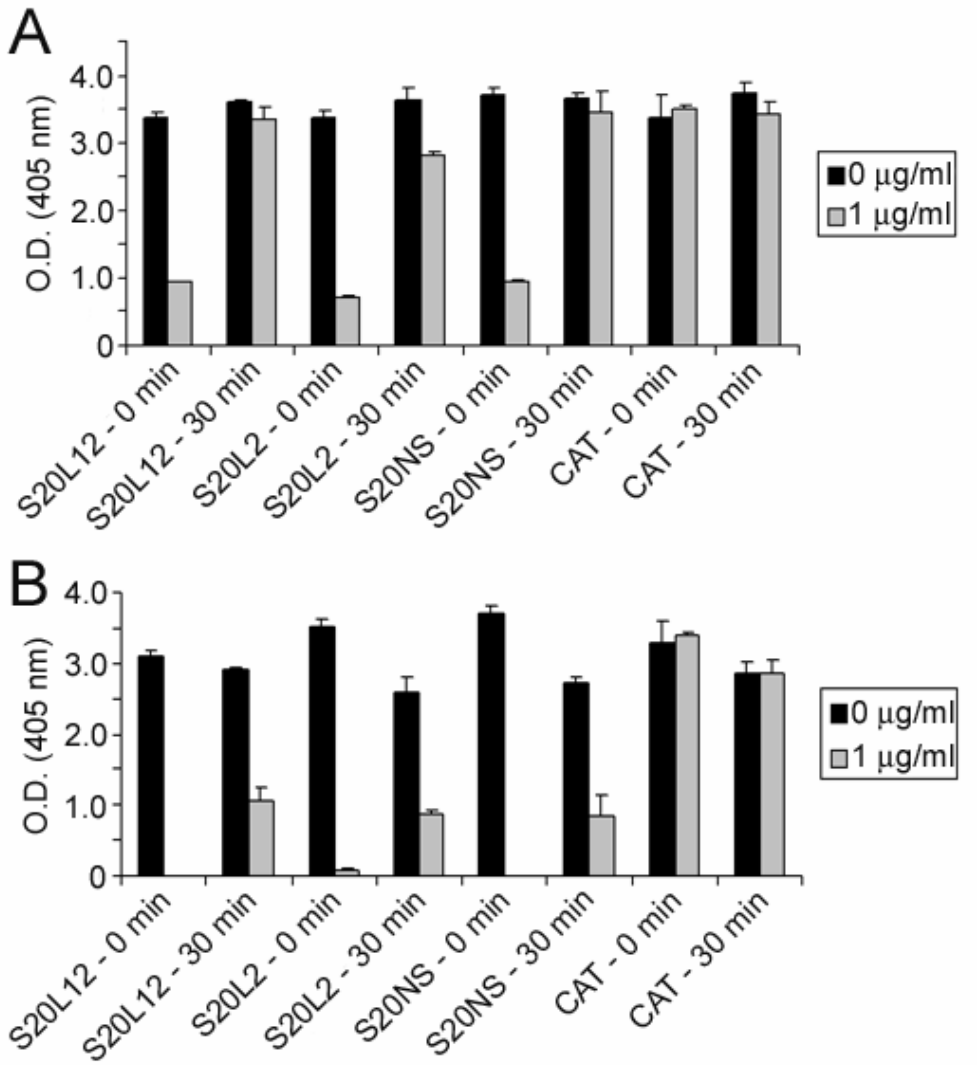


Fig 4.3 Deposition of C3 or fB in the presence of S20L12 and S20L2. S20NS, S20L12, S20L2, or CAT (1 µg/ml) were added to the agarose wells of a 96-well plate either immediately with NHS (0 min) or 30 min after NHS (30 min). The wells were washed and plate bound C3 (A) or fB (B) were detected with specific antibodies. The error bars represent the standard error of the mean O.D. value where N=3.

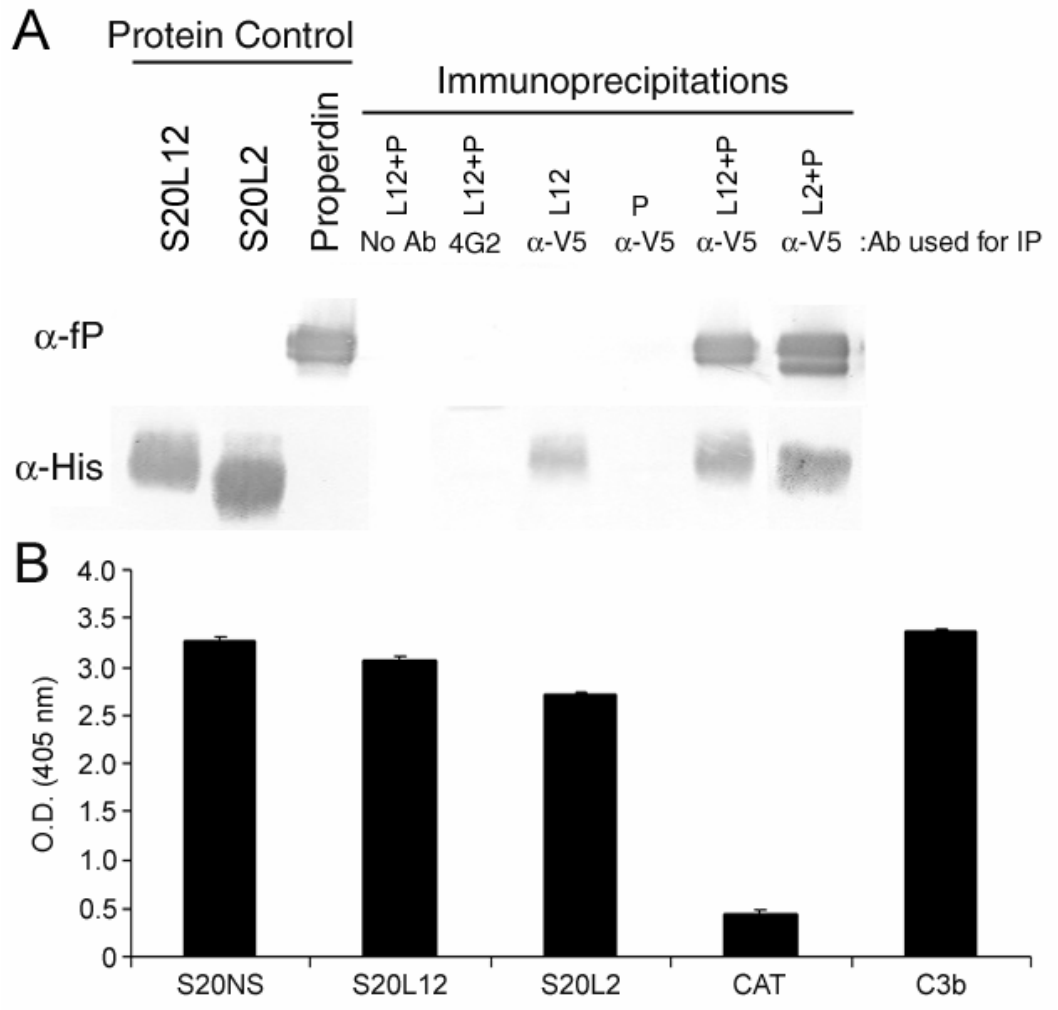


Fig 4.4 S20L12 and S20L2 directly bind properdin. ***A.*** S20L12 (L12), S20L2 (L2), properdin (P), or S20L12 and S20L2 previously incubated with properdin (L12+P and L2+P, respectively) were immunoprecipitated (IP) with a monoclonal α-V5 Ab against the C-terminal epitope tags on S20L12 and S20L2. Immunoprecipitates were then analyzed by Western Blots using specific Abs directed against properdin (α-fP) or S20L12 and S20L2 (α-His). ***B.*** Microtiter plate wells were coated with S20NS, S20L12, S20L2, CAT (negative control), or C3b (positive control). The wells were washed, blocked, and then incubated with properdin. Bound properdin was detected by ELISA. The error bars represent the standard error of the mean where N=3.

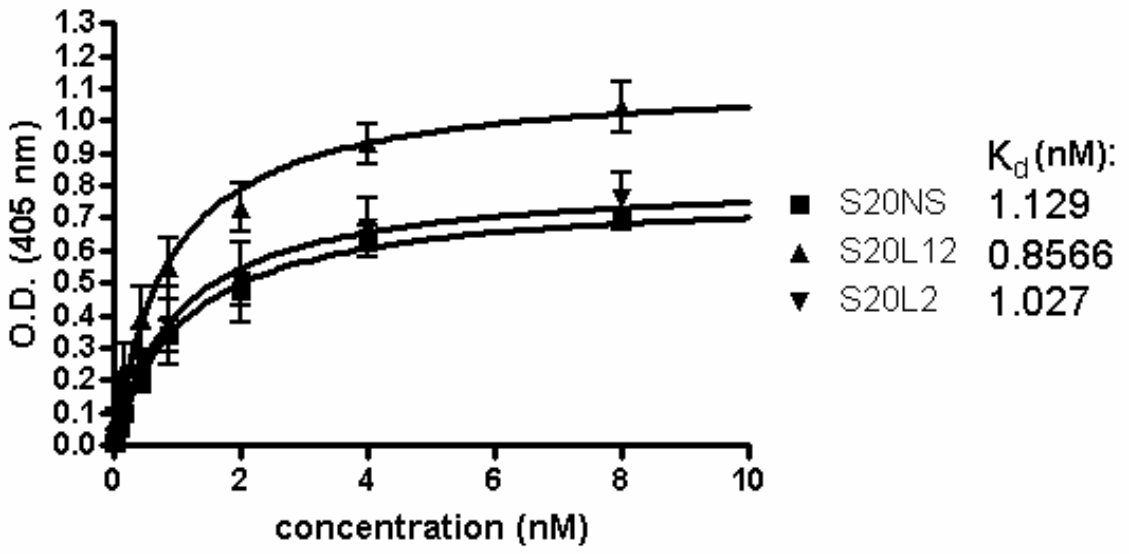


Fig 4.5 Saturation binding curves and relative binding affinities of properdin for ILP family members. Microtiter plate wells were coated with S20NS, S20L12, or S20L2. Increasing concentrations of properdin were then added to the wells, and bound properdin was detected by ELISA. The error bars represent the standard error of the mean where N=9.

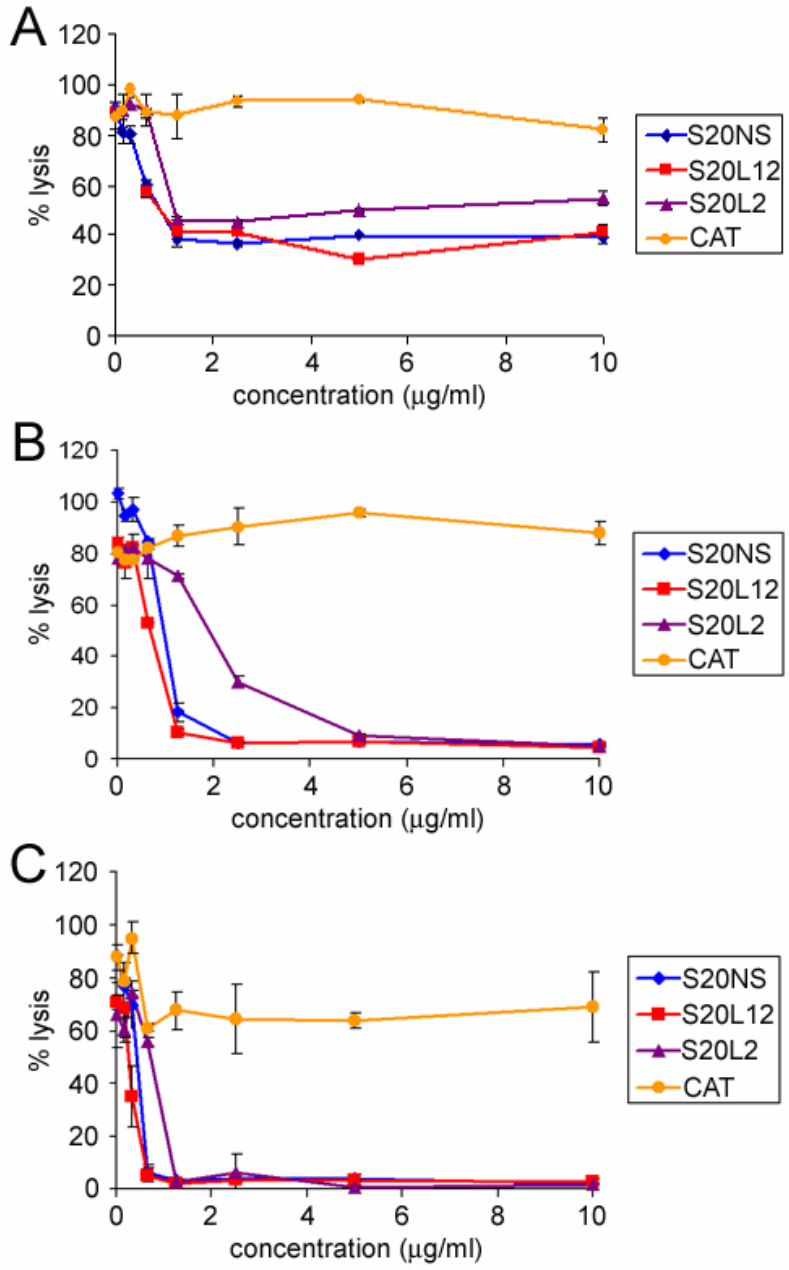


Fig 4.6 Inhibition of complement from various mammalian hosts by ILP family members. RbEs were incubated with NHS (***A***), NMS (***B***), or NPS (***C***) in presence of Mg^{2+} and increasing concentrations of S20NS, S20L12, S20L2, or CAT. After lysis, whole cells and cell debris were pelleted by centrifugation. The $O.D._{405}$ of the supernatants was determined and the % lysis calculated. The error bars represent the standard error of the mean where N=3.

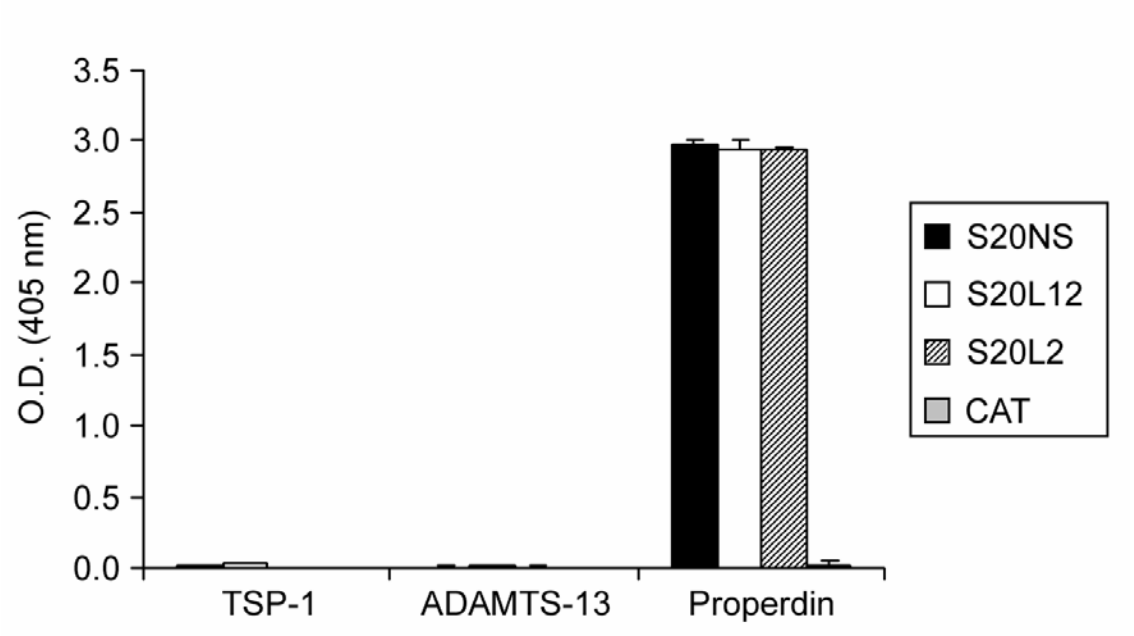


Fig 4.7 Binding of ILP family members to different TSR-containing proteins. Microtiter wells were coated with TSP-1, ADAMTS-13, or properdin. The wells were then blocked and incubated with S20NS (1 μg/ml), S20L12 (1 μg/ml), S20L2 (1 μg/ml), or CAT (1 μg/ml). Bound S20NS, S20L12, S20L2, or CAT was detected with a mouse monoclonal α-V5 antibody. The error bars represent the standard error of the mean where N=3.

4.7 References

1. Adams, J. C. and R. P. Tucker. 2000. The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development. *Dev Dyn*. 218: 280-99.

2. Alarcon-Chaidez, F. J., U. U. Muller-Doblies, and S. Wikel. 2003. Characterization of a recombinant immunomodulatory protein from the salivary glands of *Dermacentor andersoni. Parasite Immunol*. 25: 69-77.

3. Andrade, B. B., C. R. Teixeira, A. Barral, and M. Barral-Netto. 2005. Haematophagous arthropod saliva and host defense system: a tale of tear and blood. *An Acad Bras Cienc*. 77: 665-93.

4. Brossard, M. and S. K. Wikel. 2004. Tick immunobiology. *Parasitology*. 129 Suppl: S161-76.

5. Cal, S., A. J. Obaya, M. Llamazares, C. Garabaya, V. Quesada, and C. Lopez-Otin. 2002. Cloning, expression analysis, and structural characterization of seven novel human ADAMTSs, a family of metalloproteinases with disintegrin and thrombospondin-1 domains. *Gene*. 283: 49-62.

6. Couvreur, B., J. Beaufays, C. Charon, K. Lahaye, F. Gensale, V. Denis, B. Charloteaux, Y. Decrem, P. P. Prevot, M. Brossard, L. Vanhamme, and E. Godfroid. 2008. Variability and Action Mechanism of a Family of Anticomplement Proteins in *Ixodes ricinus. PLoS ONE*. 3: e1400.

7. Daix, V., H. Schroeder, N. Praet, J. P. Georgin, I. Chiappino, L. Gillet, K. de Fays, Y. Decrem, G. Leboulle, E. Godfroid, A. Bollen, P. P. Pastoret, L. Gern, P. M. Sharp, and A. Vanderplasschen. 2007. *Ixodes* ticks belonging to the *Ixodes ricinus* complex encode a family of anticomplement proteins. *Insect Mol Biol*. 16: 155-66.

8. Das, S., G. Banerjee, K. DePonte, N. Marcantonio, F. S. Kantor, and E. Fikrig. 2001. Salp25D, an *Ixodes scapularis* antioxidant, is 1 of 14 immunodominant antigens in engorged tick salivary glands. *J Infect Dis*. 184: 1056-64.

9. Dennis, D. T., Piesman, J. F., *Overview of Tick-Borne Infections of Humans*, In *Tick-Borne Diseases of Humans*, D.T.D. Jesse L. Goodman, Daniel E. Sonenshine, Editor. 2005, American Society for Microbiology Press: Washingtion, DC. p. 3-11.

10. Francischetti, I. M., V. My Pham, B. J. Mans, J. F. Andersen, T. N. Mather, R. S. Lane, and J. M. Ribeiro. 2005. The transcriptome of the salivary glands of the female western black-legged tick *Ixodes pacificus* (Acari: Ixodidae). *Insect Biochem Mol Biol*. 35: 1142-61.

11. Higgins, J. M., H. Wiedemann, R. Timpl, and K. B. Reid. 1995. Characterization of mutant forms of recombinant human properdin lacking single thrombospondin type I repeats. Identification of modules important for function. *J Immunol*. 155: 5777-85.

12. Hovius, J. W., N. Ramamoorthi, C. Van't Veer, K. A. de Groot, A. M. Nijhof, F. Jongejan, A. P. van Dam, and E. Fikrig. 2007. Identification of Salp15 homologues in *Ixodes ricinus* ticks. *Vector Borne Zoonotic Dis*. 7: 296-303.

13. Kotsyfakis, M., S. Karim, J. F. Andersen, T. N. Mather, and J. M. Ribeiro. 2007. Selective cysteine protease inhibition contributes to blood-feeding success of the tick *Ixodes scapularis. J Biol Chem*. 282: 29256-63.

14. Lawler, J. 2000. The functions of thrombospondin-1 and-2. *Curr Opin Cell Biol*. 12: 634-40.

15. Lawrie, C. H., S. E. Randolph, and P. A. Nuttall. 1999. *Ixodes* ticks: serum species sensitivity of anticomplement activity. *Exp Parasitol*. 93: 207-14.

16. Muller-Doblies, U. U. and S. K. Wikel, *The Human Reaction to Ticks*, In *Tick-Borne Diseases of Humans*, D.T.D. Jesse L. Goodman, Daniel E. Sonenshine, Editor. 2005, American Society for Microbiology: Washington D.C. p. 102-122.

17. Narasimhan, S., R. A. Koski, B. Beaulieu, J. F. Anderson, N. Ramamoorthi, F. Kantor, M. Cappello, and E. Fikrig. 2002. A novel family of anticoagulants from the saliva of *Ixodes scapularis. Insect Mol Biol*. 11: 641-50.

18. Nolan, K. F., W. Schwaeble, S. Kaluz, M. P. Dierich, and K. B. Reid. 1991. Molecular cloning of the cDNA coding for properdin, a positive regulator of the alternative pathway of human complement. *Eur J Immunol*. 21: 771-6.

19. Paesen, G. C., P. L. Adams, K. Harlos, P. A. Nuttall, and D. I. Stuart. 1999. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. *Mol Cell*. 3: 661-71.

20. Pangburn, M. K. 1989. Analysis of the natural polymeric forms of human properdin and their functions in complement activation. *J Immunol*. 142: 202-7.

21. Perdikoulis, M. V., U. Kishore, and K. B. Reid. 2001. Expression and characterisation of the thrombospondin type I repeats of human properdin. *Biochim Biophys Acta*. 1548: 265-77.

22. Ribeiro, J. M. 1987. *Ixodes dammini*: salivary anti-complement activity. *Exp Parasitol*. 64: 347-53.

23. Ribeiro, J. M., F. Alarcon-Chaidez, I. M. Francischetti, B. J. Mans, T. N. Mather, J. G. Valenzuela, and S. K. Wikel. 2006. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol*. 36: 111-29.

24. Ribeiro, J. M. and I. M. Francischetti. 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol*. 48: 73-88.

25. Santos-Martinez, M. J., C. Medina, P. Jurasz, and M. W. Radomski. 2008. Role of metalloproteinases in platelet function. *Thromb Res*. 121: 535-42.

26. Schroeder, H., V. Daix, L. Gillet, J. C. Renauld, and A. Vanderplasschen. 2007. The paralogous salivary anti-complement proteins IRAC I and IRAC II encoded by *Ixodes ricinus* ticks have broad and complementary inhibitory activities against the complement of different host species. *Microbes Infect*. 9: 247-50.

27. Soares, C. A., C. M. Lima, M. C. Dolan, J. Piesman, C. B. Beard, and N. S. Zeidner. 2005. Capillary feeding of specific dsRNA induces silencing of the *isac* gene in nymphal *Ixodes scapularis* ticks. *Insect Mol Biol*. 14: 443-52.

28. Sonenshine, D. E., 1991. *Biology of Ticks*. Oxford University Press, New York, NY.

29. Sonenshine, D. E., *The Biology of Tick Vectors of Human Disease*, In *Tick-Borne Diseases of Human*, D.T.D. Jesse L. Goodman, Daniel E. Sonenshine, Editor. 2005, American Society for Microbiology: Washington D.C. p. 12-35.

30. Spitzer, D., L. M. Mitchell, J. P. Atkinson, and D. E. Hourcade. 2007. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J Immunol*. 179: 2600-8.

31. Tucker, R. P. 2004. The thrombospondin type 1 repeat superfamily. *Int J Biochem Cell Biol*. 36: 969-74.

32. Tyson, K., C. Elkins, H. Patterson, E. Fikrig, and A. de Silva. 2007. Biochemical and functional characterization of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Mol Biol*. 16: 469-79.

33. Valenzuela, J. G. 2004. Exploring tick saliva: from biochemistry to 'sialomes' and functional genomics. *Parasitology*. 129 Suppl: S83-94.

34. Valenzuela, J. G., R. Charlab, T. N. Mather, and J. M. Ribeiro. 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis. J Biol Chem*. 275: 18717-23.

35. Valenzuela, J. G., I. M. Francischetti, V. M. Pham, M. K. Garfield, T. N. Mather, and J. M. Ribeiro. 2002. Exploring the sialome of the tick *Ixodes scapularis. J Exp Biol*. 205: 2843-64.

36. Vogt, W. 1974. Activation, activities and pharmacologically active products of complement. *Pharmacol Rev*. 26: 125-69.

37. Wikel, S. K. 1979. Acquired resistance to ticks: expression of resistance by C4-deficient guinea pigs. *Am J Trop Med Hyg*. 28: 586-90.

38. Wikel, S. K. 1996. Host immunity to ticks. *Annu Rev Entomol*. 41: 1-22.

CHAPTER 5

Evaluation of the anti-complement ILP family during tick feeding

Katharine Tyson[1], Nandhini Ramamoorthi[2], Erol Fikrig[2], and Aravinda de Silva[1]

[1]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, NC 27599 and [2]Section of Rheumatology, Department of Medicine, Yale University School of Medicine, New Haven, CT 06510

5.1 Abstract

I. scapularis ticks secrete numerous salivary proteins that inhibit host hemostasis, inflammation, and immunity. A large family of *I. scapularis* salivary anti-complement proteins, the ILP family, inhibits the alternative complement pathway, preventing the generation of multiple anaphylatoxins and the lysis of tick cells by complement. Several members of the ILP family specifically inhibit complement activation by directly binding the positive regulator of the alternative complement pathway, properdin. This interaction prevents the assembly of C3 convertases and causes the dissociation of pre-assembled convertases. Currently, it is unknown if the activities of ILP family members are required for tick feeding. In this work, we have evaluated the importance of *salp20* and related ILP family member gene expression during tick feeding through gene silencing by RNAi. Microinjection of siRNAs into nymphal ticks, regardless of the target gene specificity,

resulted in reduced tick attachment. As an alternative approach to evaluate the importance of the ILP family during tick feeding, we also immunized mice with recombinant insect expressed ILPs and then fed ticks on the immunized mice. Nymphal *I. scapularis* ticks fed successfully and efficiently on mice with antibodies directed against multiple salivary ILPs.

5.2 Introduction

Members of the ILP family, which includes Isac, Salp20, S20Lclone 12, S20Lclone 2, Irac-1, Irac-2, and IxACs, inhibit the alternative complement pathway, preventing various host responses that could be detrimental to the tick (2, 3, 10, 11). Salp20, S20Lclone12, S20Lclone 2, Irac-1, Irac-2, and multiple IxACs specifically bind properdin, preventing C3 convertase formation and causing decay acceleration of preformed convertases (2, 3, 10) (Chapter 3). Recent work suggests that multiple ILP family members are co-expressed and secreted into a host during tick feeding (2, 3). These family members likely display antigenic variation, allowing them to evade host immune recognition, which ultimately permits the tick to effectively inhibit the alternative complement pathway and feed successfully (2). However, it is currently unclear if the ILP family is required for tick feeding.

RNAi is an effective tool that has been used successfully to knockdown the expression of various tick salivary proteins, including histamine-binding proteins, Salp14, Salp15, and Isac (1, 4, 6-8). Using RNAi, we examined the role of Salp20 and additional ILP family members during tick feeding. We first silenced *salp20* and ILP family member genes in nymphal ticks by microinjection of siRNAs corresponding to conserved regions among the ILP family members. We then evaluated any differences between mock-treated and siRNA-injected ticks during attachment and feeding. In addition to RNAi, we also immunized mice

with S20NS, S20L12, and S20L2 individually or in combination to generate antibody responses against the ILPs. After immunization, we fed ticks on the immunized and mock-treated mice and observed any differences in tick attachment and feeding.

5.3 Materials and Methods

Mice and Ticks

BALB/c mice, 4-6 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). Nymphal *I. scapularis* ticks used for RNAi experiments were provided courtesy of E. Fikrig (Yale Univeristy, New Haven, CT). Mock-treated and siRNA-injected nymphal ticks were placed on BALB/c mice and allowed to feed for 60 hrs. Ticks were then removed and dissected to obtain salivary glands and guts. Nymphal *I. scapularis* ticks that were fed on immunized mice were raised as previously described by Sonenshine (9). Nymphal ticks were placed on immunized BALB/c mice and allowed to feed to repletion. Replete ticks that had fallen off the mice were then collected and weighed.

RNA interference

RNAi experiments in *I. scapaularis* nymphs were performed following a modified protocol of Narasimhan et al. (4). Duplex siRNAs corresponding to conserved regions among ILP family members and duplex nonsense siRNAs (Table 5.1) were synthesized (Invitrogen, Carlsbad, CA). dsRNA corresponding to full-length *salp20* was provided courtesy of E. Fikrig (Yale University, New Haven, CT). Groups of 25 nymphal ticks were injected with approximately 4.6 nl of a mixture of siRNAs (~1 x 10^{15} molecules/μl) or 2.3 nl of dsRNA (~1 x 10^{10} molecules/μl) in the ventral torso of the idiosoma using a micromanipulator (Narishige, Tokyo) connected to a Nanojet II microinjector (Drummond

Scientific, Broomall, PA). The needles attached to the micromanipulator were 3.5 inch capillaries (Drummond Scientific, Broomall, PA) drawn to a fine point with a micropipette puller (Sutter Instruments, Novato, CA). Mock- treated ticks were injected with 2.3 nl of buffer (10 mM Tris-HCl, pH 8.0, 20mM NaCl, 1 mM EDTA). After the injections, nymphal ticks were allowed to rest 3-4 hrs at 23^{o}C in a humidity chamber. The ticks were then placed BALB/c mice and allowed to feed for 60 hrs. After feeding, the ticks were removed and dissected to obtain salivary glands and guts.

RT-PCR to confirm gene silencing

In order to validate successful silencing of *salp20* with siRNAs and dsRNA, we preformed RT-PCR on RNA extracted from the dissected salivary glands and guts of dsRNA-treated ticks. Five pairs of salivary glands or 3 guts from siRNA, dsRNA, or mock treated fed ticks were dissected and placed in 150 μl of Trizol (Invitrogen, Carlsbad, CA). Glands were subsequently homogenized and RNA was extracted following the manufacturer's directions. Contaminating DNA was eliminated from purified RNA samples using the TURBO DNA-free kit (Ambion, Austin, TX) according to the protocol of the manufacturer. cDNA was then generated from DNA-free RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and the following primer sets: *salp20*F and *salp20*R, which were provided by E. Fikrig (Yale University, New Haven, CT), and *β-actin* F – 5`-GATGACCCAGATCATGTTCG-3` and *β-actin* R – 5`-GCCGATGGTGATCACCTG-3`. PCR products were the analyzed by agarose gel electrophoresis for the presence of *salp20* and *actin*.

Immunizations with S20NS, S20L12, and S20L2

BALB/c mice were immunized subcutaneously with 10 μg of S20NS, S20L12, and S20L2 either individually or in combination in a 100 μl emulsion of PBS and Freund's complete adjuvant (Sigma, St. Louis, MO). Mice were then boosted subcutaneously 2 and 4 weeks after the initial immunization with 10 μg of S20NS, S20L12, and S20L2 individually or in combination in 100 μl of PBS and Freund's incomplete. Mice were bled 1 week after the initial immunization and each boost by tail bleeds. Serum was purified from the collected blood using microtainer serum separators (Becton Dickinson, Franklin Lakes, NJ) following the manufacturer's instructions. Nymphal ticks were placed on the mice 2 weeks after the final boost and allowed to feed to repletion. Replete ticks were then collected and weighed.

Western blot analysis for antibody detection and specificity

For Western blot analysis, ~100 ng of recombinant purified S20NS, S20L12, S20L2, and CAT, and 25 μl of culture media from insect cells expressing recombinant Salp20 without the C-terminal V5-epitope and 6X-His tags (S20S) were electrophoresed under reducing conditions by 10% SDS-PAGE. Samples were then transferred to nitrocellulose membranes by semi-dry transfer at 380 mA for 60 min. Membranes were blocked with 1X Tris-buffered saline, 0.2% Tween-20 (TBST), 5% milk for 12-16 hrs at 4^{o}C. Mouse anti-V5 monoclonal antibody (Invitrogen, Carlsbad, CA) or mouse sera from immunized mice were diluted 1:5000 and 1:100, respectively, in TBST and incubated with the membranes for 1 hr at room temperature. Membranes were next incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma, St. Louis, MO) diluted 1:10000 in TBST for 45 min at room temperature. Bound antibody was detected by chemiluminescence using the

ECL Plus detection kit (Amersham Biosciences (GE Healthcare), Buckinghamshire, UK) following the manufacturer's instructions.

ELISAs for antibody detection and specificity

For ELISAs, microtiter plates were coated with 100 ng/well S20NS, S20L12, S20L2, or CAT in 0.1 M Carbonate Buffer for 12 hrs at 4^{o}C. After coating, the wells were blocked for 1 hr at 37^{o}C with 1X TBS, 0.1% BSA. Sera from immunized mice were diluted 1:50 in 1X TBS, 0.1% BSA and incubated in the blocked wells 1 hr at 37^{o}C. The wells were washed with TBST and subsequently incubated with an AP-conjugated goat anti-mouse antibody diluted 1:1000 in 1X TBS, 0.1% BSA. Wells were washed and bound antibodies were detected by measuring the O.D.$_{405}$ after the addition of PNPP (Sigma, St. Louis, MO).

5.4 Results

RNA interference in nymphal *I. scapularis* ticks

In order to assess the importance and necessity of Salp20 and other ILP family members during tick feeding, we silenced *salp20* expression, and potentially other ILP family member gene expression, with siRNAs and fed siRNA-treated ticks on naïve mice. Three siRNAs were generated corresponding to conserved regions among the ILP family members (Table 5.1). Since these siRNAs were conserved among different family members, they would likely silence multiple ILP genes. Groups of 30 nymphal ticks were microinjected with either buffer (mock) or a mixture of the three siRNAs (*salp20* siRNAs), allowed to rest, and then placed on naïve mice to feed. All of the mock-injected ticks attached to the mice and fed normally. Surprisingly, only 11 of the 30 *salp20* siRNA-injected ticks successfully attached to mice. After feeding for 5 days, salivary glands and

guts were dissected from the fed nymphal ticks. RNA was extracted from the salivary glands and guts and subjected to RT-PCR to determine the expression levels of *salp20* and *actin*. Two groups of pooled salivary gland pairs and pooled guts from mock-injected ticks expressed both *salp20* and *actin*, while two groups of pooled salivary gland pairs from *salp20* siRNA-injected ticks expressed only *actin* and not *salp20* (Fig 5.1). Interestingly, *salp20* and *actin* expression were detected in the guts of *salp20* siRNA-injected ticks. These results suggest that silencing of *salp20* expression, and potentially other ILP family members, by RNAi reduces the ability of *I. scapularis* nymphs to successfully attach to a host.

In order to establish the lack of attachment we observed in the previous experiment was the result of *salp20* siRNA specificity, we repeated the experiment and injected 4 groups of 15 ticks with buffer (mock), full-length *salp20* dsRNA (*salp20* dsRNA), *salp20* siRNAs, or nonsense siRNAs (Table 5.1). When the injected nymphal ticks were placed on mice, mock-injected and *salp20* dsRNA-injected ticks attached successfully (Table 5.2). However, the *salp20* siRNA-injected and the nonsense siRNA-injected ticks failed to attach (Table 5.2), indicating the presence of siRNA, regardless of target specificity, results in reduced *I. scapularis* attachment rates.

Mouse immunizations with recombinant S20NS, S20L12, and S20L2

As an alternative approach for evaluating the importance and necessity of the ILP family during tick feeding and to examine the antigenic properties of ILP members, we immunized mice with recombinant S20NS, S20L12, and S20L2 either individually or in combination and subsequently fed nymphal ticks on the immunized mice. Mice immunized with S20NS, S20L12, and S20L2 individually or in combination developed robust antibody

responses against the immunizing recombinant proteins and additional ILP family members. Antibodies in sera from mice immunized with a combination of S20NS, S20L12, and S20L2 reacted against all three recombinant proteins when compared to pre-immune sera, while antibodies in sera from mice immunized with S20NS, S20L12, or S20L2 individually reacted against the recombinant protein used for the immunizations and additional ILP family members when compared to pre-immune sera (Fig 5.2A and data not shown). Since all of the mice were immunized with recombinant proteins containing C-terminal V5-epitope and 6X-His tags, antibodies may have developed against the tags resulting in cross-reactivity. In support of this possibility all immunized mouse sera reacted weakly with CAT, which contains the C-terminal epitope tags (Fig 5.2A). However, antibodies specific for the recombinant proteins without the tags were apparent as serum from a mouse immunized with S20NS detected a doublet by Western blot analysis in a sample containing culture media from insect cells expressing Salp20 without the C-terminal tags (S20S) (Fig 5.2B). Additionally, the proportion of antibodies specific for the V5-epitope and 6X-His tags was likely minimal since serum from the mouse immunized with S20NS barely detected CAT (Fig 5.2B).

After we determined immunized mice developed antibody responses against the recombinant ILPs, we placed nymphal *I. scapularis* ticks on the immunized mice. All ticks attached and fed successfully to repletion on all immunized mice. Fully engorged ticks that fed on immunized mice displayed no significant average weight differences from engorged ticks that fed on PBS-injected (mock) mice (Fig 5.3). These results suggest that the antibody responses generated against the recombinant ILPs do not prevent successful *I. scapularis* attachment and feeding.

5.5 Discussion

In this study, we assessed the importance and necessity of Salp20 and related ILP family members during tick feeding by first performing RNAi assays. For these assays, we microinjected nymphal ticks with siRNAs corresponding to conserved regions among the ILP family members and silenced *salp20* expression, and possibly other ILP gene expression. Surprisingly, we were unable to detect differences in the number of ticks successfully attaching to mice when *salp20* siRNA-injected ticks were compared to ticks that were microinjected with nonsense siRNAs. These results indicate that the presence of siRNAs alone within ticks affects the ability of the tick to successfully attach to a host. One explanation for this observed result is that high concentrations of siRNAs were injected into the ticks, perhaps resulting in abnormal tick behaviors. In order to detect specific effects of silencing *salp20* and related ILP gene expression, these experiments should be repeated with multiple dilutions of siRNAs to determine the tick attachment and feeding abilities at the appropriate concentration of siRNA that causes efficient specific gene silencing without displaying nonspecific side effects.

Our approach to silence *salp20* and other related ILP family member genes with siRNAs was unique in that all successful RNAi studies in ticks thus far have typically been performed with full-length dsRNAs corresponding to the target gene, as opposed to siRNAs (1, 4, 6-8). The stability and longevity of siRNAs within ticks is unknown. Silencing of *salp20* expression by microinjection of full-length *salp20* dsRNA in nymphal *I. scapularis* ticks also had no effect on tick attachment, feeding times, or fed weights (personal communication, N. Ramamoorthi). Since *salp20* dsRNA potentially silenced only *salp20* expression, other ILP genes may have been expressed resulting in no observed effects during

tick attachment and feeding. Even though we successfully silenced *salp20* expression with both siRNAs and dsRNA, we may observe more consistent and definitive results in tick attachment and feeding if we perform these studies using multiple full-length dsRNAs corresponding to several ILP family members.

Soares et al. demonstrated that capillary feeding of *isac* dsRNA effectively silenced *isac* expression and led to reduced nymphal tick weights during feeding, resulting in ineffective pathogen transmission (8). However, in their approach, Soares et al. prefed nymphal ticks 72 hr before feeding them *isac* dsRNA. During that 72 hrs, the ticks successfully attached and cemented themselves in place, making their removal extremely difficult. In fact, during removal from the host, tick mouthparts are often damaged, which ultimately prevents further successful feeding and may even lead to premature death. In their studies, Soares et al. may have damaged tick mouthparts during removal from the host, resulting in reduced tick weights when the ticks were allowed to feed again after *isac* dsRNA administration. Furthermore, as Soares et al. administered only *isac* dsRNA to nymphal ticks, they may not have effectively silenced the entire ILP family. More pronounced differences in feeding times and weights may be apparent between mock-treated and dsRNA-treated ticks if the entire ILP family is effectively silenced.

Besides RNAi, we also developed specific antibodies against ILP family members to determine the importance of the ILP family during tick feeding and to additionally assess the antigenicity of various family members. When mice were immunized with S20NS, S20L12, and S20L2 individually or in combination, they developed robust antibody responses against the ILPs. However, these antibody responses did not prevent successful tick feeding as comparable numbers of fed nymphal ticks from all immunized mice and PBS-injected

(mock) mice displayed similar average weights. One explanation for this result is that if the ILP family members indeed display antigenic variation, the antibodies generated in the immunized mice may have neutralized specific ILPs but not all family members secreted in tick saliva during feeding, allowing the ticks to feed successfully. Couvreur et al. have demonstrated that antibodies in the serum from a mouse immunized with a single recombinant *I. ricinus* ILP detected and neutralized the activity of only the immunizing protein and not related family members, suggesting antigenic variation between family members (3). In addition, Daix et al. determined that monoclonal antibodies directed against Irac-1 were only capable of recognizing Irac-1, and not Irac-2. Likewise, Irac-2 monoclonal antibodies only recognized Irac-2, and not Irac-1 (3). In the studies described here, antibodies from mice immunized with individual ILPs, S20NS, S20L12, and S20L2, were able to recognize all recombinant ILP proteins, suggesting antigenic variation may not be a likely possibility. However, all of the recombinant ILP proteins possessed V5-epitope and 6X-His tags, possibly resulting in the generation of cross-reactive anti-V5 and anti-His antibodies. We did observe some cross-reactive anti-V5 and anti-His antibodies as sera from all immunized mice were capable of reacting with CAT, which is not an ILP but contains C-terminal V5-epitope and 6X-His tags. In order to draw conclusions about the antigenicity of various ILP family members and the necessity and importance of the ILP family during tick feeding, we must generate either monoclonal antibodies against specific family members or polyclonal sera lacking antibodies against the C-terminal tags present on all recombinant ILPs.

Besides difficulties with the antibody responses, we also may not have observed any differences in average fed tick weights between ticks that fed on mock-treated and

immunized mice because the ticks used for this study were nymphal ticks. Narasimhan et al. demonstrated that silencing *salp14* expression in adult *I. scapularis* ticks resulted in pronounced differences in fed tick weights between dsRNA-injected ticks and mock-treated ticks (4). However, when Pedra et al. repeated these studies in nymphal ticks, no differences in fed tick weights were observed between *salp14* dsRNA-injected and mock-treated ticks, indicating the feeding characteristics and tick rejection mechanisms are different between adults and nymphs (5). Nymphal ticks are very small, making weight determinations difficult. In addition, female and male nymphs appear identical, but females usually consume much larger blood meals than males (9). In our studies, it is highly likely that male and female nymphs were distributed unevenly between feeding groups. Therefore, significant differences in fed tick weights between dsRNA-injected and mock-treated and between ticks feeding on immunized versus mock-treated mice may be hard to distinguish. Both the RNAi and immunization experiments may provide clearer results if performed with adult *I. scapularis* ticks. Once these experiments are eventually optimized, and if we are able to determine the ILP family is vital for successful tick feeding, various family members may be attractive targets for anti-tick vaccines.

5.6 Contributions

In this work, I designed and performed all of the experiments with the following exceptions. Nandhini Ramamoorthi, a postdoctoral fellow in the laboratory of Erol Fikrig, aided me in the design of the RNAi studies and assisted me with the microinjections of ticks with dsRNA and RT-PCR of dissected tick tissues. Nandhini Ramamoorthi also provided full-length *salp20* dsRNA as a control in the RNAi experiments. Erol Fikrig provided

nymphal ticks and laboratory space for me to perform the RNAi studies, and Aravinda de Silva helped with the design of the experiments.

siRNA Name	Sequence
Salp20_siRNA_157	5`-GCACAGUAUAGGAAUUCAA-3`
Salp20_siRNA_157R	5`-UUGAAUUCCUAUACUGUGC-3`
Salp20_siRNA_117	5`-CUACGAACGUCAAUAUAGA-3`
Salp20_siRNA_117R	5`-UCUAUAUUGACGUUCGUAG-3`
Salp20_siRNA_156	5`-GGCACAGUAUAGGAAUUCA-3`
Salp20_siRNA_156R	5`-UGAAUUCCUAUACUGUGCC-3`
nonsense_siRNA_157_rev	5`-AACUUAAGGAUAUGACACG-3`
nonsense_siRNA_157R_rev	5`-CGUGUCAUAUCCUUAAGUU-3`
nonsense_siRNA_117_rev	5`-AGAUAUAACUGCAAGCAUC-3`
nonsense_siRNA_117R_rev	5`-GAUGCUUGCAGUUAUAUCU-3`
nonsense_siRNA_156_rev	5`-ACUUAAGGAUAUGACACGG-3`
nonsense_siRNA_156R_rev	5`-CCGUGUCAUAUCCUUAAGU-3`

Table 5.1 siRNAs used for RNA interference in nymphal *I. scapularis* ticks.

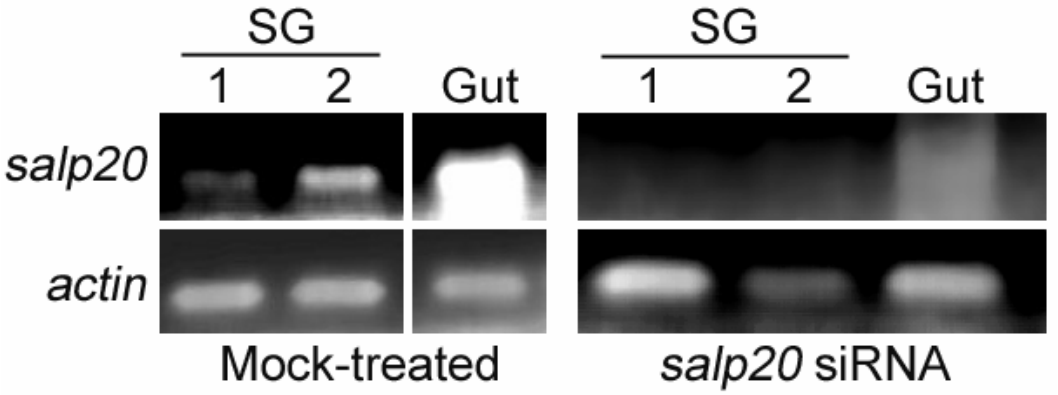


Fig 5.1 RT-PCR of RNA from the salivary glands and guts of mock- and *salp20*-siRNA injected *I. scapularis* nymphs. RNA was extracted from two groups (1 & 2) of 5 pairs of salivary glands (SG) and 3 guts (Gut) of nymphal buffer-injected (mock-treated) or *salp20*-siRNA injected (salp20 siRNA) *I. scapularis* ticks that fed on mice for 5 days. The RNA was subjected to RT-PCR with *salp20*- and *actin*-specific primers.

Treatment	# attached/total number injected
Mock	15/15
salp20 dsRNA	13/15
salp20 siRNAs	0/15
nonsense siRNAs	0/15

Table 5.2 Numbers of nymphal *I. scapularis* ticks that attached to mice after dsRNA microinjections.

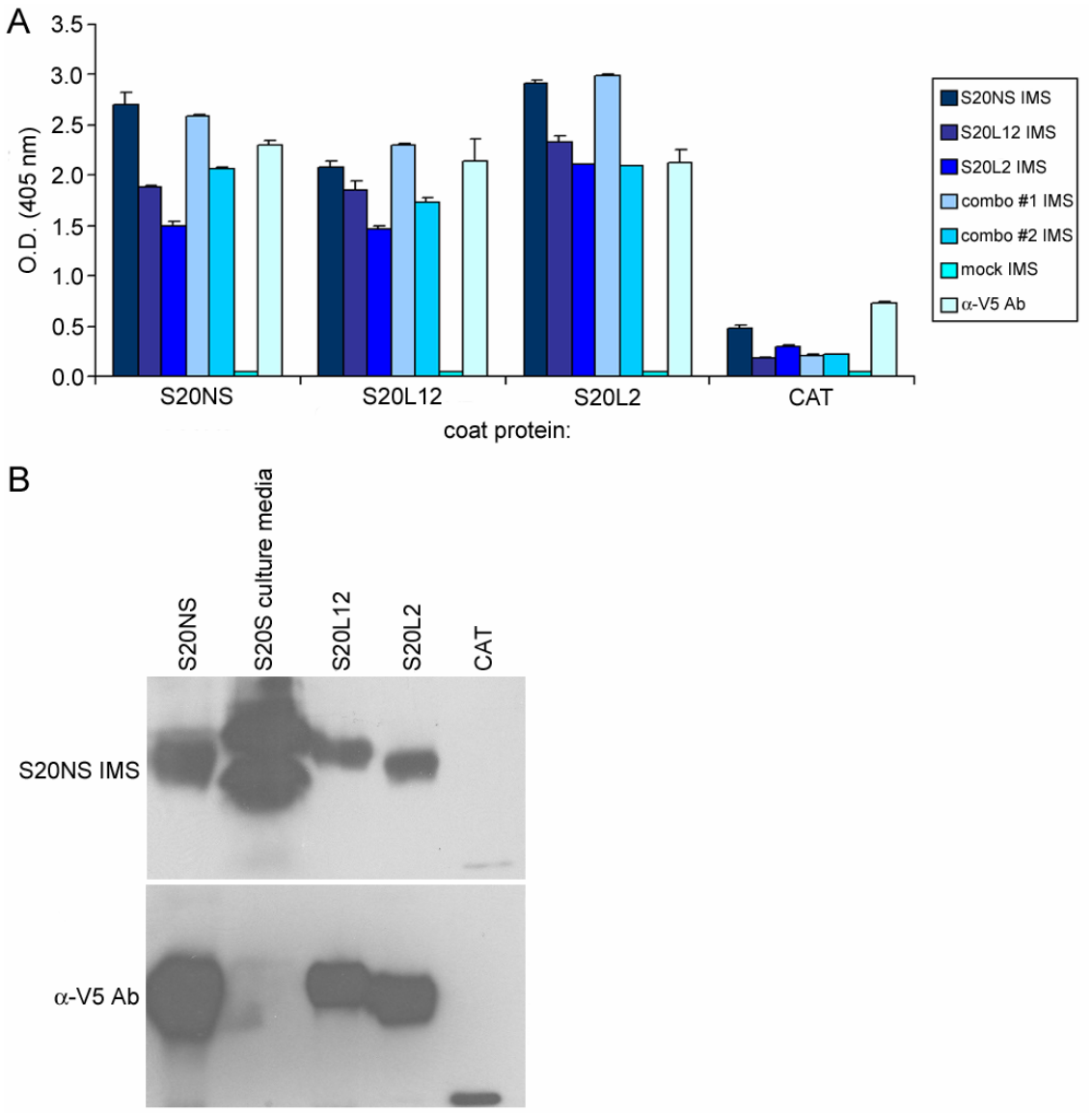


Fig 5.2 Antibody responses and specificities from mice immunized with recombinant ILP proteins. ***A.*** Microtiter plates were coated with S20NS, S20L12, and S20L2 and then incubated with immune mouse sera (diluted 1:100) from mice that were immunized and boosted subcutaneously with 10 μg of recombinant insect-derived S20NS, S20L12, and S20L2 individually (S20NS IMS, S20L12 IMS, S20L2 IMS) or in combination (combo #1 IMS, combo #2 IMS). Bound antibodies were detected by ELISA with a secondary AP-conjugated goat α-mouse IgG. ***B.*** Approximately 100 ng of S20NS, S20L12, S20L2, CAT, and 25 μl of culture media from stably transfected insect cells expressing S20S were probed with immune serum from a mouse immunized with S20NS (1:100) or with a monoclonal mouse α-V5 antibody (1:5000) and a secondary HRP-conjugated goat α-mouse IgG.

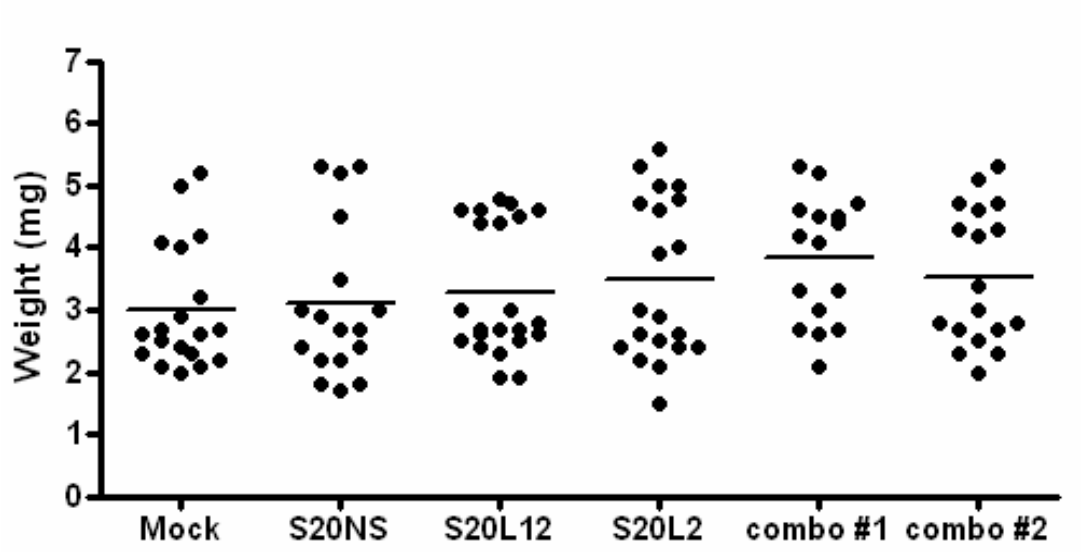


Fig 5.3 Weights of nymphal *I. scapularis* ticks fed on mice immunized with recombinant ILP proteins. Groups of engorged nymphal *I. scapularis* ticks that fed on mice immunized with S20NS, S20L12, and S20L2 individually (S20NS, S20L12, S20L2) or in combination (combo #1, combo#2) were weighed. Nymphal ticks that fed on mice injected with PBS (Mock) were also weighed. Average tick weights in each group are represented by the horizontal line.

5.7 References

1. Aljamali, M. N., A. D. Bior, J. R. Sauer, and R. C. Essenberg. 2003. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum. Insect Mol Biol*. 12: 299-305.

2. Couvreur, B., J. Beaufays, C. Charon, K. Lahaye, F. Gensale, V. Denis, B. Charloteaux, Y. Decrem, P. P. Prevot, M. Brossard, L. Vanhamme, and E. Godfroid. 2008. Variability and action mechanism of a family of anticomplement proteins in *Ixodes ricinus. PLoS ONE*. 3: e1400.

3. Daix, V., H. Schroeder, N. Praet, J. P. Georgin, I. Chiappino, L. Gillet, K. de Fays, Y. Decrem, G. Leboulle, E. Godfroid, A. Bollen, P. P. Pastoret, L. Gern, P. M. Sharp, and A. Vanderplasschen. 2007. *Ixodes* ticks belonging to the *Ixodes ricinus* complex encode a family of anticomplement proteins. *Insect Mol Biol*. 16: 155-66.

4. Narasimhan, S., R. R. Montgomery, K. DePonte, C. Tschudi, N. Marcantonio, J. F. Anderson, J. R. Sauer, M. Cappello, F. S. Kantor, and E. Fikrig. 2004. Disruption of *Ixodes scapularis* anticoagulation by using RNA interference. *Proc Natl Acad Sci U S A*. 101: 1141-6.

5. Pedra, J. H., S. Narasimhan, K. Deponte, N. Marcantonio, F. S. Kantor, and E. Fikrig. 2006. Disruption of the salivary protein 14 in *Ixodes scapularis* nymphs and impact on pathogen acquisition. *Am J Trop Med Hyg*. 75: 677-82.

6. Ramakrishnan, V. G., M. N. Aljamali, J. R. Sauer, and R. C. Essenberg. 2005. Application of RNA interference in tick salivary gland research. *J Biomol Tech*. 16: 297-305.

7. Ramamoorthi, N., S. Narasimhan, U. Pal, F. Bao, X. F. Yang, D. Fish, J. Anguita, M. V. Norgard, F. S. Kantor, J. F. Anderson, R. A. Koski, and E. Fikrig. 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature*. 436: 573-7.

8. Soares, C. A., C. M. Lima, M. C. Dolan, J. Piesman, C. B. Beard, and N. S. Zeidner. 2005. Capillary feeding of specific dsRNA induces silencing of the *isac* gene in nymphal *Ixodes scapularis* ticks. *Insect Mol Biol*. 14: 443-52.

9. Sonenshine, D. E., 1991. *Biology of Ticks*. Oxford University Press, New York, NY.

10. Tyson, K., C. Elkins, H. Patterson, E. Fikrig, and A. de Silva. 2007. Biochemical and functional characterization of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Mol Biol*. 16: 469-79.

11. Valenzuela, J. G., R. Charlab, T. N. Mather, and J. M. Ribeiro. 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis. J Biol Chem.* 275: 18717-23.

CHAPTER 6

Discussion

Hematophagus ectoparasitic arthropods, including ticks, are major pest of animals and humans, and transmit numerous bacterial, viral, and protozoan pathogens that cause devastating diseases. Arthropod control strategies typically involve the use of insecticides, which present many disadvantages including environmental pollution, human health risks, and the development of insecticide resistant arthropods. *I. scapularis*, the blacklegged tick, is an extensively studied arthropod since it transmits a variety of pathogens, including the causative agent of Lyme disease. Elucidating proteins and mechanisms that are important for successful blood feeding in *I. scapularis* will provide further insight into the feeding process of the tick, and potentially other blood feeding arthropods. The future characterization of proteins and mechanisms important for successful arthropod blood feeding may aid in the development of alternative strategies to control arthropods and pathogen transmission.

6.1 Characterization of *I. scapularis* anti-complement proteins

I. scapularis and *I. ricinus* ticks express a large family of anti-complement proteins, the ILP family, that specifically inhibit the alternative complement pathway (2, 3, 23, 26, 28). ILPs uniquely interact with properdin, a positive regulator of the C3 convertase, displacing it from the convertase, which ultimately leads to decay acceleration of the C3 convertase (2) (Chapter 3) (Fig 6.1). In the future, we plan on further characterizing the interaction between

ILPs and properdin. Properdin is primarily composed of 6 TSRs flanked by short N- and C-terminal regions (7, 16). These TSRs are important for mediating the interaction of properdin with Bb, C3b, and the C3 convertase, and for binding surfaces (10, 13, 21). Specifically, the TSRs have been shown to interact with sulfated glycoconjugates and glycosaminglycans (8, 13). The possibility exists that the numerous N- and O-linked carbohydrates of ILPs mediate their interactions with properdin. In support of this possibility, we were unable to detect a direct interaction between an *E. coli* expressed glutathione S-transferase (GST)-Salp20 fusion protein that lacked post-translational modifications (data not shown). However, the lack of interaction between properdin and GST-Salp20 may be the result of the large GST tag, ~ 25 kDa, which possibly prevents the appropriate folding and activity of Salp20. Future studies are aimed at evaluating the properdin binding capabilities of insect derived recombinant ILPs lacking N- and O-linked glycans that will be generated either through various enzymatic deglycosylations or through protein production in the presence of glycosylation inhibitors.

6.2 Determination of the purpose of ILP family production

Couvreur et al. and Daix et al. demonstrated that multiple ILPs are coexpressed during tick feeding (2, 3). Since individual ILPs typically displayed partial inhibition of the alternative complement pathway (Chapter 4), we believed multiple ILPs acted synergistically when coexpressed during tick feeding, resulting in complete inhibition of the alternative pathway. However, when mixed together, multiple *I. scapularis* ILPs displayed partial inhibition of the alternative complement pathway, similar to activity observed with individual ILPs (Chapter 4). As an alternative possibility justifying ILP coexpression, individual ILPs

may potentially display host specificity as both *I. scapularis* and *I. ricinus* feed on numerous animal hosts. Yet, we detected no differences in the inhibitory activity of individual *I. scapularis* ILPs when incubated with various host animal serum sources (Chapter 4), comparable to the results obtained with *I. ricinus* ILPs in similar experiments (2, 25). Another alternative possibility explaining ILP coexpression is that different ILPs display multiple functions besides complement inhibition by potentially interacting with other TSR-containing proteins in addition to properdin. In these studies, we demonstrated that different ILPs specifically interact with only properdin and not other TSR-containing proteins. However, as only two other proteins containing TSRs, ADAMTS-13 and TSP-1, were assayed, ILPs may still be capable of displaying multiple functions, unrelated to ADAMTS-13 and TSP-1, that facilitate blood feeding in hosts.

Since ILPs potentially contain numerous N- and O-linked carbohydrate residues, these proteins may also be capable of inhibiting the mannose-binding lectin complement pathway. The MBL pathway is initiated when MBL binds high density terminal mannose residues on activating surfaces, resulting in the recruitment and activation of MASPs (6, 15, 24, 30, 31). The carbohydrate residues present on different ILPs may bind MBL, preventing it from recognizing sugars on activating tick surfaces. We are currently interested in determining if different ILPs bind MBL and possibly prevent activation of the MBL complement pathway in addition to inhibition of the alternative complement pathway.

Couvreur et al. have recently demonstrated that individual *I. ricinus* ILPs display antigenic variation, which potentially explains ILP coexpression during tick feeding (2). As *I. ricinus* ticks, like *I. scapularis* ticks, feed on a host for several days, the host has ample time to develop to immune responses, including antibody responses, against tick salivary

proteins. Furthermore, *I. ricinus* and *I. scapularis* ticks may repeatedly feed on the same hosts as the ticks live within the vicinity of their animal hosts. After an initial exposure to tick salivary antigens, the animal host may develop antibodies against the antigens, preventing a second successful tick feeding. In order to circumvent these antibody responses and feed successfully without rejection, the tick may produce antigenically variable salivary proteins. Future studies are directed at evaluating the antigenic variability of the different *I. scapularis* ILPs.

Besides the possibility that ILPs are antigenically variable, ILPs may also display slight variations in their functions. Even though S20L12, S20L2, and S20NS bind properdin with similar affinities, they may bind different domains of the protein. Within properdin, TSR-5 mediates the C3b interaction while TSR-6 facilitates polymerization, resulting in the formation of active properdin tetramers, trimers, and dimers (10, 19, 21). It is possible that different ILPs bind different TSRs of properdin, preventing its interaction with C3b or its polymerization, both of which would result in functional inactivation. Determining the specific domains of properdin that are bound by different ILPs will provide further insight into the functions and purpose of multiple ILP family members. Regardless of the outcomes of our future studies, characterizing differences that exist between ILP family members will hopefully elucidate the overall function of the *I. scapularis* anti-complement protein family and other various immunosuppressive tick salivary protein families. Regardless of the outcomes of our future studies, characterizing differences that exist between ILP family members will hopefully elucidate the overall function of the *I. scapularis* anti-complement protein family and other various immunosuppressive tick salivary protein families.

6.3 Importance of the ILP family during tick feeding and pathogen transmission

As *I. scapularis* produces multiple anti-complement proteins with identical functions that are likely coexpressed during tick feeding, the ILPs are probably vitally important for successful tick feeding. Soares et al. have demonstrated that silencing of *isac* by RNAi results in reduced *I. scapularis* fed nymphal tick weights and inefficient pathogen transmission (26). However, the results of Soares et al. are not definitive as several variables that existed in the execution of the experiments, unrelated to the effects of RNAi, may have caused the observed reduced tick weights. Therefore, a thorough evaluation of the necessity and importance of the ILP family during tick feeding is still needed.

Microinjection of *salp20*-specific siRNAs, as well as nonspecific siRNAs, into nymphal *I. scapularis* ticks resulted in reduced tick attachment, while the microinjection of full-length *salp20* dsRNA had no effect on tick feeding. Additionally, the generation of antibodies directed against specific ILPs had no effect on *I. scapularis* nymphal tick feeding (Chapter 5). These results suggest that the ILP family is potentially not necessary for successful nymphal tick feeding. However, as these experiments were not properly optimized, no definitive conclusions should be drawn. In the future, the optimal composition, delivery method, and concentration of *salp20*-specific siRNAs or full-length dsRNAs that effectively silence ILP gene expression during tick feeding need to be determined. Once those variables are established, these RNAi studies in *I. scapularis* may provide more definitive results. Immunization studies may also be more definitive if mice are immunized with more than three recombinant proteins, potentially generating antibodies against most, if not all, of the ILP family members. If we successfully optimize these studies and still observe no significant effects on nymphal tick feeding, we should then pursue these

studies in *I. scapularis* adults as currently, effects on tick feeding times and fed tick weights by RNAi mediated gene silencing have primarily been observed with adult ticks.

In addition to their possible roles for successful tick feeding, ILP family members may also be vital for efficient transmission of *B. burgdorferi*. Ramamoorthi et al. recently demonstrated that RNAi mediated silencing of *salp15*, and inhibitor of T-cell proliferation and IL-2 production, had no effect on successful nymphal tick feeding but prevented the efficient transmission of *B. burgdorferi* to a murine host (22). Sukumaran et al. also recently determined that RNAi mediated silencing of *salp16*, an *I. scapularis* salivary protein with unknown functions, prevents the successful migration of *Anaplasma phagocytophilum*, the causative agent of human anaplasmosis, from the tick gut to the salivary glands during feeding and tick infection (27). Since S20NS prevented complement-mediated killing of serum sensitive *B. garnii* spirochetes by NHS *in vitro* (Chapter 2), S20NS, and other ILPs, may aid in successful pathogen transmission *in vivo* by preventing complement activation. Future studies are aimed at testing this possibility.

6.4 The *I. scapularis* genome project and ILPs

The goal of the *I. scapularis* Genome Project, a collaborative effort between the international community of tick researchers and two genome sequencing centers, is to perform whole genome shotgun sequencing of *I. scapularis* to approximately six-fold coverage of the genome (11). Compared to sequenced fly genomes, *I. scapularis* has a larger genome (2.1×10^3 Mbp), which is uniquely organized into 27% highly repetitive, 39% moderately repetitive and 34% unique DNA (29). Currently, more than 18 million trace reads, representing approximately five-fold coverage of the genome, have been deposited at

the National Center for Biotechnology Information (NCBI) trace archive (18). In addition, 20 *Ixodes* BAC clones, 370,000 BAC-end reads, and more than 80,000 ESTs have also been sequenced. By mid-2008, sequencing, assembly, and annotation of six-fold coverage of the *Ixodes* genome are expected. The complete *I. scapularis* genome will provide valuable information about the origination and regulation of different ILP family members and likely help with the identification and of other salivary protein gene families.

6.5 Development of an anti-tick vaccine

As ticks are continual pests of humans, pets, and livestock, transmit numerous pathogens to animals, and cause devastating effects in livestock populations, numerous efforts have been employed to control tick feeding and pathogen transmission. Current tick control strategies involve the use of acaricides, but acaricides present multiple disadvantages such as food contamination, environmental pollution, human health risks, and the emergence of acaricide-resistant ticks (4, 5, 17). Vaccination strategies directed against specific tick-transmitted pathogens are somewhat impractical as ticks transmit such a wide variety of pathogens. Therefore, vaccines directed against various tick antigens that potentially prevent successful tick feeding and pathogen transmission present an attractive alternative to the current tick control strategies. The feasibility of anti-tick vaccines is supported by the development of TickGARD, a vaccine based on the recombinant concealed *B. microplus* tick gut protein, Bm86, which induces strong antibody responses in immunized cattle, resulting in reduced survival of feeding *B. microplus* adults (32). However, as Bm86 is a concealed antigen, repeated immunizations must be administered to induce effective tick blocking antibody titers. Labuda et al. have recently demonstrated that vaccination of mice with a

recombinant *R. appendiculatus* protein identified in the midgut and salivary glands, 64TRP, protects mice from tick-borne encephalitis virus infections as well as prevents ticks from acquiring the virus from infected mice during feeding, indicating the plausibility of the use of tick antigens as pathogen-transmission blocking vaccines (14).

Exposed tick salivary antigens are attractive anti-tick vaccine candidates since repeated tick feedings on immunized animals would boost the immune responses generated by initial immunizations, eliminating the need for repeated immunizations. Furthermore, the generation of immune responses directed against various immunosuppressive tick salivary proteins would potentially prevent successful tick feeding and block pathogen transmission. The ILP family may be useful in the development of anti-tick and pathogen transmission blocking vaccines as members are exposed in the host during feeding. However, if the different ILP family members display antigenic variation, and moreover, if members of other immunosuppressive tick salivary protein families also display antigenic variation, the development of a multivalent anti-tick vaccine directed against numerous exposed salivary antigens will be necessary.

6.6 Use of ILPs as therapeutic agents in complement mediated diseases

Inappropriate activation or uncontrolled regulation of the complement cascades leads to the development of numerous inflammatory and autoimmune diseases (24). Multiple groups have demonstrated with the use of fB and fD deficient mice that the alternative complement pathway is involved in the development of several diseases including rheumatoid arthritis, lupus nephritis, ischemia-reperfusion injury, anti-phospholipid

syndrome, experimental allergic encephalomyelitis, and pulmonary disease (12). Currently, numerous efforts are directed at developing effective complement-specific therapies.

The ILPs are potentially appealing complement-specific therapeutics as they are small proteins that are likely to be stable *in vivo* since they are naturally secreted into hosts by ticks. Furthermore, since the ILPs specifically inhibit the alternative complement pathway by binding properdin, the classical and MBL pathways would still be functional in ILP treated individuals, which would provide partial complement-mediated immunity against invading microorganisms. The use of tick proteins as therapeutics seems very feasible as Paveglio et al. have recently established that *I. scapularis* Salp15 prevents the development of experimental murine asthma (20). In addition, Hepburn et al. demonstrated that *O. moubata* OmCI, an inhibitor of the complement pathway that directly binds C5, prevents the development of the experimental autoimmune myasthenia gravis, a neuromuscular disorder, in rats (9). We are currently investigating the potential of S20NS as a therapeutic agent that inhibits the development of alternative pathway complement-mediated diseases *in vivo*.

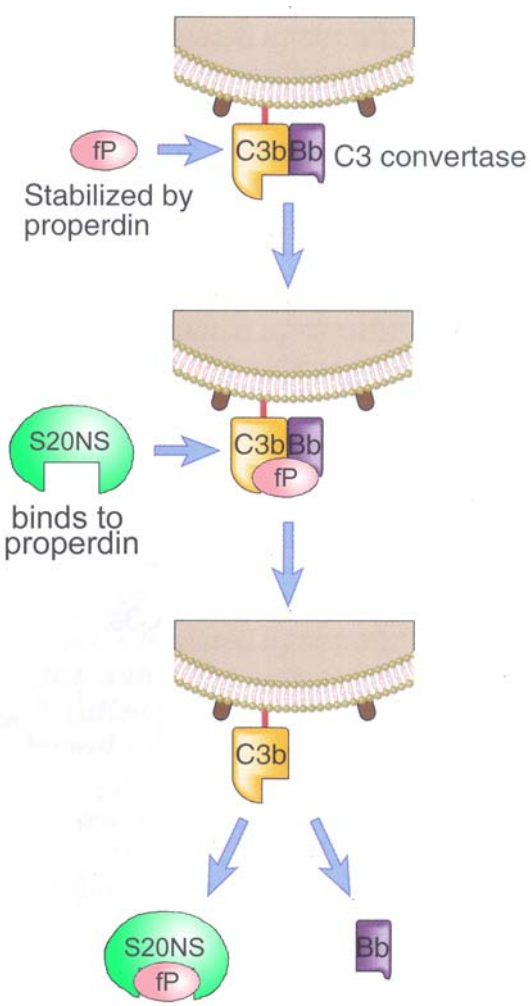


Fig 6.1 Proposed model of ILP (S20NS) complement inhibitory activity. The C3 convertase (C3bBb) is stabilized by properdin, which binds the convertase and significantly increases its half-life. During tick feeding, ILP proteins (S20NS) secreted into the host in tick saliva directly interact with properdin, removing it from the C3 convertase. Once properdin is removed, the C3 convertase rapidly decays and surface bound exposed C3b is available for fI mediated degradation. Figure adapted from (1).

6.7 References

1. Abbas, A. K., Lichtman A. H., Pober J. S., 2000. *Cellular and Molecular Immunology*. 4th ed. W.B. Saunders Company, Philadelphia, PA.

2. Couvreur, B., J. Beaufays, C. Charon, K. Lahaye, F. Gensale, V. Denis, B. Charloteaux, Y. Decrem, P. P. Prevot, M. Brossard, L. Vanhamme, and E. Godfroid. 2008. Variability and Action Mechanism of a Family of Anticomplement Proteins in *Ixodes ricinus*. *PLoS ONE*. 3: e1400.

3. Daix, V., H. Schroeder, N. Praet, J. P. Georgin, I. Chiappino, L. Gillet, K. de Fays, Y. Decrem, G. Leboulle, E. Godfroid, A. Bollen, P. P. Pastoret, L. Gern, P. M. Sharp, and A. Vanderplasschen. 2007. *Ixodes* ticks belonging to the *Ixodes ricinus* complex encode a family of anticomplement proteins. *Insect Mol Biol*. 16: 155-66.

4. de la Fuente, J. and K. M. Kocan. 2006. Strategies for development of vaccines for control of ixodid tick species. *Parasite Immunol*. 28: 275-83.

5. de la Fuente, J., K. M. Kocan, and E. F. Blouin. 2007. Tick vaccines and the transmission of tick-borne pathogens. *Vet Res Commun*. 31 Suppl 1: 85-90.

6. Gadjeva, M., S. Thiel, and J. C. Jensenius. 2001. The mannan-binding-lectin pathway of the innate immune response. *Curr Opin Immunol*. 13: 74-8.

7. Goundis, D. and K. B. Reid. 1988. Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. *Nature*. 335: 82-5.

8. Guo, N. H., H. C. Krutzsch, E. Negre, T. Vogel, D. A. Blake, and D. D. Roberts. 1992. Heparin- and sulfatide-binding peptides from the type I repeats of human thrombospondin promote melanoma cell adhesion. *Proc Natl Acad Sci U S A*. 89: 3040-4.

9. Hepburn, N. J., A. S. Williams, M. A. Nunn, J. C. Chamberlain-Banoub, J. Hamer, B. P. Morgan, and C. L. Harris. 2007. *In vivo* characterization and therapeutic efficacy of a C5-specific inhibitor from the soft tick *Ornithodoros moubata*. *J Biol Chem*. 282: 8292-9.

10. Higgins, J. M., H. Wiedemann, R. Timpl, and K. B. Reid. 1995. Characterization of mutant forms of recombinant human properdin lacking single thrombospondin type I repeats. Identification of modules important for function. *J Immunol*. 155: 5777-85.

11. Hill, C. A. and S. K. Wikel. 2005. The *Ixodes scapularis* Genome Project: an opportunity for advancing tick research. *Trends Parasitol*. 21: 151-3.

12. Holers, V. M. and J. M. Thurman. 2004. The alternative pathway of complement in disease: opportunities for therapeutic targeting. *Mol Immunol*. 41: 147-52.

13. Holt, G. D., M. K. Pangburn, and V. Ginsburg. 1990. Properdin binds to sulfatide [Gal(3-SO4)beta 1-1 Cer] and has a sequence homology with other proteins that bind sulfated glycoconjugates. *J Biol Chem*. 265: 2852-5.

14. Labuda, M., A. R. Trimnell, M. Lickova, M. Kazimirova, G. M. Davies, O. Lissina, R. S. Hails, and P. A. Nuttall. 2006. An antivector vaccine protects against a lethal vector-borne pathogen. *PLoS Pathog*. 2: e27.

15. Morgan, B. P., Harris, C. L., 1999. *Complement regulatory proteins*. Academic Press, San Diego, CA.

16. Nolan, K. F., W. Schwaeble, S. Kaluz, M. P. Dierich, and K. B. Reid. 1991. Molecular cloning of the cDNA coding for properdin, a positive regulator of the alternative pathway of human complement. *Eur J Immunol*. 21: 771-6.

17. Nuttall, P. A., A. R. Trimnell, M. Kazimirova, and M. Labuda. 2006. Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. *Parasite Immunol*. 28: 155-63.

18. Pagel Van Zee, J., N. S. Geraci, F. D. Guerrero, S. K. Wikel, J. J. Stuart, V. M. Nene, and C. A. Hill. 2007. Tick genomics: The *Ixodes* genome project and beyond. *Int J Parasitol*. 37: 1297-305.

19. Pangburn, M. K. 1989. Analysis of the natural polymeric forms of human properdin and their functions in complement activation. *J Immunol*. 142: 202-7.

20. Paveglio, S. A., J. Allard, J. Mayette, L. A. Whittaker, I. Juncadella, J. Anguita, and M. E. Poynter. 2007. The tick salivary protein, Salp15, inhibits the development of experimental asthma. *J Immunol*. 178: 7064-71.

21. Perdikoulis, M. V., U. Kishore, and K. B. Reid. 2001. Expression and characterisation of the thrombospondin type I repeats of human properdin. *Biochim Biophys Acta*. 1548: 265-77.

22. Ramamoorthi, N., S. Narasimhan, U. Pal, F. Bao, X. F. Yang, D. Fish, J. Anguita, M. V. Norgard, F. S. Kantor, J. F. Anderson, R. A. Koski, and E. Fikrig. 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature*. 436: 573-7.

23. Ribeiro, J. M., F. Alarcon-Chaidez, I. M. Francischetti, B. J. Mans, T. N. Mather, J. G. Valenzuela, and S. K. Wikel. 2006. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol*. 36: 111-29.

24. Ricklin, D. and J. D. Lambris. 2007. Complement-targeted therapeutics. *Nat Biotechnol*. 25: 1265-75.

25. Schroeder, H., V. Daix, L. Gillet, J. C. Renauld, and A. Vanderplasschen. 2007. The paralogous salivary anti-complement proteins IRAC I and IRAC II encoded by *Ixodes ricinus* ticks have broad and complementary inhibitory activities against the complement of different host species. *Microbes Infect*. 9: 247-50.

26. Soares, C. A., C. M. Lima, M. C. Dolan, J. Piesman, C. B. Beard, and N. S. Zeidner. 2005. Capillary feeding of specific dsRNA induces silencing of the *isac* gene in nymphal *Ixodes scapularis* ticks. *Insect Mol Biol*. 14: 443-52.

27. Sukumaran, B., S. Narasimhan, J. F. Anderson, K. DePonte, N. Marcantonio, M. N. Krishnan, D. Fish, S. R. Telford, F. S. Kantor, and E. Fikrig. 2006. An *Ixodes scapularis* protein required for survival of *Anaplasma phagocytophilum* in tick salivary glands. *J Exp Med*. 203: 1507-17.

28. Tyson, K., C. Elkins, H. Patterson, E. Fikrig, and A. de Silva. 2007. Biochemical and functional characterization of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Mol Biol*. 16: 469-79.

29. Ullmann, A. J., C. M. Lima, F. D. Guerrero, J. Piesman, and W. C. t. Black. 2005. Genome size and organization in the blacklegged tick, *Ixodes scapularis* and the Southern cattle tick, *Boophilus microplus*. *Insect Mol Biol*. 14: 217-22.

30. Walport, M. J. 2001. Complement. First of two parts. *N Engl J Med*. 344: 1058-66.

31. Walport, M. J. 2001. Complement. Second of two parts. *N Engl J Med*. 344: 1140-4.

32. Willadsen, P., P. Bird, G. S. Cobon, and J. Hungerford. 1995. Commercialisation of a recombinant vaccine against *Boophilus microplus*. *Parasitology*. 110 Suppl: S43-50.