

STUDY OF IMMUNOMODULATORY ACTIVITY AND ANTI-COAGULATION FACTORS
FROM THE SALIVARY GLAND OF THE BLACK FLY, *SIMULIUM VITTATUM*

by

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(Under the Direction of Donald E. Champagne)

ABSTRACT

Vector arthropods modulate many physiological responses of hosts in order to acquire a blood meal. They create wounds that initiate hosts' hemostatic responses to stop bleeding. To counteract this response, vectors inject saliva containing many anti-hemostatic factors. However, injection of saliva, (recognized as foreign by the hosts) also elicits host immune responses to remove such materials, which may reduce the anti-hemostatic activity. Vector saliva therefore contains factors that modulate host immune responses. As vectors create wounds and modulate hosts' physiological responses, they may serendipitously aid establishment of pathogenic infections.

Black flies belong to the Simuliidae, a family of lower Diptera, and are important vectors of onchocerciasis (river blindness) and vesicular stomatitis virus, but also cause a direct impact including death of animals due to simuliotoxicosis. However, the effects of black fly salivary gland components have not been extensively investigated.

This dissertation describes immunomodulatory properties of the salivary glands of the black fly, *Simulium vittatum*, using mouse models. Black fly salivary gland extract (SGE) inhibited mitogen-stimulated T-cell and B-cell proliferation. T-cell proliferation was inhibited

more strongly than B-cell proliferation. SGE induced apoptosis specifically in CD4⁺ and CD8⁺ T cells. The component responsible for the inhibition was determined to be a protein larger than 50 kDa. SGE enhanced production of nitric oxide (NO) from LPS-stimulated macrophages at low concentrations and inhibited it at a high concentration. As NO-producing macrophages can inhibit T-cell proliferation, altogether these results imply that SGE specifically inhibits the T-cell mediated adaptive immune response. NO-enhancing effects of SGE on macrophages could also explain the fatal effect of simuliotoxicosis.

This dissertation also describes functions of putative anti-coagulation factors in *S. vittatum* salivary glands. Two Kunitz proteins (SV-66 and SV-170) were expressed in a bacterial system, and recombinant SV-66 exhibited anti-coagulation activity. Mutant forms revealed that the residues in the reactive-site loop (RSL) are important in the anti-coagulation activity, as is typical for canonical Kunitz family proteins. SV-66 inhibited a wide range of serine proteases, but with relatively weak affinity. These results indicate that SV-66 could inhibit coagulation as well as modulating local inflammation.

INDEX WORDS: Black fly, Saliva, Salivary gland, Immunology, Immunomodulation, Protease inhibitor, Anti-clot, Anti-coagulation, Hemostasis, Kunitz

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DEDICATION

I dedicate this work to my parents and ancestors as well as all the insects and mice I used for my study. My life and this work depend on them, and no artificial methods can replace them.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Black flies (Diptera: Simuliidae) not only are vectors of pathogens such as the filarial parasites, *Onchocerca* spp. and vesicular stomatitis virus, but also cause a direct impact on livestock and wildlife as their massive attacks sometimes cause death due to toxic effects of the salivary injection. Blood-feeding arthropods inject saliva during blood-feeding process to facilitate successful blood feeding. The amount of saliva injected is quite small, but it contains a cocktail of factors that counteract host physiological responses to prevent blood loss (i.e., hemostasis), which are effective enough to accomplish blood feeding. From vector salivary glands, a number of such factors have been described, many to the level of mRNA and amino acid sequences, and in some cases more detailed studies have revealed 3-D structures and molecular interaction with cognate ligands or substrates. Most of these studies have been undertaken using major vector species such as mosquitoes and ticks due to their importance in global health. Vector saliva is also known to modulate the host immune response, presumably to benefit the vectors themselves, but which may also help transmission of pathogens. Despite the importance of black flies to human and veterinary health, anti-hemostatic factors and immunomodulatory functions in the black fly salivary glands have not been well documented. This dissertation investigates both immunomodulatory functions and anti-coagulation factors in the salivary glands of the black fly, *Simulium vittatum*.

Black flies

Black flies (family Simuliidae) are small, stout-bodied flies, among which many species require a blood meal for egg maturation. Black flies belong to lower Diptera, formerly

considered to be a suborder Nematocera (Bertone *et al.*, 2008) (nemato = thread, cera = horn: thread horn), in which adults of most species have long antennae (longer than head and thorax combined). However, black fly adults have cigar-shaped antennae shorter than the head. Black flies belong to the suborder Culicomorpha, which includes most of the major vector families in the lower Diptera, specifically the Culicidae (mosquitoes), Simuliidae and Ceratopogonidae (biting midges or ‘no-see-um’), excluding only the Psychodidae (subfamily Phlebotominae: sand flies). Although relationships between the families in this suborder have not been well resolved, monophyly of the Culicomorpha is well supported, meaning that Culicomorpha contains all the lineages deriving from a common ancestor (Bertone *et al.*, 2008; Friedrich and Tautz, 1997; Yeates and Wiegmann, 1999). There are additional blood-feeding lineages not of medical importance in the Culicomorpha (eg., Corethrellidae). The presence of multiple blood-feeding families in the Culicomorpha and its monophyletic relationship may indicate that blood-feeding occurred only once in the common ancestor, and some families lost the trait later (Grimaldi and Engel, 2005).

As black flies require flowing water for larval and pupal habitats, adults are also found near riparian zones (Adler and McCreadie, 2002). The black fly larvae play an important role in stream ecosystems as they may occur in huge numbers under optimal conditions, and they serve as food source for predatory animals (Currie and Adler, 2008). Adult females of more than 90% of black fly species require a blood meal for egg maturation, but autogenous species (where females do not require blood meal to complete at least one gonotrophic cycle) also exist (Adler and McCreadie, 2002). *Simulium vittatum*, which is used in this study, is autogenous for the first gonotrophic cycle, but requires blood meal for further egg maturation. Probably this aspect made maintenance of laboratory culture simpler—no need of blood feeding for egg production, and the

black flies maintain blood feeder status (see references in which the black flies were used for blood feeding experiments (Mead *et al.*, 2004a; Mead *et al.*, 2004b; Mead *et al.*, 2000; Smith *et al.*, 2009)). Moreover, Brockhouse and Adler (2002) assessed the genetic diversity of this laboratory colony using the specimen collected in 1999 and found that the colony maintain original diversity then at 18 years of continuous culture. The study indicates that the colony may still maintain the original genetic diversity and represents a natural population of the black fly.

Problems caused by black flies

Since some pest species of black flies occur in huge numbers, they can be a nuisance to humans and domestic animals. Their massive attack can even cause exanguination in domestic animals (Adler and McCreadie, 2002).

Black flies are known to vector pathogens. Historically, the most important to humans is the nematode, *Onchocerca volvulus*, the causative agent of “river blindness” (human ocular onchocerciasis). Black flies vector other *Onchocerca* species that cause onchocerciasis of bovines and deer (Adler and McCreadie, 2002; Crosskey, 1993). Nematode worms in the genus *Dirofilaria* are also transmitted by black flies (Adler and McCreadie, 2002; Crosskey, 1993). Other than nematode parasites, black flies vector protozoan parasites, *Leukocytozoon* species, which cause malaria-like diseases in avian hosts, sometimes called “turkey malaria” or “duck fever” (Adler and McCreadie, 2002), and Vesicular Stomatitis Virus (VSV) both biologically (Mead *et al.*, 2004a) and mechanically (Smith *et al.*, 2009).

Not only do black flies transmit pathogens and cause a nuisance, but also they can cause death by their bites, by a host response called simuliotoxicosis (Currie and Adler, 2008). Mechanisms of simuliotoxicosis are not well characterized, but are likely to be caused by a toxic effect of black fly saliva injected upon feeding, which leads to acute toxemia or anaphylactic

shock (Adler and McCreadie, 2002; Currie and Adler, 2008). Records indicate simuliotoxicosis can have an severe impact on livestock; it killed 22,000 animals in 1923 in Europe and thousands of livestock in more than 80 years between 1886 and 1970s on the Canadian prairies (Adler and McCreadie, 2002). Sublethal attacks can also have economic impact by weight loss, reduced milk production, malnutrition etc. (Currie and Adler, 2008).

Study of vector and black fly salivary glands:

Over the past thirty years, there has been an increased interest in studying vector salivary gland components. Initially, efforts were put into characterizing anti-hemostatic components. Subsequently, study of anti-inflammatory or immunomodulatory functions followed. Study of black fly salivary gland components have traced the same path, but the number of publications were significantly more scarce compared to other major vector species (eg., *Aedes aegypti* mosquito). Studies of black fly salivary anti-hemostatic activity can be traced as far back as Yang and Davies (1974), who described delayed plasma clotting time with addition of salivary gland extract (SGE). Subsequent studies determined a few factors that inhibit coagulation and cause vasodilation (Abebe *et al.*, 1995; Abebe *et al.*, 1996; Cupp *et al.*, 1998; Jacobs *et al.*, 1990), but only the vasodilator and anti-thrombin activities have been characterized as far as the cDNA sequence. Moreover, only two studies have been published on the immunomodulatory function of black fly salivary gland components prior to this study (Cross *et al.*, 1994b; Cross *et al.*, 1993). Thus, salivary gland components of black fly have been significantly understudied. However, recent publication of the salivary gland transcriptome coupled with some proteomic analysis (together constituting the sialome) (Andersen *et al.*, 2009) will certainly open the door that has been shut for a long time.

Anti-hemostatic factors in the salivary gland of blood-feeding arthropods:

Hemostasis consists of three complex and redundant processes: platelet aggregation, vasoconstriction and coagulation. Study of salivary gland products of blood-feeding arthropods has identified many anti-hemostatic molecules. Although a small fraction of all blood feeding species have been studied, nearly all studied species have at least one platelet aggregation inhibitor, one anti-coagulant and one hypotensive factor (Ribeiro and Francischetti, 2003).

1. Platelet-aggregation inhibitors:

Platelet-aggregation inhibitors in blood-feeding arthropod saliva

Apyrase activity has been found in the widest variety of blood feeding arthropod studied to date (Ribeiro and Francischetti, 2003). Apyrases are enzymes that hydrolyze ATP and ADP into AMP and orthophosphates. ATP and ADP are released from injured cells into blood. ADP is a potent agonist of platelet aggregation. Therefore, apyrases act primarily as platelet aggregation inhibitors. There are three types of apyrases found from blood feeding arthropod salivary glands: [1] a 5'-nucleotidase family enzyme as in mosquitoes (Champagne *et al.*, 1995) and triatomine bugs (Faudry *et al.*, 2004), [2] a novel type enzyme found in the bed bug *Cimex lectularius* (Valenzuela *et al.*, 1998) and sand flies (Valenzuela *et al.*, 2001) and [3] a CD39 type apyrase in the flea *Xenopsylla cheopis* (Andersen *et al.*, 2007). These are structurally unrelated but have the same function, likely due to convergent evolution to attain anti-platelet function. Interesting to note is that mosquitoes are more closely related to sand flies than to bed bugs, but mosquitoes and sand flies evolved to have different apyrases to gain the same function.

Beside apyrases, inhibitors of collagen-induced platelet aggregation have been characterized in salivary glands of *Ae. aegypti* (aegyptin) and *Anopheles stephensi* (anopheline antiplatelet protein: AAPP) salivary glands (Calvo *et al.*, 2007b; Yoshida *et al.*, 2008). These

have been identified as 30-kDa allergens in mosquito sialomes, for example, Arca *et al.* (2007), Arca *et al.* (2005), Francischetti *et al.* (2002)b, Ribeiro *et al.* (2007), Ribeiro *et al.* (2004).

In addition, other anti-platelet aggregation factors have been described from other vector species. For example, pallidipin is an inhibitor of collagen-mediated platelet-aggregation from *Triatoma pallidipennis* (Noeske-Jungblut *et al.*, 1994), and *Rhodnius prolixus* aggregation inhibitor 1 (RPAI-1) binds to ADP to inhibit platelet aggregation (Francischetti *et al.*, 2000). Both are lipocalin-family proteins, but each acquired different mode of function.

Moreover, direct inhibitors of platelet surface glycoprotein IIb/IIIa (integrin α_{IIb}/β_3) binding to fibrinogen, which activates platelet aggregation, have also found from blood-feeding arthropod saliva (Francischetti, 2009). These inhibitors typically contain an RGD (Arg-Gly-Asp) domain (Francischetti, 2009), but inhibitors that do not contain the RGD domain have also been found. Such an inhibitor found in tabanid salivary glands is actually structurally related to 5' nucleotidase family of apyrases, but apparently lacks the enzyme function (Reddy *et al.*, 2000). Another inhibitor lacking the RGD-domain has been found from the soft tick, *Ornithodoros moubata* and is a Kunitz family protein (Karczewski and Connolly, 1997; Karczewski *et al.*, 1994). For more detailed review of platelet aggregation inhibitors, see the recent review by Francischetti (Francischetti, 2009).

Platelet-aggregation inhibitors in black fly saliva

Black flies also have apyrase activity in their SGE (Cupp *et al.*, 1993). The apyrase activity in black fly salivary glands had been described to be more similar to sand fly apyrases than mosquito apyrases in terms of ADP preference over ATP as substrate (Cupp and Cupp, 1997; Cupp *et al.*, 1993). However, the divalent cation requirement is not restricted to Ca^{2+} like sand fly apyrases, and the transcriptome analysis indicates that the putative apyrase has

significant amino acid sequence identity to mosquito apyrases, but not to sand fly's, implying that functional similarity is convergent due to a similar mode of feeding (Andersen *et al.*, 2009; Cupp and Cupp, 1997).

Homologues of the 30-kDa inhibitors of collagen-induced platelet aggregation have also been found in the black fly sialome (Andersen *et al.*, 2009). It is noteworthy that no homologue was found in sand fly sialomes (Anderson *et al.*, 2006; Kato *et al.*, 2006; Oliveira *et al.*, 2006; Valenzuela *et al.*, 2004). The fact that black flies and mosquitoes have the same type of apyrase and (putative) inhibitor of collagen-induced platelet aggregation supports the hypothesis that mosquitoes and black flies share a common blood-feeding ancestor (Grimaldi and Engel, 2005).

2. Anti-coagulation factors:

Anti-coagulation factors in blood-feeding arthropod saliva

Blood-feeding arthropods have evolved a variety of anti-coagulation factors, probably because there are multiple components in the coagulation pathways. Although many activating and inactivating factors are present in the coagulation system, most inhibitors have been determined to inhibit activation of factor X (FX) or prothrombin, or directly inhibit FXa or thrombin, the factors shared by both the intrinsic and extrinsic pathways.

To briefly explain the coagulation cascade (See Figure 1.1), the extrinsic pathway (also called “tissue factor pathway”) initiates with binding of circulating FVIIa to tissue factor (TF) expressed in subendothelial cells at an injury site to form the ‘tenase’ complex in the presence of a phospholipid surface (likely to be negatively-charged, in case of activated platelets) and Ca^{2+} , which activates FX to its active form FXa (Furie and Furie, 1988; Mann, 1999). FXa activates prothrombin to thrombin, which is the final step enzyme that catalyzes fibrinogen to insoluble fibrin in the presence of Na^{+} (Di Cera, 2008). Thrombin also catalyzes FV and FVIII to active

forms FVa and FVIIIa, respectively. FXa, FVa, phospholipid surface and Ca^{2+} form the prothrombinase complex, which activates prothrombin to thrombin ~300,000 times more efficiently than FXa alone (Francischetti *et al.*, 2009). The intrinsic pathway (also called “contact pathway”) initiates with FXII, which is self-activated upon contact with negatively charged surfaces (Schmaier, 2008). FXIIa activates FXI to FXIa which in turn activates FIX to FIXa. FIXa forms an intrinsic ‘tenase’ complex with FVIIIa, which is activated by a small amount of thrombin present in blood, phospholipid surface and Ca^{2+} . Although the intrinsic and extrinsic pathways are distinct, factors within each pathway can interact to synergistically activate factors in the alternate pathway, especially during the amplification phase. In fact, intrinsic tenase is activated following activation of extrinsic tenase and is fifty times more efficient as an activator of FX (Mann, 1999). Therefore, inhibiting even one factor may slow down significantly formation of the final product, a fibrin clot.

The anticoagulation factors vary even among mosquitoes; culicine mosquitoes belonging to the genus *Aedes* and *Culex* have salivary anti-FXa activity, and the responsible component in *Ae. aegypti* was determined to be a serine protease inhibitor (serpin) (Stark and James, 1995, 1996), while mosquitoes belonging to the genus *Anopheles* do not have anti-FXa activity in their salivary glands but have anti-thrombin activity instead (Stark and James, 1996). The thrombin inhibitor in *Anopheles albimanus* was determined to be a novel peptide, anophelin (Valenzuela *et al.*, 1999). This difference in culicine and anopheline mosquitoes seems to be consistent in other mosquito species according to several mosquito sialomes (Arca *et al.*, 2007; Arca *et al.*, 2005; Calvo *et al.*, 2004; Calvo *et al.*, 2007a; Ribeiro *et al.*, 2007; Ribeiro *et al.*, 2004; Valenzuela *et al.*, 2003).

Triatomine bugs utilize quite different molecules for anti-coagulation. Nitrophorin 2 (NP2) from the kissing bug *Rhodnius prolixus*, which is known as nitric oxide (NO) carrier (see below), also acts as anti-coagulation factor (Ribeiro *et al.*, 1995). NP2 inhibits coagulation by binding to FIX and FIXa to prevent FXa generation by the intrinsic ‘tenase’ complex (Isawa *et al.*, 2000). The intrinsic tenase complex interacts with the extrinsic pathway as well, as FIX and FVIII are activated by the extrinsic tenase complex and thrombin, respectively. Another salivary lipocalin from *Triatoma* species is known to be a thrombin inhibitor (Noeske-Jungblut *et al.*, 1995). This lipocalin, triabin from *Triatoma pallidipennis*, has been determined to specifically inhibit thrombin by binding at the fibrinogen-recognition exosite (exosite I) (Fuentes-Prior *et al.*, 1997).

Both hard and soft ticks utilize a number of Kunitz family protease inhibitors, which may have single or multiple Kunitz domains, for anti-clotting activity (Francischetti *et al.*, 2009). The Kunitz family proteins are well-studied polypeptides whose primary function is inhibition of serine proteases (Ascenzi *et al.*, 2003). A Kunitz domain is quite compact and typically consists of about 60 amino acid residues including 6 cysteines, which form three disulfide bridges. The Kunitz family proteins are found from a variety of organisms ranging from animals and plants to prokaryotes, which implies ancient origin and function in diverse biological systems. For example, a well-characterized tick Kunitz family anti-coagulant is ixolaris from *Ixodes scapularis* (Corral-Rodriguez *et al.*, 2009; Francischetti *et al.*, 2002a; Monteiro *et al.*, 2008; Monteiro *et al.*, 2005). Ixolaris contains two Kunitz domains and binds to the heparin-binding exosite of FX and FXa by the N-terminal Kunitz domain and to FVIIa in FVIIa/TF/FXa complex by the C-terminal Kunitz domain (Corral-Rodriguez *et al.*, 2009; Francischetti *et al.*, 2002a). Another multi-Kunitz domain protein, penthalaris, also described from *I. scapularis* salivary

gland, seemed to have similar mode of action to ixolaris (Corral-Rodriguez *et al.*, 2009; Francischetti *et al.*, 2004). A number of other Kunitz family proteins in tick salivary glands have been identified as anticoagulants, and their mode of action varies quite widely ranging from canonical binding inhibition (by reactive site loop: RSL) to inhibition by C- or N-terminal end (not by RSL) (Corral-Rodriguez *et al.*, 2009). Phylogenetic comparison between hard (family Ixodidae) and soft tick (family Argasidae) proteins showed that the diversification of the inhibitors is lineage specific, indicating that these proteins evolved respective functions after diversification of the two families (Mans *et al.*, 2008a). In addition, a total of 297 proteins containing Kunitz domains, or Kunitz-related proteins, which potentially have anti-coagulation functions have been found from tick sialomes (Francischetti *et al.*, 2009). Interestingly, salivary gland transcriptomes of biting midges (Diptera: Ceratopogonidae; a member of the Culicomorpha) contain transcripts encoding Kunitz family protease inhibitors in their sialomes, which might have an anti-coagulation function (Campbell *et al.*, 2005; Russell *et al.*, 2009).

Metalloproteases found in hard ticks in salivary glands of *Ixodes* species have been demonstrated to hydrolyze fibrin (Decrem *et al.*, 2008; Francischetti *et al.*, 2003). Additional novel proteins from blood-feeding arthropod saliva have been determined to have anti-coagulation properties (example: thrombostatin from the horn fly *Haematobia irritans* (Zhang *et al.*, 2002)), but their mode of action will require functional assays because of the lack of known homologues.

Anti-coagulation factors in black fly saliva

An early study reported that black fly salivary gland homogenate had anti-coagulation activity (Yang and Davies, 1974). Later studies described independent factors that have anti-FXa (Jacobs *et al.*, 1990), anti-thrombin (Abebe *et al.*, 1995) and anti-FV (Abebe *et al.*, 1996)

activities. These studies have been performed using semi-purified salivary gland extract, and only the anti-thrombin (simulidin) has been described to N-terminal sequence (and cDNA sequence in a patent application) (Abebe *et al.*, 1995; Cupp and Cupp, 1998).

The black fly sialome contains a transcript (SV-7) encoding a protein identical to the N-terminal sequence of simulidin (Andersen *et al.*, 2009). SV-7 is classified in the D7/odorant-binding protein family, which includes D7 proteins found in lower Diptera. In mosquitoes, there are long and short forms of D7 proteins. The short form binds to biogenic amines (Calvo *et al.*, 2006; Mans *et al.*, 2007). The long form has two odorant-binding domains, which bind to biogenic amines through the C-terminal domain and cysteinyl leukotrienes through the N-terminal domain (Calvo *et al.*, 2009). Simulidin is similar to short form D7 proteins, but the binding ability is unknown, and the mechanism of anti-thrombin activity has not been elucidated. It may utilize a novel mechanism, as another short D7-like protein (hamadarin) from *Anopheles stephensi* has been described to be an inhibitor of reciprocal activation of FXII and prekallikrein and bradykinin production by binding to FXII, FXIIa and high molecular weight kininogen (Isawa *et al.*, 2002). The black fly sialome contains additional short and long form D7 homologues, with as yet uncharacterized functions, although binding to biogenic amines and cysteinyl leukotrienes is suspected (Andersen *et al.*, 2009).

No sequence information for the black fly anti-FXa and anti-FV is known. In the sialome study, Kunitz-domain containing peptides (SV-66 and SV-170) have been suggested to account for the anti-FXa described in the previous studies (Andersen *et al.*, 2009; Jacobs *et al.*, 1990). As mentioned above, many Kunitz family proteins from tick salivary glands are known to be anti-coagulants. The specific anti-FXa in black fly saliva, however, remains to be elucidated.

3. Hypotensive factors:

Hypotensive factors in blood-feeding arthropod saliva

Hypotensive factors (causing vasodilation or inhibiting vasoconstriction) found from blood-feeding arthropods are also diverse. Within mosquitoes, a tachykinin peptide, sialokinin, is present in the salivary gland of *Ae. aegypti* (Champagne and Ribeiro, 1994), and is not found in any *Anopheles* sialomes (Arca *et al.*, 2005; Calvo *et al.*, 2004; Calvo *et al.*, 2007a; Valenzuela *et al.*, 2003). Vasodilative activity suggestive of a tachykinin peptide is also found in *Ochlerotatus triseriatus* salivary glands (Ribeiro *et al.*, 1994). In *Anopheles* a catechol oxidase/peroxidase was found to have a vasodilatory function by degrading catecholamine vasoconstrictors like norepinephrine (Ribeiro and Nussenzveig, 1993; Ribeiro *et al.*, 1994).

The triatomine bug *Rhodnius prolixus* has heme-containing lipocalin proteins (nitrophorins) that carry the vasodilative gas, NO (Ribeiro *et al.*, 1993). Other triatomine bugs in the genus *Triatoma* do not have the heme-containing lipocalin, but triatomine bugs have many lipocalins in their salivary glands that may sequester vasoconstrictor molecules such as serotonin and norepinephrine (including amine-binding protein in *Rhodnius*) (Andersen *et al.*, 2003; Assumpcao *et al.*, 2008; Kato *et al.*, 2009; Santos *et al.*, 2007). Yet another type of nitrophorin, described from the bed bug *C. lectularius*, is a heme-containing inositol phosphatase, not a lipocalin (Valenzuela *et al.*, 1998; Valenzuela *et al.*, 1995).

Many lipocalins are also found in both hard and soft tick sialomes (Chmelar *et al.*, 2008; Francischetti *et al.*, 2008a; Francischetti *et al.*, 2008b; Francischetti *et al.*, 2005; Mans *et al.*, 2008a; Ribeiro *et al.*, 2006), among which some have been demonstrated to be histamine- and serotonin-binding lipocalins (Mans *et al.*, 2008b; Paesen *et al.*, 2000) and others to be eicosanoid-binding lipocalins (Mans and Ribeiro, 2008a, b). The lipocalins can affect multiple

aspects of hemostasis and inflammation, as the molecules sequestered by them may have multiple functions. For example, moubatin from the soft tick *O. moubata* and a related lipocalin from *O. savignyi* bind to thromboxane A₂, which has both platelet aggregation and vasoconstriction functions (Mans and Ribeiro, 2008b).

Unlike mosquitoes, the New World sand flies in the genus *Lutzomyia* have a novel peptide and one of the most potent vasodilators, maxadilan (MAX) (Lerner *et al.*, 1991). Old World sand flies in the genus *Phlebotomus*, however, lack MAX and have the small molecules adenosine and 5'-AMP (Ribeiro *et al.*, 1999; Ribeiro and Modi, 2001). In addition, novel Kazal domain-containing protein vasodilators have been found from horse flies (Takac *et al.*, 2006; Xu *et al.*, 2008).

Hypotensive factors in black fly saliva

Black fly SGE has been shown to induce rapid and persistent erythema, and the erythema-inducing component from *S. vittatum* was determined to be a protein of a size of 15351 Da (Cupp *et al.*, 1994). This protein was characterized to the genetic level, cloned and expressed as an active peptide (Cupp *et al.*, 1998). This protein, called *S. vittatum* erythema protein or SVEP, is one of the most potent vasodilators known, seems to act on ATP-dependent K⁺ channels, and has no homology to any known proteins (Cupp *et al.*, 1998).

As summarized above, anti-hemostatic factors in blood-feeding arthropod saliva are diverse and seem to exploit every molecular aspect of hemostasis. Therefore, it is expected that additional characterization of saliva in black flies will potentially reveal many more novel anti-hemostatic factors.

Immunomodulatory functions in black fly salivary gland:

Overview of vertebrate immune response:

Vertebrates have a very sophisticated defense mechanism to eliminate invading microorganisms that potentially cause pathology. The arsenal of innate immunity, such as phagocytic cells and complement proteins in plasma, is always ready. The innate immune system recognizes foreign invaders by their surface molecular pattern that is unique and conserved in non-host organisms, such as lipopolysaccharide (LPS) and lipoteichoic acid, by germ-line coded receptors and secreted proteins (Akira and Takeda, 2004; Joiner, 1988; Kumar *et al.*, 2009). The adaptive immune system is very efficient and specific, but requires experience (exposure) to foreign materials that are recognized as “dangerous” to the host (Matzinger, 2002). Like hemostasis, innate and adaptive immune systems are not completely separate entities. Activation occurs reciprocally. Briefly, for example, a phagocytic cell recognizes a microorganism by its surface pattern with cognate receptors (like toll-like receptor 4 (TLR4) with coreceptor CD14 for LPS (Akira and Takeda, 2004; Kumar *et al.*, 2009)) and phagocytose it. The phagocytic cell then processes proteins of the microorganism and presents fragments of the proteins loaded on its surface major histocompatibility complex (MHC) molecules. CD4⁺ T lymphocytes recognize specific protein fragments in the context of MHC class II molecules and are activated to undergo clonal expansion and to express effector functions such as secreting interferon- γ (IFN- γ), which in turn activates phagocytic cells to kill internalized microorganisms by, for example, increasing production of NO (Abbas, 2005). In the case that the pathogen invades host cell, as viruses do, the invaded cell presents fragments of processed proteins on the MHC class I molecules. CD8⁺ T cells recognize the specific peptide-MHC class I complex and are activated for clonal expansion and effector function such as lysing infected cells (Abbas, 2005). Effector CD4⁺ T cells may also

stimulate B cells to produce antibodies efficiently. When antibodies bind to surface proteins on the surface of microorganisms to make an “immune complex” the classical pathway of complement system is activated, which leads to assembly of the membrane attack complex (MAC) on the membrane of the microorganism to cause lysis (Abbas, 2005). In addition, fragments of complement proteins (C3a, C5a) act as chemoattractants and activating factors for granulocytes, mast cells and macrophages (Klos *et al.*, 2009).

While the major function of CD8⁺ T cell is lysis of infected cells, three types of CD4⁺ T helper cell subsets (Th cells), which play major roles in adaptive immunity, have been recognized. The three subsets are Th1, Th2 and Th17 subsets, which will be briefly discussed below.

Th cell subsets are unique in producing distinct sets of cytokines and biological functions. The differentiation of Th subsets is likely manipulated by antigen presenting cells (APCs). Requirements for activation of naive Th cells include: 1) prolonged contact between an APC and a naive Th cell that forms an “immunological synapse”; and 2) secretion of cytokines from APCs (Abbas, 2005). The immunological synapse consists of a complex interaction between receptors, ligands, costimulatory molecules and adhesion molecules. When the activation of APCs is biased to type-1, APCs produce interleukin-18 (IL-18) and IL-12, which promote differentiation of a Th1 cell type. Th1 cells produce interferon- γ (IFN- γ), IL-2, IL-3 and tumor necrosis factor- β (TNF- β) that basically lead to cell-mediated immunity where B cells are activated to produce opsonizing (enhancing phagocytosis) antibody (IgG_{2a} in mice), macrophages are activated to produce oxygen and nitrogen radicals to enhance killing of engulfed invaders, and CD8⁺ T cells are activated for their cytolytic function (Fietta and Delsante, 2009). Th2 cells are induced by APCs secreting IL-4, IL-6 and IL-13, and secrete IL-4, IL-5, IL-13, IL-10 and IL-25 to promote a

humoral response where B cells are activated to switch antibody isotypes to IgE and IgG₁ (in mice), which leads to phagocyte-independent destruction of extracellular invaders; eosinophils, basophils and mast cells are activated to release parasite-destroying mediators (such as histamine and leukotrienes from mast cells) (Fietta and Delsante, 2009). The Th17 subset develops in the presence of transforming growth factor- β (TGF- β), IL-1 β , IL-6 and IL-23 from APCs and secretes IL-17, IL-21 and IL-22, which have functions in limited control of intracellular parasites and crucial roles in eliminating extracellular parasites, specifically gram-negative bacteria, fungi and some protozoa as well as in tissue inflammation and autoimmunity (Fietta and Delsante, 2009). The cytokines related to each Th subset antagonize development or function of other subsets. The Th1 cytokine IFN- γ inhibits development of Th2 phenotype, and Th2 cytokines IL-4, IL-10 and IL-13 antagonize secretion of IL-12 from APCs thus inhibiting Th1 differentiation. Both IFN- γ and IL-4 antagonize Th17, and TGF- β inhibits both Th1 and Th2. The interaction of cytokines in differentiation and inhibition of Th subsets is summarized in Figure 1.2. There is yet another CD4⁺ T cell subpopulation, regulatory T cells (Treg) whose development and functions are complex and extremely important, but they will not be discussed here as no study has been done concerning the effect of blood-feeding arthropod saliva on Treg populations.

T lymphocytes exist in a “naive” state until they are activated by encountering a foreign peptide in the context of MHC molecules with appropriate costimulatory molecules and cytokines as was described already. The activation of T cells is solely dependent on antigen-presenting cells (APCs) that express MHC molecules. Virtually all cell types that express MHC class II molecules can stimulate helper T cells, but researchers found that different cell types stimulate T cells differently. Thus, certain cell types are classified as “professional” APCs. The term “professional APC” typically includes B lymphocytes, macrophages and dendritic cells

(DCs), among which DCs are considered to be the most important cell type that can activate naive T cells to commit and differentiate into effector or memory status (Trombetta and Mellman, 2005). However, macrophages and B cells can also have a role in activating naive T cells (Rodriguez-Pinto, 2005; Trombetta and Mellman, 2005). B cells are efficient in internalizing antigens bound to B-cell receptor (BCR: membrane-bound antibody) and present peptides of the specific epitopes on the MHC class II molecules, which activates Th cells and in turn enhances the antigen producing ability of the B cells (Rodriguez-Pinto, 2005).

Macrophages are also able to activate naive T cells, but are especially important as major effector cells in the innate immune system as they have an exceptional capacity to internalize microbes and antigens (Fujiwara and Kobayashi, 2005; Trombetta and Mellman, 2005).

Macrophages reside in each tissue and are the first immune cell type that pathogens encounter. Upon activation by, for example, pathogen-specific stimuli (e.g., LPS) or T-cell mediated stimuli (e.g., IFN- γ), macrophages enhance their capacity to kill internalized microbes and promote inflammation by secreting proinflammatory mediators, such as TNF- α and IL-12 (Fujiwara and Kobayashi, 2005). This capacity is important for clearing pathogens at the site of infection. After clearing microbes, macrophages also act to resolve the inflammation, for example by secreting the anti-inflammatory cytokines (IL-10 and TGF- β) (Fujiwara and Kobayashi, 2005). Thus, manipulating the function of macrophages could regulate local inflammation.

Immunomodulation by blood-feeding arthropod saliva:

It has been shown that vector SGE or salivary components have immunomodulatory functions (Titus *et al.*, 2006). The most extensive research has been, and continues to be done for ticks and sand flies. For example, saliva of the tick *Ixodes scapularis*, the vector of *Borrelia burgdorferi* (the causative agent of Lyme disease) has been shown to have multiple

immunomodulatory functions. A few examples among many components found in the tick salivary glands are: the anti-complement proteins Isac and Salp20 inhibit the alternative pathway of complement activation (Tyson *et al.*, 2007; Valenzuela *et al.*, 2000); prostaglandin E₂ (PGE₂) inhibits dendritic cell (DC) maturation and function, disrupting activation of antigen-specific CD4⁺ T cell (Sa-Nunes *et al.*, 2007); and Salp15 not only protects *B. burgdorferi* from antibody-mediated killing, but also suppresses T-cell activation through the T cell receptor by binding to the coreceptor CD4, and modifies DC function by interacting with the surface receptor lectin, DC-SIGN (Garg *et al.*, 2006; Hovius *et al.*, 2008; Ramamoorthi *et al.*, 2005).

There is some direct evidence that vector saliva affects transmission of pathogens. A classical example has been observed in the sand fly-*Leishmania*-mouse model. Sand flies (Diptera: Psychodidae) are the vectors of *Leishmania* spp, and *Leishmania* parasites are always co-inoculated with sand fly saliva in natural infection. Thus, the hypothesis has been postulated that sand fly saliva has effects on *Leishmania* infection. In fact, infectivity of *Leishmania major* was significantly greater when co-inoculated in mice with New World sand fly, *Lutzomyia longipalpis*, salivary gland lysate (SGL) than when *L. major* was inoculated alone (Titus and Ribeiro, 1988). These experiments used immunologically naive mice with no prior exposure to *Leishmania* or sand fly saliva (or SGL). However, in nature most hosts would have had a history of exposure to uninfected sand fly bites. In contrast to naive mice, pre-exposure to *L. longipalpis* SGL conferred a partial protection to mice against *L. amazonensis* infection (Thiakaki *et al.*, 2005). Moreover, a particular component in *L. longipalpis* salivary gland, the vasodilator maxadilan (MAX), has been shown to confer protection against *L. major* infection in mice when the mice are vaccinated against MAX (Morris *et al.*, 2001). Similar protection has been observed when using the Old World sand fly *Phlebotomus papatasi* (a natural vector of *L. major*) salivary

gland sonicate (SGS). Pre-exposure with the SGS conferred protection to mice against *L. major* infection (Belkaid *et al.*, 1998). This protection is characterized by biasing the host response to Th1 cytokine secretion and delayed-type hypersensitivity against salivary gland components at the site of infection (Belkaid *et al.*, 1998). As is the case for *L. longipalpis* SGL, a particular component of the salivary gland was determined to confer protection (see below).

A similar phenomenon in tick-borrelia transmission also has been demonstrated. Repeated prior infestation with pathogen-free ticks conferred protection to mice against subsequent *B. burgdorferi* infection by tick bite (Wikel *et al.*, 1997).

Sialomic studies have spurred the determination of salivary components that confer protection against or exacerbation of *L. major* infection. A group utilized a technique called DNA vaccination where recombinant DNA is injected to the host, and the host cells (APCs) encode the DNA into target protein (antigen) and activate antigen-specific adaptive immune responses. Their study using individual salivary components of *P. papatasi* by the DNA vaccination revealed that mice immunized against 15-kDa *P. papatasi* salivary protein (PpSP15) developed protection against subsequent challenge with *L. major* with SGE injection, while mice immunized against another protein, PpSP44, showed exacerbation of infection upon challenge with *L. major* (Oliveira *et al.*, 2008). This observation demonstrated that vector saliva is a complex mixture that contains components which may give opposing effect when used individually.

Mosquitoes are more closely related to black flies than they are to ticks or sand flies, and immunomodulatory effects of their salivary gland components may be more relevant to the black fly system. One of the earliest studies described using rat mast cells (Bissonnette *et al.*, 1993).

Ae. aegypti SGE inhibited secretion of a proinflammatory cytokine, tumor necrosis factor- α (TNF- α), and cytotoxicity of rat peritoneal mast cells, but did not inhibit antigen-induced histamine release *in vitro* (Bissonnette *et al.*, 1993). The factor(s) responsible for the mast cell modulation was determined to be a protein with a molecular weight of > 10 kDa (Bissonnette *et al.*, 1993). Studies using mouse systems report that *Ae. aegypti* SGE generally reduce *in vitro* splenocyte proliferation under various stimulatory conditions (including concanavalin A (Con A), ovalbumin (OVA) or OVA peptide fragment for naive, OVA-primed or OVA peptide-specific T cell transgenic mice (DO11.10)—all in a BALB/c background, respectively) (Cross *et al.*, 1994a; Wasserman *et al.*, 2004). Wanasen *et al.* (2004) conducted similar experiments using a different strain of mice (C3H/HeJ) and observed similar trends. Cytokine release in the splenocyte culture supernatant was also modulated by *Ae. aegypti* SGE. The general trend was the reduction of levels of various cytokines (Cross *et al.*, 1994a; Wanasen *et al.*, 2004; Wasserman *et al.*, 2004). The most comprehensive study has been done by Wasserman *et al.* using OVA-stimulated DO11.10 mouse splenocytes. *Ae. aegypti* SGE reduced *in vitro* secretion of IL-2, IFN- γ , IL-12, granulocyte-macrophages colony stimulation factor (GM-CSF), tumor necrosis factor- α (TNF- α), IL-4, IL-5 and IL-10, although GM-CSF and IL-10 were reduced only with a high concentration of SGE (Wasserman *et al.*, 2004). A study examined the cytokine levels from mice previously bitten (once) by mosquitoes showed decreased levels of Th1 cytokines (IL-2 and IFN- γ) and increased levels of Th2 cytokines (IL-4 and IL-10), and this phenomenon was mimicked by injecting the salivary vasodilator sialokinin (Zeidner *et al.*, 1999). The overall trend of *Ae. aegypti* SGE effects on cytokine release is biased to a Th2 response, which is generally beneficial for intracellular pathogens like viruses. Two groups observed cell death caused by *Ae. aegypti* SGE *in vitro* in splenic CD4⁺ and CD8⁺ cells, to a

much lower degree in splenic B220⁺ B cells, and not in a DC cell line (Wanasen *et al.*, 2004; Wasserman *et al.*, 2004). Wasserman *et al.* also described inhibition of LPS-stimulated splenocyte proliferation (B-cell proliferation) (Wasserman *et al.*, 2004). Recently, effects of *Ae. aegypti* SGE on infection with West Nile Virus (WNV) have been described (Schneider *et al.*, 2007; Schneider *et al.*, 2006). Inoculation of WNV with SGE following mosquito bite increased mouse mortality (Schneider *et al.*, 2006). Coinoculation of SGE with Sindbis virus modified cytokine levels in mouse skin in a manner which indicated biasing toward a Th2 response by increasing IL-4 and IL-10 mRNA levels (Schneider *et al.*, 2004). Moreover, pre-exposure to uninfected *Ae. aegypti* bites enhanced mortality following subsequent WNV infection, which was associated with increased level of IL-10 mRNA in mice (Schneider *et al.*, 2007). On the other hand, non-infective bites of the malaria mosquito *An. stephensi* conferred partial protection against subsequent infection with the mouse malarial parasite *Plasmodium yoelii* by reducing parasite load (Donovan *et al.*, 2007). This protection was associated with increased levels of IFN- γ and IL-12p40 mRNA and decreased levels of IL-4 mRNA at the bite site, liver and spleen (Donovan *et al.*, 2007). Only one salivary component that has specific immunomodulatory function was described recently from *Ae. aegypti* salivary gland (Boppana *et al.*, 2009). This protein, which has been previously annotated as 30 kDa allergen-like protein in the sialome, biased the Th function toward a Th2 phenotype both *in vitro* and *in vivo* (Boppana *et al.*, 2009). This is the first known protein from the mosquito salivary gland that modifies CD4⁺ T cell function. Identifying the component(s) that has functions to modulate host immune responses, however, may be beneficial to study pathogen transmission and develop strategies to block infection.

Immunomodulation by black fly saliva (SGE):

There are only two studies available on the immunomodulatory effects of black fly SGE, both using a BALB/c mouse model. Cross *et al.* described that SGE of the black fly, *S. vittatum* reduced both expression of I-A (mouse MHC class II) in splenocytes from the mice injected with SGE 48 hours prior to the preparation of the cells in comparison to those from the sham protein-injected mice (Cross *et al.*, 1993). However, the difference has not been observed when the splenocytes were incubated with SGE or the sham protein for 48 hours (Cross *et al.*, 1993). Moreover, there was no significant difference in I-A expression in axillary lymph node cells and epidermal cells with or without *in vitro* incubation (Cross *et al.*, 1993). They also observed that SGE reduced proliferation of splenocytes by mitogen stimulation (by LPS or Con A) where the effects were irrespective to priming status (Cross *et al.*, 1993). They further observed that SGE injection resulted in an increase in antibody titer and antibody-secreting cells against sheep erythrocytes (Cross *et al.*, 1993). Another study using BALB/c mice repeatedly injected with SGE (sensitization) described reduced IL-5 and IL-10 secretion by OVA-stimulated *ex vivo* splenocytes from SGE injected mice compared to splenocytes from saline-injected mice (Cross *et al.*, 1994b). The latter study also described a lower percentage of blood eosinophils in SGE-injected mice than those in saline-injected mice (Cross *et al.*, 1994b). The same study further described that pre-exposure of splenocytes from naive mice to black fly SGE reduced *ex vivo* responsiveness to IL-2 and IL-4, and lower IL-4 utilization (indicated by higher residual IL-4 in IL-4-stimulated culture supernatant) (Cross *et al.*, 1994b). The correlation between the low IL-4 utilization and the low IL-4 secretion is unclear.

Goals of this dissertation:

Prior research has demonstrated that black fly (*S. vittatum*) SGE has a variety of antihemostatic and immunomodulatory effects on the vertebrate host, but much remained to be determined about the specific salivary components responsible for these effects, and about effects on specific immune cell types. Better understanding of these properties of SGE could suggest novel means to reduce transmission of the pathogens that are vectored by black flies and identify novel antihemostatic components which may have a potential for therapeutic or research use. Accordingly, I conducted my dissertation research to fulfill the following goals:

Goal 1 (Chapter 2)

Characterize the factors and mechanisms in *S. vittatum* SGE responsible for inhibition of mouse splenocyte proliferation and possible mechanism of the inhibition. To achieve this goal, mouse splenocytes were treated with various concentrations of SGE and stimulated with Con A, and proliferation was assessed by a colorimetric method and flow cytometry. Differential inhibition of CD4⁺ and CD8⁺ T cell subsets was also assessed by flow cytometry.

Characterization of the responsible component(s) was also examined for the size and chemical properties (heat stability and digestion by a protease). As a possible mechanism, induction of apoptosis was hypothesized, and the induction of apoptosis was assessed as follows: mouse splenocytes were incubated with various concentrations of SGE, and size change and the caspase-3 activation were assessed by flow cytometry.

Goal 2 (Chapter 3)

Now that we know that black fly SGE inhibits T cell proliferation and causes apoptosis specifically on T cells using BALB/c mice, which were used in previous studies, it is important to reveal that the inhibition of proliferation occurs with other cell types and other genetic

background. Thus, inhibition of splenocyte proliferation was assessed for Con A and LPS-stimulated culture. C3H/HeN mice, which are considered to show more natural immune response than BALB/c, were used. The proliferation was assessed by ³H-thymidine incorporation. In addition, effects of SGE on phagocytic cells were assessed using peritoneal macrophages. Harvested macrophages were treated with SGE and stimulated with LPS, and release of NO was measured by the Griess method.

Aim 3 (Chapter 4)

In the *S. vittatum* sialome, transcripts encoding Kunitz serine protease inhibitor family proteins were found. To characterize the functions of these proteins as putative coagulation inhibitors, I cloned and expressed recombinant forms of these proteins. I assessed the anticoagulation activity by plasma clotting time using recalcification time assay. I further characterized their active sites by creating point-mutated forms, and assessing activity. To define specificity of the Kunitz protein, the recombinant protein was tested against 15 serine proteases and the IC₅₀ was determined for the proteases that were significantly inhibited.

References:

- Abbas, A.K., 2005. Cellular and Molecular Immunology, 5th ed. Elsevier Saunders, Philadelphia, PA.
- Abebe, M., Cupp, M.S., Champagne, D., Cupp, E.W., 1995. Simulidin: a black fly (*Simulium vittatum*) salivary gland protein with anti-thrombin activity. *Journal of Insect Physiology* 41, 1001-1006.
- Abebe, M., Ribeiro, J.M.C., Cupp, M.S., Cupp, E.W., 1996. Novel anticoagulant from salivary glands of *Simulium vittatum* (Diptera: Simuliidae) inhibits activity of coagulation factor V. *Journal of Medical Entomology* 33, 173-176.
- Adler, P.H., McCreadie, J.W., 2002. Black Flies (*Simuliidae*), in: Mullen, G., Durden, L. (Eds.), *Medical and Veterinary Entomology*. Elsevier Science, San Diego, CA, pp. 185-202.
- Akira, S., Takeda, K., 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4, 499-511.
- Andersen, J.F., Francischetti, I.M., Valenzuela, J.G., Schuck, P., Ribeiro, J.M., 2003. Inhibition of hemostasis by a high affinity biogenic amine-binding protein from the saliva of a blood-feeding insect. *J Biol Chem* 278, 4611-4617.
- Andersen, J.F., Hinnebusch, B.J., Lucas, D.A., Conrads, T.P., Veenstra, T.D., Pham, V.M., Ribeiro, J.M., 2007. An insight into the sialome of the oriental rat flea, *Xenopsylla cheopis* (Rots). *BMC Genomics* 8, 102.
- Andersen, J.F., Pham, V.M., Meng, Z., Champagne, D.E., Ribeiro, J.M., 2009. Insight into the Sialome of the Black Fly, *Simulium vittatum*. *J Proteome Res*.
- Anderson, J.M., Oliveira, F., Kamhawi, S., Mans, B.J., Reynoso, D., Seitz, A.E., Lawyer, P., Garfield, M., Pham, M., Valenzuela, J.G., 2006. Comparative salivary gland transcriptomics of sandfly vectors of visceral leishmaniasis. *BMC Genomics* 7, 52.
- Arca, B., Lombardo, F., Francischetti, I.M., Pham, V.M., Mestres-Simon, M., Andersen, J.F., Ribeiro, J.M., 2007. An insight into the sialome of the adult female mosquito *Aedes albopictus*. *Insect Biochem Mol Biol* 37, 107-127.
- Arca, B., Lombardo, F., Valenzuela, J.G., Francischetti, I.M., Marinotti, O., Coluzzi, M., Ribeiro, J.M., 2005. An updated catalogue of salivary gland transcripts in the adult female mosquito, *Anopheles gambiae*. *J Exp Biol* 208, 3971-3986.
- Ascenzi, P., Bocedi, A., Bolognesi, M., Spallarossa, A., Coletta, M., De Cristofaro, R., Menegatti, E., 2003. The bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor): a milestone protein. *Curr Protein Pept Sci* 4, 231-251.
- Assumpcao, T.C., Francischetti, I.M., Andersen, J.F., Schwarz, A., Santana, J.M., Ribeiro, J.M., 2008. An insight into the sialome of the blood-sucking bug *Triatoma infestans*, a vector of Chagas' disease. *Insect Biochem Mol Biol* 38, 213-232.

- Belkaid, Y., Kamhawi, S., Modi, G., Valenzuela, J., Noben-Trauth, N., Rowton, E., Ribeiro, J., Sacks, D.L., 1998. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. *J Exp Med* 188, 1941-1953.
- Bertone, M.A., Courtney, G.W., Wiegmann, B.M., 2008. Phylogenetics and temporal diversification of the earliest true flies (Insecta: Diptera) based on multiple nuclear genes. *Syst Entomol* 33, 668-687.
- Bissonnette, E.Y., Rossignol, P.A., Befus, A.D., 1993. Extracts of mosquito salivary gland inhibit tumour necrosis factor alpha release from mast cells. *Parasite Immunol* 15, 27-33.
- Boppana, V.D., Thangamani, S., Adler, A.J., Wikel, S.K., 2009. SAAG-4 is a novel mosquito salivary protein that programmes host CD4 T cells to express IL-4. *Parasite Immunol* 31, 287-295.
- Brockhouse, C.L., Adler, P.H., 2002. Cytogenetics of laboratory colonies of simulium vittatum cytospecies IS-7 (Diptera: Simuliidae). *J Med Entomol* 39, 293-297.
- Calvo, E., Andersen, J., Francischetti, I.M., de, L.C.M., deBianchi, A.G., James, A.A., Ribeiro, J.M., Marinotti, O., 2004. The transcriptome of adult female *Anopheles darlingi* salivary glands. *Insect Mol Biol* 13, 73-88.
- Calvo, E., Dao, A., Pham, V.M., Ribeiro, J.M., 2007a. An insight into the sialome of *Anopheles funestus* reveals an emerging pattern in anopheline salivary protein families. *Insect Biochem Mol Biol* 37, 164-175.
- Calvo, E., Mans, B.J., Andersen, J.F., Ribeiro, J.M., 2006. Function and evolution of a mosquito salivary protein family. *J Biol Chem* 281, 1935-1942.
- Calvo, E., Mans, B.J., Ribeiro, J.M., Andersen, J.F., 2009. Multifunctionality and mechanism of ligand binding in a mosquito antiinflammatory protein. *Proc Natl Acad Sci U S A* 106, 3728-3733.
- Calvo, E., Tokumasu, F., Marinotti, O., Villeval, J.L., Ribeiro, J.M., Francischetti, I.M., 2007b. Aegyptin, a novel mosquito salivary gland protein, specifically binds to collagen and prevents its interaction with platelet glycoprotein VI, integrin alpha2beta1, and von Willebrand factor. *J Biol Chem* 282, 26928-26938.
- Campbell, C.L., Vandyke, K.A., Letchworth, G.J., Drolet, B.S., Hanekamp, T., Wilson, W.C., 2005. Midgut and salivary gland transcriptomes of the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae). *Insect Mol Biol* 14, 121-136.
- Champagne, D.E., Ribeiro, J.M., 1994. Sialokinin I and II: vasodilatory tachykinins from the yellow fever mosquito *Aedes aegypti*. *Proc Natl Acad Sci U S A* 91, 138-142.

- Champagne, D.E., Smartt, C.T., Ribeiro, J.M., James, A.A., 1995. The salivary gland-specific apyrase of the mosquito *Aedes aegypti* is a member of the 5'-nucleotidase family. *Proc Natl Acad Sci U S A* 92, 694-698.
- Chmelar, J., Anderson, J.M., Mu, J., Jochim, R.C., Valenzuela, J.G., Kopecky, J., 2008. Insight into the sialome of the castor bean tick, *Ixodes ricinus*. *BMC Genomics* 9, 233.
- Corral-Rodriguez, M.A., Macedo-Ribeiro, S., Barbosa Pereira, P.J., Fuentes-Prior, P., 2009. Tick-derived Kunitz-type inhibitors as antihemostatic factors. *Insect Biochem Mol Biol* 39, 579-595.
- Cross, M.L., Cupp, E.W., Enriquez, F.J., 1994a. Differential modulation of murine cellular immune responses by salivary gland extract of *Aedes aegypti*. *Am J Trop Med Hyg* 51, 690-696.
- Cross, M.L., Cupp, E.W., Enriquez, F.J., 1994b. Modulation of murine cellular immune responses and cytokines by salivary gland extract of the black fly *Simulium vittatum*. *Trop Med Parasitol* 45, 119-124.
- Cross, M.L., Cupp, M.S., Cupp, E.W., Galloway, A.L., Enriquez, F.J., 1993. Modulation of murine immunological responses by salivary gland extract of *Simulium vittatum* (Diptera: Simuliidae). *J Med Entomol* 30, 928-935.
- Crosskey, R.W., 1993. Blackflies (*Simuliidae*), in: Lane, R.P., Crosskey, R.W. (Eds.), *Medical Insects and Arachnids*, 1 ed, New York, NY, pp. 241-287.
- Cupp, E.W., Cupp, M.S., 1997. Black fly (Diptera:Simuliidae) salivary secretions: importance in vector competence and disease. *J Med Entomol* 34, 87-94.
- Cupp, M.S., Cupp, E.W., 1998. Antithrombin protein and DNA sequences from black fly, USA.
- Cupp, M.S., Cupp, E.W., Ramberg, F.B., 1993. Salivary Gland Apyrase in Black Flies (*Simulium vittatum*). *Journal of Insect Physiology* 39, 817-821.
- Cupp, M.S., Ribeiro, J.M., Champagne, D.E., Cupp, E.W., 1998. Analyses of cDNA and recombinant protein for a potent vasoactive protein in saliva of a blood-feeding black fly, *Simulium vittatum*. *J Exp Biol* 201, 1553-1561.
- Cupp, M.S., Ribeiro, J.M., Cupp, E.W., 1994. Vasodilative activity in black fly salivary glands. *Am J Trop Med Hyg* 50, 241-246.
- Currie, D.C., Adler, P.H., 2008. Global diversity of black flies (Diptera : Simuliidae) in freshwater. *Hydrobiologia* 595, 469-475.
- Decrem, Y., Beaufays, J., Blasioli, V., Lahaye, K., Brossard, M., Vanhamme, L., Godfroid, E., 2008. A family of putative metalloproteases in the salivary glands of the tick *Ixodes ricinus*. *FEBS J* 275, 1485-1499.
- Di Cera, E., 2008. Thrombin. *Mol Aspects Med* 29, 203-254.

- Donovan, M.J., Messmore, A.S., Scrafford, D.A., Sacks, D.L., Kamhawi, S., McDowell, M.A., 2007. Uninfected mosquito bites confer protection against infection with malaria parasites. *Infect Immun* 75, 2523-2530.
- Faudry, E., Lozzi, S.P., Santana, J.M., D'Souza-Ault, M., Kieffer, S., Felix, C.R., Ricart, C.A., Sousa, M.V., Vernet, T., Teixeira, A.R., 2004. *Triatoma infestans* apyrases belong to the 5'-nucleotidase family. *J Biol Chem* 279, 19607-19613.
- Fietta, P., Delsante, G., 2009. The effector T helper cell triade. *Riv Biol* 102, 61-74.
- Francischetti, I.M., 2009. Platelet aggregation inhibitors from hematophagous animals. *Toxicon*.
- Francischetti, I.M., Mans, B.J., Meng, Z., Gudderra, N., Veenstra, T.D., Pham, V.M., Ribeiro, J.M., 2008a. An insight into the sialome of the soft tick, *Ornithodoros parkeri*. *Insect Biochem Mol Biol* 38, 1-21.
- Francischetti, I.M., Mather, T.N., Ribeiro, J.M., 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, 869-875.
- Francischetti, I.M., Mather, T.N., Ribeiro, J.M., 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, 886-898.
- Francischetti, I.M., Meng, Z., Mans, B.J., Gudderra, N., Hall, M., Veenstra, T.D., Pham, V.M., Kotsyfakis, M., Ribeiro, J.M., 2008b. An insight into the salivary transcriptome and proteome of the soft tick and vector of epizootic bovine abortion, *Ornithodoros coriaceus*. *J Proteomics* 71, 493-512.
- Francischetti, I.M., My Pham, V., Mans, B.J., Andersen, J.F., Mather, T.N., Lane, R.S., Ribeiro, J.M., 2005. The transcriptome of the salivary glands of the female western black-legged tick *Ixodes pacificus* (Acari: Ixodidae). *Insect Biochem Mol Biol* 35, 1142-1161.
- Francischetti, I.M., Ribeiro, J.M., Champagne, D., Andersen, J., 2000. Purification, cloning, expression, and mechanism of action of a novel platelet aggregation inhibitor from the salivary gland of the blood-sucking bug, *Rhodnius prolixus*. *J Biol Chem* 275, 12639-12650.
- Francischetti, I.M., Sa-Nunes, A., Mans, B.J., Santos, I.M., Ribeiro, J.M., 2009. The role of saliva in tick feeding. *Front Biosci* 14, 2051-2088.
- Francischetti, I.M., Valenzuela, J.G., Andersen, J.F., Mather, T.N., Ribeiro, J.M., 2002a. 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, 3602-3612.
- Francischetti, I.M., Valenzuela, J.G., Pham, V.M., Garfield, M.K., Ribeiro, J.M., 2002b. Toward a catalog for the transcripts and proteins (sialome) from the salivary gland of the malaria vector *Anopheles gambiae*. *J Exp Biol* 205, 2429-2451.

- Friedrich, M., Tautz, D., 1997. Evolution and phylogeny of the Diptera: a molecular phylogenetic analysis using 28S rDNA sequences. *Syst Biol* 46, 674-698.
- Fuentes-Prior, P., Noeske-Jungblut, C., Donner, P., Schleuning, W.D., Huber, R., Bode, W., 1997. Structure of the thrombin complex with triabin, a lipocalin-like exosite-binding inhibitor derived from a triatomine bug. *Proc Natl Acad Sci U S A* 94, 11845-11850.
- Fujiwara, N., Kobayashi, K., 2005. Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy* 4, 281-286.
- Furie, B., Furie, B.C., 1988. The molecular basis of blood coagulation. *Cell* 53, 505-518.
- Garg, R., Juncadella, I.J., Ramamoorthi, N., Ashish, Ananthanarayanan, S.K., Thomas, V., Rincon, M., Krueger, J.K., Fikrig, E., Yengo, C.M., Anguita, J., 2006. Cutting edge: CD4 is the receptor for the tick saliva immunosuppressor, Salp15. *J Immunol* 177, 6579-6583.
- Grimaldi, D., Engel, M.S., 2005. *Evolution of the Insects*. Cambridge University Press, New York.
- Hovius, J.W., de Jong, M.A., den Dunnen, J., Litjens, M., Fikrig, E., van der Poll, T., Gringhuis, S.I., Geijtenbeek, T.B., 2008. Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization. *PLoS Pathog* 4, e31.
- Isawa, H., Yuda, M., Orito, Y., Chinzei, Y., 2002. A mosquito salivary protein inhibits activation of the plasma contact system by binding to factor XII and high molecular weight kininogen. *J Biol Chem* 277, 27651-27658.
- Isawa, H., Yuda, M., Yoneda, K., Chinzei, Y., 2000. The insect salivary protein, prolixin-S, inhibits factor IXa generation and Xase complex formation in the blood coagulation pathway. *J Biol Chem* 275, 6636-6641.
- Jacobs, J.W., Cupp, E.W., Sardana, M., Friedman, P.A., 1990. Isolation and characterization of a coagulation factor Xa inhibitor from black fly salivary glands. *Thromb Haemost* 64, 235-238.
- Joiner, K.A., 1988. Complement evasion by bacteria and parasites. *Annu Rev Microbiol* 42, 201-230.
- Karczewski, J., Connolly, T.M., 1997. The interaction of disagregin with the platelet fibrinogen receptor, glycoprotein IIb-IIIa. *Biochem Biophys Res Commun* 241, 744-748.
- Karczewski, J., Endris, R., Connolly, T.M., 1994. Disagregin is a fibrinogen receptor antagonist lacking the Arg-Gly-Asp sequence from the tick, *Ornithodoros moubata*. *J Biol Chem* 269, 6702-6708.
- Kato, H., Anderson, J.M., Kamhawi, S., Oliveira, F., Lawyer, P.G., Pham, V.M., Sangare, C.S., Samake, S., Sissoko, I., Garfield, M., Sigutova, L., Volf, P., Doumbia, S., Valenzuela, J.G., 2006. High degree of conservancy among secreted salivary gland proteins from two

- geographically distant *Phlebotomus duboscqi* sandflies populations (Mali and Kenya). BMC Genomics 7, 226.
- Kato, H., Jochim, R.C., Sakoda, R., Iwata, H., Gomez, E.A., Valenzuela, J.G., Hashiguchi, Y., 2009. A repertoire of the dominant transcripts from the salivary glands of the blood-sucking bug, *Triatoma dimidiata*, a vector of Chagas disease. Infect Genet Evol.
- Klos, A., Tenner, A.J., Johswich, K.O., Ager, R.R., Reis, E.S., Kohl, J., 2009. The role of the anaphylatoxins in health and disease. Mol Immunol 46, 2753-2766.
- Kumar, H., Kawai, T., Akira, S., 2009. Toll-like receptors and innate immunity. Biochem Biophys Res Commun 388, 621-625.
- Lerner, E.A., Ribeiro, J.M., Nelson, R.J., Lerner, M.R., 1991. Isolation of maxadilan, a potent vasodilatory peptide from the salivary glands of the sand fly *Lutzomyia longipalpis*. J Biol Chem 266, 11234-11236.
- Mann, K.G., 1999. Biochemistry and physiology of blood coagulation. Thromb Haemost 82, 165-174.
- Mans, B.J., Andersen, J.F., Francischetti, I.M., Valenzuela, J.G., Schwan, T.G., Pham, V.M., Garfield, M.K., Hammer, C.H., Ribeiro, J.M., 2008a. Comparative sialomics between hard and soft ticks: implications for the evolution of blood-feeding behavior. Insect Biochem Mol Biol 38, 42-58.
- Mans, B.J., Calvo, E., Ribeiro, J.M., Andersen, J.F., 2007. The crystal structure of D7r4, a salivary biogenic amine-binding protein from the malaria mosquito *Anopheles gambiae*. J Biol Chem 282, 36626-36633.
- Mans, B.J., Ribeiro, J.M., 2008a. A novel clade of cysteinyl leukotriene scavengers in soft ticks. Insect Biochem Mol Biol 38, 862-870.
- Mans, B.J., Ribeiro, J.M., 2008b. Function, mechanism and evolution of the moubatin-clade of soft tick lipocalins. Insect Biochem Mol Biol 38, 841-852.
- Mans, B.J., Ribeiro, J.M., Andersen, J.F., 2008b. Structure, function, and evolution of biogenic amine-binding proteins in soft ticks. J Biol Chem 283, 18721-18733.
- Matzinger, P., 2002. The danger model: a renewed sense of self. Science 296, 301-305.
- Mead, D.G., Gray, E.W., Noblet, R., Murphy, M.D., Howerth, E.W., Stallknecht, D.E., 2004a. Biological transmission of vesicular stomatitis virus (New Jersey serotype) by *Simulium vittatum* (Diptera: Simuliidae) to domestic swine (*Sus scrofa*). J Med Entomol 41, 78-82.
- Mead, D.G., Howerth, E.W., Murphy, M.D., Gray, E.W., Noblet, R., Stallknecht, D.E., 2004b. Black fly involvement in the epidemic transmission of vesicular stomatitis New Jersey virus (Rhabdoviridae: Vesiculovirus). Vector Borne Zoonotic Dis 4, 351-359.

- Mead, D.G., Ramberg, F.B., Besselsen, D.G., Mare, C.J., 2000. Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice. *Science* 287, 485-487.
- Monteiro, R.Q., Rezaie, A.R., Bae, J.S., Calvo, E., Andersen, J.F., Francischetti, I.M., 2008. Ixolaris binding to factor X reveals a precursor state of factor Xa heparin-binding exosite. *Protein Sci* 17, 146-153.
- Monteiro, R.Q., Rezaie, A.R., Ribeiro, J.M., Francischetti, I.M., 2005. Ixolaris: a factor Xa heparin-binding exosite inhibitor. *Biochem J* 387, 871-877.
- Morris, R.V., Shoemaker, C.B., David, J.R., Lanzaro, G.C., Titus, R.G., 2001. Sandfly maxadilan exacerbates infection with *Leishmania major* and vaccinating against it protects against *L. major* infection. *J Immunol* 167, 5226-5230.
- Noeske-Jungblut, C., Haendler, B., Donner, P., Alagon, A., Possani, L., Schleuning, W.D., 1995. Triabin, a highly potent exosite inhibitor of thrombin. *J Biol Chem* 270, 28629-28634.
- Noeske-Jungblut, C., Kratzschmar, J., Haendler, B., Alagon, A., Possani, L., Verhallen, P., Donner, P., Schleuning, W.D., 1994. An inhibitor of collagen-induced platelet aggregation from the saliva of *Triatoma pallidipennis*. *J Biol Chem* 269, 5050-5053.
- Oliveira, F., Kamhawi, S., Seitz, A.E., Pham, V.M., Guigal, P.M., Fischer, L., Ward, J., Valenzuela, J.G., 2006. From transcriptome to immunome: identification of DTH inducing proteins from a *Phlebotomus ariasi* salivary gland cDNA library. *Vaccine* 24, 374-390.
- Oliveira, F., Lawyer, P.G., Kamhawi, S., Valenzuela, J.G., 2008. Immunity to distinct sand fly salivary proteins primes the anti-*Leishmania* immune response towards protection or exacerbation of disease. *PLoS Negl Trop Dis* 2, e226.
- Paesen, G.C., Adams, P.L., Nuttall, P.A., Stuart, D.L., 2000. Tick histamine-binding proteins: lipocalins with a second binding cavity. *Biochim Biophys Acta* 1482, 92-101.
- Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X.F., Fish, D., Anguita, J., Norgard, M.V., Kantor, F.S., Anderson, J.F., Koski, R.A., Fikrig, E., 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature* 436, 573-577.
- Reddy, V.B., Kouniga, K., Mariano, F., Lerner, E.A., 2000. Chrysoptin is a potent glycoprotein IIb/IIIa fibrinogen receptor antagonist present in salivary gland extracts of the deerfly. *J Biol Chem* 275, 15861-15867.
- Ribeiro, J.M., Alarcon-Chaidez, F., Francischetti, I.M., Mans, B.J., Mather, T.N., Valenzuela, J.G., Wikel, S.K., 2006. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol* 36, 111-129.
- Ribeiro, J.M., Arca, B., Lombardo, F., Calvo, E., Phan, V.M., Chandra, P.K., Wikel, S.K., 2007. An annotated catalogue of salivary gland transcripts in the adult female mosquito, *Aedes aegypti*. *BMC Genomics* 8, 6.

- Ribeiro, J.M., Charlab, R., Pham, V.M., Garfield, M., Valenzuela, J.G., 2004. An insight into the salivary transcriptome and proteome of the adult female mosquito *Culex pipiens quinquefasciatus*. *Insect Biochem Mol Biol* 34, 543-563.
- Ribeiro, J.M., Francischetti, I.M., 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol* 48, 73-88.
- Ribeiro, J.M., Hazzard, J.M., Nussenzveig, R.H., Champagne, D.E., Walker, F.A., 1993. Reversible binding of nitric oxide by a salivary heme protein from a bloodsucking insect. *Science* 260, 539-541.
- Ribeiro, J.M., Katz, O., Pannell, L.K., Waitumbi, J., Warburg, A., 1999. Salivary glands of the sand fly *Phlebotomus papatasi* contain pharmacologically active amounts of adenosine and 5'-AMP. *J Exp Biol* 202, 1551-1559.
- Ribeiro, J.M., Modi, G., 2001. The salivary adenosine/AMP content of *Phlebotomus argentipes* Annandale and Brunetti, the main vector of human kala-azar. *J Parasitol* 87, 915-917.
- Ribeiro, J.M., Nussenzveig, R.H., 1993. The salivary catechol oxidase/oxidase activities of the mosquito *Anopheles albimanus*. *J Exp Biol* 179, 273-287.
- Ribeiro, J.M., Nussenzveig, R.H., Tortorella, G., 1994. Salivary vasodilators of *Aedes triseriatus* and *Anopheles gambiae* (Diptera: Culicidae). *J Med Entomol* 31, 747-753.
- Ribeiro, J.M., Schneider, M., Guimaraes, J.A., 1995. Purification and characterization of prolixin S (nitrophorin 2), the salivary anticoagulant of the blood-sucking bug *Rhodnius prolixus*. *Biochem J* 308 (Pt 1), 243-249.
- Rodriguez-Pinto, D., 2005. B cells as antigen presenting cells. *Cell Immunol* 238, 67-75.
- Russell, C.L., Heesom, K.J., Arthur, C.J., Helps, C.R., Mellor, P.S., Day, M.J., Torsteinsdottir, S., Bjornsdottir, T.S., Wilson, A.D., 2009. Identification and isolation of cDNA clones encoding the abundant secreted proteins in the saliva proteome of *Culicoides nubeculosus*. *Insect Mol Biol* 18, 383-393.
- Sa-Nunes, A., Bafica, A., Lucas, D.A., Conrads, T.P., Veenstra, T.D., Andersen, J.F., Mather, T.N., Ribeiro, J.M., Francischetti, I.M., 2007. Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in *Ixodes scapularis* saliva. *J Immunol* 179, 1497-1505.
- Santos, A., Ribeiro, J.M., Lehane, M.J., Gontijo, N.F., Veloso, A.B., Sant'Anna, M.R., Nascimento Araujo, R., Grisard, E.C., Pereira, M.H., 2007. The sialotranscriptome of the blood-sucking bug *Triatoma brasiliensis* (Hemiptera, Triatominae). *Insect Biochem Mol Biol* 37, 702-712.
- Schmaier, A.H., 2008. The elusive physiologic role of Factor XII. *J Clin Invest* 118, 3006-3009.

- Schneider, B.S., McGee, C.E., Jordan, J.M., Stevenson, H.L., Soong, L., Higgs, S., 2007. Prior exposure to uninfected mosquitoes enhances mortality in naturally-transmitted West Nile virus infection. *PLoS One* 2, e1171.
- Schneider, B.S., Soong, L., Girard, Y.A., Campbell, G., Mason, P., Higgs, S., 2006. Potentiation of West Nile encephalitis by mosquito feeding. *Viral Immunol* 19, 74-82.
- Schneider, B.S., Soong, L., Zeidner, N.S., Higgs, S., 2004. *Aedes aegypti* salivary gland extracts modulate anti-viral and TH1/TH2 cytokine responses to sindbis virus infection. *Viral Immunol* 17, 565-573.
- Smith, P.F., Howerth, E.W., Carter, D., Gray, E.W., Noblet, R., Mead, D.G., 2009. Mechanical transmission of vesicular stomatitis New Jersey virus by *Simulium vittatum* (Diptera: Simuliidae) to domestic swine (*Sus scrofa*). *J Med Entomol* 46, 1537-1540.
- Stark, K.R., James, A.A., 1995. A factor Xa-directed anticoagulant from the salivary glands of the yellow fever mosquito *Aedes aegypti*. *Exp Parasitol* 81, 321-331.
- Stark, K.R., James, A.A., 1996. Salivary gland anticoagulants in culicine and anopheline mosquitoes (Diptera: Culicidae). *J Med Entomol* 33, 645-650.
- Takac, P., Nunn, M.A., Meszaros, J., Pechanova, O., Vrbjar, N., Vlasakova, P., Kozanek, M., Kazimirova, M., Hart, G., Nuttall, P.A., Labuda, M., 2006. Vasotab, a vasoactive peptide from horse fly *Hybomitra bimaculata* (Diptera, Tabanidae) salivary glands. *J Exp Biol* 209, 343-352.
- Thiakaki, M., Rohousova, I., Volfova, V., Volf, P., Chang, K.P., Soteriadou, K., 2005. Sand fly specificity of saliva-mediated protective immunity in *Leishmania amazonensis*-BALB/c mouse model. *Microbes Infect* 7, 760-766.
- Titus, R.G., Bishop, J.V., Mejia, J.S., 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, 131-141.
- Titus, R.G., Ribeiro, J.M., 1988. Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. *Science* 239, 1306-1308.
- Trombetta, E.S., Mellman, I., 2005. Cell biology of antigen processing *in vitro* and *in vivo*. *Annu Rev Immunol* 23, 975-1028.
- Tyson, K., Elkins, C., Patterson, H., Fikrig, E., de Silva, A., 2007. Biochemical and functional characterization of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Mol Biol* 16, 469-479.
- Valenzuela, J.G., Belkaid, Y., Rowton, E., Ribeiro, J.M., 2001. The salivary apyrase of the blood-sucking sand fly *Phlebotomus papatasi* belongs to the novel *Cimex* family of apyrases. *J Exp Biol* 204, 229-237.

- Valenzuela, J.G., Charlab, R., Galperin, M.Y., Ribeiro, J.M., 1998. Purification, cloning, and expression of an apyrase from the bed bug *Cimex lectularius*. A new type of nucleotide-binding enzyme. *J Biol Chem* 273, 30583-30590.
- Valenzuela, J.G., Charlab, R., Mather, T.N., Ribeiro, J.M., 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis*. *J Biol Chem* 275, 18717-18723.
- Valenzuela, J.G., Francischetti, I.M., Pham, V.M., Garfield, M.K., Ribeiro, J.M., 2003. Exploring the salivary gland transcriptome and proteome of the *Anopheles stephensi* mosquito. *Insect Biochem Mol Biol* 33, 717-732.
- Valenzuela, J.G., Francischetti, I.M., Ribeiro, J.M., 1999. Purification, cloning, and synthesis of a novel salivary anti-thrombin from the mosquito *Anopheles albimanus*. *Biochemistry* 38, 11209-11215.
- Valenzuela, J.G., Garfield, M., Rowton, E.D., Pham, V.M., 2004. Identification of the most abundant secreted proteins from the salivary glands of the sand fly *Lutzomyia longipalpis*, vector of *Leishmania chagasi*. *J Exp Biol* 207, 3717-3729.
- Valenzuela, J.G., Walker, F.A., Ribeiro, J.M., 1995. A salivary nitrophorin (nitric-oxide-carrying hemoprotein) in the bedbug *Cimex lectularius*. *J Exp Biol* 198, 1519-1526.
- Wanassen, N., Nussenzveig, R.H., Champagne, D.E., Soong, L., Higgs, S., 2004. Differential modulation of murine host immune response by salivary gland extracts from the mosquitoes *Aedes aegypti* and *Culex quinquefasciatus*. *Med Vet Entomol* 18, 191-199.
- Wasserman, H.A., Singh, S., Champagne, D.E., 2004. Saliva of the Yellow Fever mosquito, *Aedes aegypti*, modulates murine lymphocyte function. *Parasite Immunol* 26, 295-306.
- Wikel, S.K., Ramachandra, R.N., Bergman, D.K., Burkot, T.R., Piesman, J., 1997. Infestation with pathogen-free nymphs of the tick *Ixodes scapularis* induces host resistance to transmission of *Borrelia burgdorferi* by ticks. *Infect Immun* 65, 335-338.
- Xu, X., Yang, H., Ma, D., Wu, J., Wang, Y., Song, Y., Wang, X., Lu, Y., Yang, J., Lai, R., 2008. Toward an understanding of the molecular mechanism for successful blood feeding by coupling proteomics analysis with pharmacological testing of horsefly salivary glands. *Mol Cell Proteomics* 7, 582-590.
- Yang, Y.J., Davies, D.M., 1974. The saliva of adult female blackflies (Simuliidae: Diptera). *Can J Zool* 52, 749-753.
- Yeates, D.K., Wiegmann, B.M., 1999. Congruence and controversy: toward a higher-level phylogeny of Diptera. *Annu Rev Entomol* 44, 397-428.
- Yoshida, S., Sudo, T., Niimi, M., Tao, L., Sun, B., Kambayashi, J., Watanabe, H., Luo, E., Matsuoka, H., 2008. Inhibition of collagen-induced platelet aggregation by anopheline antiplatelet protein, a saliva protein from a malaria vector mosquito. *Blood* 111, 2007-2014.

Zeidner, N.S., Higgs, S., Happ, C.M., Beaty, B.J., Miller, B.R., 1999. Mosquito feeding modulates Th1 and Th2 cytokines in flavivirus susceptible mice: an effect mimicked by injection of sialokinins, but not demonstrated in flavivirus resistant mice. *Parasite Immunol* 21, 35-44.

Zhang, D., Cupp, M.S., Cupp, E.W., 2002. Thrombostasin: purification, molecular cloning and expression of a novel anti-thrombin protein from horn fly saliva. *Insect Biochem Mol Biol* 32, 321-330.

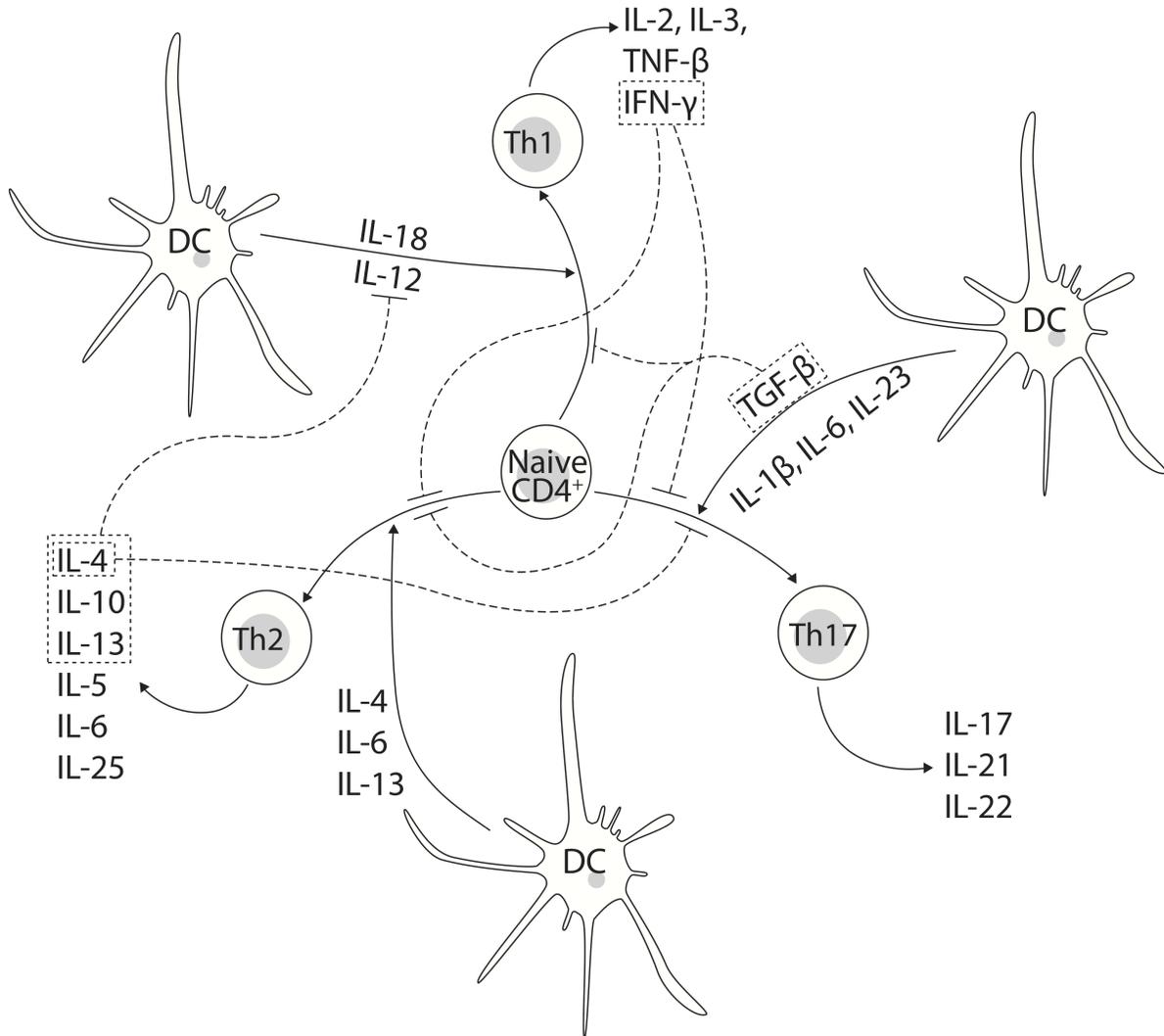


Figure 1.2: Simplified diagram of the cytokine network of Th subset activation and suppression. DC (dendritic cell) is the representative of antigen presenting cells (APCs). Arrows with solid line denote release of cytokine or development of Th subsets, and dashed lines denote inhibition/suppression of Th subset development or cytokine secretion. DC: dendritic cell (represents antigen-presenting cell (APC)). Cell-to-cell contact between DC (APC) and T cells are not described in this diagram. See text for details.

CHAPTER 2

BLACK FLY SALIVARY GLAND EXTRACT INHIBITS PROLIFERATION AND INDUCES APOPTOSIS IN MURINE SPLENOCYTES¹

¹ Tsujimoto, H., Gray, E.W. and Champagne, D.E., 2010, *Parasite Immunology*, 32(4):275-284.
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Summary

Black flies are known to be vectors of pathogens including *Onchocerca volvulus*, which causes human onchocerciasis, and Vesicular Stomatitis Virus. Their salivary secretion has been shown to contain a complex cocktail of anti-haemostatic factors and immunomodulatory activities, which may contribute to efficient transmission of the pathogens. Black fly salivary gland extract (SGE) inhibits mitogen-stimulated mouse splenocyte proliferation, including proliferation of both CD4⁺ and CD8⁺ T cells. The factor responsible for the inhibition was determined to be a protein (or protein complex) of a size larger than 50 kDa. Moreover, exposure to SGE results in activation of caspase 3 and characteristic morphological changes in CD4⁺ and CD8⁺ T cells, suggesting that induction of apoptosis could, at least in part, be responsible for this inhibition.

Keywords: apoptosis, black fly, saliva, T lymphocyte

Introduction

Black flies (Diptera: Simuliidae) are a family of nematoceran flies closely related to mosquitoes and biting midges. Immature stages of black flies require running water, and consequently most adult black flies occur in riparian zones. Not only are adult black flies a nuisance and cause of allergic reactions, but they also can vector viruses, protozoan and helminthic parasites to humans and livestock (Adler and McCreadie, 2002; Crosskey, 1993; Cupp, 1996). Of prominent importance in human and livestock health is that they vector *Onchocerca* spp (including *Onchocerca volvulus*, the causative agent of human onchocerciasis or ‘river blindness’) and Vesicular Stomatitis Virus (VSV) (Cupp, 1996; Mead *et al.*, 2004; Mead *et al.*, 2000). Transmission occurs when black flies create wounds upon blood feeding on a host, and pathogens carried by black flies gain entry to vertebrate hosts through the wound.

When black flies feed on a host's blood, they need to overcome the haemostatic responses, which is accomplished using a salivary secretion containing a pharmacologically active cocktail of anti-haemostatic compounds. To date, several anti-haemostatic factors or activities have been identified from black fly saliva or salivary glands. Initially, black fly salivary glands were shown to contain an inhibitor of activated coagulation factor X (FXa) (Jacobs *et al.*, 1990). Subsequently, an anti-platelet aggregation factor apyrase activity, which hydrolyses the platelet aggregation agonist ADP (and ATP) to AMP and orthophosphate, was described (Cupp *et al.*, 1993). Further study of anti-coagulation activities determined the presence of anti-thrombin and anti-FV activities that are caused by different factors (Abebe *et al.*, 1995; Abebe *et al.*, 1996). Black fly bites are also known to cause a very persistent erythema (Cupp *et al.*, 1994). This was determined to be caused by a potent vasodilator, *Simulium vittatum* erythema protein (SVEP) (Cupp *et al.*, 1998). Moreover, black fly salivary gland contains a hyaluronidase activity that may facilitate dissemination of the saliva around the bite site by breaking down the extracellular matrix component, hyaluronic acid (Ribeiro *et al.*, 2000; Volfova *et al.*, 2008). Transcriptomic analysis combined with proteomic analysis of black fly salivary gland soluble proteins revealed many transcripts and proteins with apparent homology to already described proteins, and in addition many more whose function has not been described, but which have potential anti-haemostatic functions (Andersen *et al.*, 2009).

Black fly salivary glands are also known to contain immunomodulatory activities. Cross *et al.* demonstrated that salivary gland extract (SGE) of the black fly, *S. vittatum*, when injected into mice reduced expression of I-A (mouse MHC class II) in splenocytes (Cross *et al.*, 1993). However, this reduction of I-A expression in splenocytes was only seen on the day of SGE injection. Moreover, in the same study, there was no difference in I-A expression in axillary

lymph node cells and epidermal cells between SGE-injected mice and sham protein-injected mice. Mitogen-stimulated proliferation of splenocytes, using either lipopolysaccharide (LPS) or concanavalin A (Con A), was reduced following SGE injection in SGE-sensitized mice. They also observed that SGE injection resulted in a higher antibody titre and an increased number of antibody-secreting cells following challenge with sheep erythrocytes. A subsequent study using mice repeatedly injected with SGE described reduced IL-5 and IL-10 secretion by *ex vivo* splenocytes, compared with splenocytes from saline-injected mice (Cross *et al.*, 1994). The latter study also described a lower percentage of blood eosinophils, reduced *ex vivo* splenocyte responsiveness to IL-2 and IL-4, and lower IL-4 utilization (based on higher residual IL-4 in IL-4-stimulated culture supernatant) in SGE-injected mice than in saline-injected mice.

There have been no subsequent efforts to characterize the immunomodulatory effects of black fly saliva or SGE. This study has been conducted to characterize further the component(s) responsible for immunomodulation and identify target cell populations. SGE of the Dengue vector mosquito, *Aedes aegypti*, has been shown to have activity to cause cell death in mouse splenocytes (Wanasek *et al.*, 2004; Wasserman *et al.*, 2004) and has the potential activity of causing apoptosis in human peripheral blood mononucleated cells (PBMC) (H Wasserman, unpublished PhD dissertation). Therefore, we tested the hypothesis that black fly SGE is able to induce apoptosis in splenocytes.

Materials and Methods

Mice

BALB/c mice were purchased from National Cancer Institute (NCI), Frederick MD, and reared in the animal care facility in the University of Georgia according to the guidelines of

Institutional Animal Care and Use Committee (IACUC). Female naive mice, 6–14 weeks old, were used for splenocyte isolation.

Black flies

Adult specimens of *S. vittatum* IS-7 were provided from the black fly colony maintained at the University of Georgia. This colony is maintained following the protocol of the Cornell Automated Rearing System (Bernardo and Cupp, 1986; Gray and Noblet, 1999). The system incorporates a closed circulation trough system, where water is pumped from a lower reservoir to upper chambers where it flows over a wooden runway, creating ideal larval habitat. Associated systems have been developed to inject particulate food into the system, capture emerging adults, induce mating and provide suitable substrate for oviposition. The original material for this colony was collected in 1981 near Cambridge, NY, USA, and black flies have been maintained by uninterrupted culture since that time.

Collection of salivary glands

Simulium vittatum is an autogenous species that does not require blood meal for the first gonotrophic cycle, and it has been shown that total protein level is low, and the level of apyrase activity does not increase, until after 3 days following adult emergence (Cupp *et al.*, 1993). Therefore, adult female *S. vittatum* which have oviposited, at which point the salivary glands are ready for blood feeding, were used for salivary gland dissection. The black flies were kept at 4 °C until dissection, when they were chilled on ice, and salivary glands were dissected under a stereomicroscope in sterile HEPES saline (HS) (10 mM HEPES pH 7.0, 150 mM NaCl). Salivary glands were collected in 1.6 mL microcentrifuge tubes with 20 µL HS and stored at –70 °C until use. To isolate SGE, the salivary glands in HS were thawed, sonicated (Branson tissue sonicator, Danbury, CT, USA) at 40% duty cycle with power setting at the microtip limit, while the tube

was submerged in ice water, and centrifuged at 4°C at 14000g for 10 min. The supernatant containing soluble components was transferred to a sterile tube.

Total soluble protein content was assessed by the bicinchoninic acid method using the MicroBCA kit (Pierce, Rockford, IL, USA); an average of about 770 ng of protein was present per pair of salivary glands. Salivary gland extract was prepared from frozen salivary glands immediately before use. Preliminary experiments indicated that freeze/thawing, sonication and centrifugation in sterile buffer resulted in the elimination of culturable bacteria from the SGE without the need for additional filter sterilization, which would entail significant losses of material.

Splenocyte proliferation assay

Splenocytes were isolated from spleens of freshly killed female BALB/c mice. After removal of erythrocytes and counting, the cell suspension was adjusted to 1.25×10^6 cells/mL in RPMI-1640 supplemented with foetal bovine serum (FBS), penicillin/streptomycin, L-glutamine and gentamycin (subsequently referred to as complete RPMI: cRPMI). A volume of 100 μ L of the cell suspension was transferred to each well of a 96-well plate. After incubation of the cells at 37°C and 5% CO₂ in a CO₂ incubator, with various amounts of black fly SGE in 20 μ L of HS for 2 h, cells were stimulated with a final concentration of 5 μ g/mL Con A for an additional 56 h. For controls, cells were treated with HS without black fly SGE for 2 h and stimulated with 5 μ g/mL of Con A for the positive control or without Con A for the negative control. To measure cell proliferation, the nontoxic colorimetric reagent, alamarBlue (AbD Serotec, Raleigh, NC, USA), which has been proven to be useful for detecting proliferation and viability (Al-Nasiry *et al.*, 2007; Kwack and Lynch, 2000; Larson *et al.*, 1997), was used. This method is similar to the colorimetric method using tetrazolium salt, MTT [3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide], which was used in previous studies (Cross *et al.*, 1994; Cross *et al.*, 1993), but the alamarBlue method is much simpler and does not destroy the cells, which makes multiple measurements possible (for example, to determine a time course or optimal time point for an assay) (Al-Nasiry *et al.*, 2007; Kwack and Lynch, 2000). AlamarBlue was added at 56 h of culture, and 24 and 48 h later, the absorbance was measured at 570 and 600 nm, and the per cent reduction of alamarBlue relative to the positive control was calculated using a formula described in the product manual. Experiments were repeated three to five times for each SGE concentration; for each experiment, SGE was assayed in triplicate using splenocytes from a single mouse. AlamarBlue reduction values were compared with the Con A-stimulated positive control by a paired, two-sided t-test, using the statistical software package 'R' (<http://www.r-project.org/index.html>).

Cell division analysis

BALB/c splenocytes were isolated as described above for the splenocyte proliferation assay. Isolated cells were stained with 5 μ M of carboxyfluoresceine diacetate succinimidyl ester (CFDA-SE) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) in PBS at room temperature for 10 min. The staining reaction was stopped by the addition of an equal volume of FBS. The stained cells were washed twice with cRPMI and resuspended in cRPMI, after which the cells were counted and adjusted to the concentration of 5×10^6 cells/mL. A volume of 100 μ L of cell suspension was transferred to each well of a 96-well plate and treated with various concentrations of SGE followed by stimulation by Con A as described in the splenocyte proliferation assay. Controls also were the same as for the proliferation assay. Cells unstained with CFDA-SE also were prepared for use as a staining control for flow cytometry analysis. After 96 h of incubation in a CO₂ incubator, the cells were harvested and washed with cold

staining solution (PBS containing 0.1% BSA and 0.09% NaN₃). Then resuspended cells were incubated with anti-Fc receptor (BD Pharmingen, San Jose, CA, USA, clone 2.4G2), washed and stained with phycoerythrin (PE)-conjugated anti-mouse CD4 (BD Pharmingen, San Jose, CA, USA, clone GK1.5) and allophycocyanin (APC)-conjugated anti-mouse CD8 (BD Pharmingen, clone 53–6.7) antibodies for 20 min. After the final wash, the cells were resuspended in flow cytometry solution and analysed by flow cytometry using a CyAn flow cytometer (Beckman Coulter, Brea, CA, USA).

For the flow cytometric analysis, a gate in the forward- vs. side-scatter (FS vs. SS) plot for a size range where lymphocytes are expected to occur was created for data acquisition, and 15000 events in the gate were collected for each sample. The acquired data were analysed using the FlowJo (TreeStar, Ashland, OR, USA). The intensity of the CFDA-SE signal indicated cell division where the cells with high intensity were undivided, and cells with serially decreased intensity were interpreted as divided.

Experiments were repeated three times; for each experiment, each SGE concentration was assayed in duplicate using splenocytes from a single mouse. AlamarBlue reduction values were compared with the ConA-stimulated positive control using a paired, two-sided t-test.

Treatment of salivary gland extract by boiling and protease digestion

An aliquot of SGE was treated in boiling water for 5 min and the supernatant was collected by centrifugation. Another aliquot of SGE was digested with trypsin (400 µg/mL) (Sigma, Saint Louis, MO, USA) in HS at room temperature for 2 h. This treatment was previously shown to digest a 5 mM solution of the chromogenic substrate Na-Benzoyl-DL-arginine 4-nitroanilide HCl (Sigma) to completion. For comparison, SGE without trypsin was also incubated at room temperature for 2 h. After boiling or trypsin treatment, the treated SGE

were diluted to various concentrations in the same volume of HS before addition to each well for the mouse splenocyte proliferation assay. The activity was assessed using the splenocyte proliferation assay described above. Assays were performed in duplicate and the experiment was replicated twice.

Size estimation of the immunomodulatory factor(s)

To estimate the size of the factor(s) responsible for inhibition of mouse splenocyte proliferation, *S. vittatum* SGE was fractionated by Microcon molecular size cutoff filters (Millipore, Billerica, MA, USA) to generate >100, >50, >30, >10, >3 and <3 kDa fractions. Filtrates (materials passed through the filter) and retentates (materials retained by the filter) were tested for inhibition of Con A-stimulated mouse splenocyte proliferation as described above. Filters were washed prior to use with 500 μ L of HS, as inhibitory activity was detected in the filtrates of HS without SGE produced from unwashed filters. Each retentate/filtrate was tested in triplicate with cells from a single mouse, and the entire experiment was replicated twice with two independent mice. Treatments were compared with the Con A-stimulated positive control using a paired, two-sided t-test.

To confirm that retentates contain reduced amount of proteins smaller than the molecular size cutoff of the filters and that filtrates contain proteins, selected (100 and 50 kDa) retentates and filtrates were analysed by SDS-PAGE with a 4–20% gradient gel (Lonza, Basel, Switzerland) stained by Coomassie brilliant blue.

Apoptosis analysis

BALB/c splenocytes were prepared as described above. The concentration of the cell suspension was adjusted to 5×10^6 cells/mL and 100 μ L of the suspension was transferred to each well of a 96-well plate. The cells were treated with various concentrations of SGE in the

same volume of HS. For negative and positive controls, HS without SGE or HS containing dexamethasone (10 μ M final concentration), respectively, were used.

To detect caspase 3 activation, the fluorophore-labelled caspase 3 substrate analogue, PhiPhiLux (PPL)-G₁D₂ (OncoImmunit, Inc., Geithersburg, MD, USA), was used. Caspase 3 is an ‘executioner protease’ in apoptosis, which exists as an inactive proenzyme in cells and is activated by other caspases. Upon activation, caspase 3 cleaves the PPL-G₁D₂ substrate and the substrate fluoresces. This detection was performed as described in the ‘Application Note #1’ for the product with modification for surface marker staining. Briefly, after 5 h of incubation with the SGE or control reagent in a 96-well plate in a CO₂ incubator, cells were harvested in 1.6 mL microcentrifuge tubes, washed with PBS and incubated in the presence of anti-Fc receptor antibody in PBS for 20 min on ice. Then the cells were washed with PBS and resuspended in PPL-G₁D₂ substrate solution with FBS and incubated at 37°C for 45 min in the dark. The tubes were placed on ice, and 50 μ L of antibody staining solution containing PE-conjugated anti-mouse CD4 and APC-conjugated anti-mouse CD8 antibodies was added to each tube. After incubation on ice for 20 min, the cells were washed, resuspended in flow cytometry solution and analysed by flow cytometry. Propidium iodide (PI; 200 ng/mL final concentration) was also added prior to flow cytometry analysis to exclude dead cells.

For the flow cytometric analysis, a gate in the forward- and side-scatter (FS vs. SS) plot was set to include the region occupied by lymphocytes, and 15000 events in the gate were collected for each sample. The acquired data were analysed using the FlowJo (TreeStar, Ashland, OR, USA). Fluorescence of the PPL-G₁D₂ substrate indicates that it has been cleaved by activated caspase 3. The cells were also differentiated into T-cell subsets by binding anti-CD4 and anti-CD8 antibodies.

Apoptotic cells decrease in cell size, whereas necrotic cells increase in cell size because of osmotic pressure following loss of plasma membrane integrity. The FS and SS plot of flow cytometry indicates size (FS) and intracellular complexity (SS). Therefore, the size analysis could be performed along with caspase 3-activation analysis. A morphological change (i.e. decrease in size, detected as a shift to the left in the FS), together with activation of caspase 3, was interpreted as evidence of apoptosis.

Each experiment was performed with duplicate samples, and the entire experiment was replicated twice with two independent mice.

Results

Inhibition of splenocyte proliferation

Initially, the effect of *S. vittatum* SGE on Con A stimulation of naive BALB/c mouse splenocyte proliferation was assessed. Con A stimulates T lymphocyte proliferation in a manner independent of T-cell epitope specificity. Proliferation was detected using the colorimetric reagent, alamarBlue, which is reduced during the cells' oxidative respiration. As shown in Figure 2.1, *S. vittatum* SGE inhibited splenocyte proliferation in a dose-dependent manner. Splenocytes exposed to SGE equivalent to 0.5 gland pairs/well were significantly inhibited, and SGE at 3 and 5 pairs/well almost completely inhibited the proliferation. The concentration of SGE needed for 50% reduction in proliferation was between 0.75 and 1 gland pairs/well.

To confirm the inhibition of proliferation, and to determine the effect of SGE on T-cell subtypes, the effect of SGE on cell division was assessed by flow cytometry using a fluorescent dye, CFDA-SE or CFSE. Cell division can be detected by dilution of the dye, seen as reduction (i.e. a shift to the left) in fluorescence intensity as labelled cells divide. Figure 2.2(a) and 2.2(b) shows that SGE inhibited cell division of Con A-stimulated mouse splenocytes, with almost

complete inhibition at 3 pairs/well of black fly SGE. SGE inhibited proliferation of both CD4⁺ and CD8⁺ cells to an equal extent.

Characterization of the inhibitory factor

To test the hypothesis that the factor in black fly SGE responsible for the inhibition of splenocyte proliferation is a protein, SGE was boiled (for 5 min) or digested by the proteolytic enzyme, trypsin. Activity of the treated SGE was assessed by splenocyte proliferation assay. Boiling and protease digestion abolished the inhibitory activity (Figure 2.3), which suggests that the component responsible for the inhibition of splenocyte proliferation is a protein that is not heat-stable.

The size of the inhibitory factor was estimated by fractionating the SGE with molecular size cutoff filters, and testing filtrates and retentates by the splenocyte proliferation assay. Significant inhibition of Con A-stimulated splenocyte proliferation was observed for all the retentates, and no inhibition was observed for all the filtrates (Figure 2.4(a)). However, the inhibitory activity of the 100 kDa filter retentate tended to be slightly less, and more variable between experiments, than the other retentates, although not significantly so. SDS-PAGE analysis of the 100 and 50 kDa filtrates and retentates revealed that the former retained a significant proportion of the proteins between 50 and 100 kDa (Figure 4.4(b)). A few proteins that are even smaller than 50 kDa are also more abundant in the retentate, which suggests that in their native state, these proteins may form homo- or heteromeric complexes large enough to be retained by the 100 kDa filter. These results suggest that the component (or components) responsible for inhibition of splenocyte proliferation is larger than 50 kDa, and may be closer to 100 kDa.

Mechanism of inhibition – apoptosis

Mouse splenocytes were treated with varying amounts of black fly SGE or, in the case of the positive control, the pro-apoptotic reagent dexamethasone (10 μ M) for 5 h, after which they were pulsed with the fluorogenic caspase 3 substrate, PPL-G₁D₂, stained with anti-CD4 and anti-CD8 antibodies, and analysed by flow cytometry. The initial size gating (forward-scatter vs. side-scatter: FS vs. SS) revealed two distinct populations that differed in size (Figure 2.5(a)). Apoptotic cells are known to shrink, whereas necrotic cells swell because of osmotic pressure caused by loss of plasma membrane integrity. The percentage of the smaller population was increased in the positive control, and progressively more so in the SGE-treated splenocytes in comparison with the negative control (Figure 2.5(a)).

When cells in the lymphocyte gate were analysed, PPL fluorescence of both CD4-positive cells and CD8-positive cells was significantly increased, indicating cleavage of the substrate because of caspase 3 activation (Figure 2.5(b)). In addition, CD4-, CD8-double negative cells were not affected (Figure 2.5(b)). This result suggests that black fly SGE selectively induces apoptosis specifically in CD4- and CD8-positive T lymphocytes.

When ‘small’ and ‘large’ populations were analysed separately for PPL fluorescence, about 90% of ‘small’ cells were PPL fluorescence positive, while only 7% of ‘large’ cells were PPL fluorescence positive (not shown). This result supports the conclusion that the smaller cells are undergoing apoptosis.

Discussion

It has long been known that black fly SGE has immunomodulatory functions (Cross *et al.*, 1994; Cross *et al.*, 1993), including the inhibition of the polyclonal activation of T-cell proliferation by the mitogen Con A. This study replicates (Figure 2.1) and extends this

observation. It also indicates that the effect may occur in immunologically naive hosts, and not only in sensitized hosts as was shown previously (Cross *et al.*, 1994).

The inhibition of splenocyte proliferation by black fly SGE was confirmed by flow cytometry analysis using the fluorescent dye CFDA-SE. The dye is incorporated in live cells, and is diluted by half with each cell division, allowing direct assessment of the extent of cellular proliferation by flow cytometry analysis. This approach allowed us to extend significantly the observation that black fly SGE inhibits Con A-simulated proliferation, by simultaneously labelling splenocytes for the T-cell subset markers, CD4 and CD8. Black fly SGE inhibited proliferation of both CD4⁺ (helper T cells) and CD8⁺ (cytotoxic T cells), with a similar reduction at one salivary gland pair (SGP)/well and complete inhibition at three and five SGP/well, which suggests that CD4⁺ and CD8⁺ T-cell subpopulations are equally susceptible to the effects of black fly SGE (Figure 2.2(a) and 2.2(b)). These results imply that black fly saliva will have inhibitory effects on T lymphocytes in the vicinity of the bite, where the concentration of injected saliva is relatively high. These results also confirm that black fly SGE inhibits splenocyte proliferation, and that our alamarBlue assay (or the MTT assay of Cross *et al.* (1993)) did not just detect a difference between non-dividing activated cells and resting cells.

As the nature of the salivary component responsible for the inhibition remains unknown, we tested the hypothesis that the inhibitor is a protein. The inhibitory activity was abolished by boiling and by trypsin digestion, which confirms the polypeptide nature of the inhibitor. Molecular size cutoff filters were used to fractionate the SGE into a series of size ranges. Bioassay of retentates indicated that activity was completely retained by the 50 kDa filter and smaller size classes; activity was only slightly (and not significantly) reduced only with the 100 kDa filter. SDS-PAGE showed that even the 100 kDa filter strongly enriched proteins larger than

50 kDa. This result could indicate that many salivary proteins exist in multimeric complexes, large enough to be retained by the 100 kDa filter, in their native state. Taken together, these results indicate that the component(s) in black fly SGE that is responsible for inhibition of mouse splenocyte proliferation is a relatively large (>50kDa, and probably 100 kDa in its native conformation) heat-unstable protein or protein complex. The size estimation might not be indicative of a single unit polypeptide as the protein could be homo- or hetero-multimeric in native conformation. Furthermore, post-translational modification of the inhibitory factor could contribute to its apparent large size, although it is premature to make any inference on this point. Identification of the factor in more detail needs to be elucidated.

As one potential mechanism of T-cell proliferation inhibition, induction of apoptosis in mouse splenocytes by black fly SGE was examined. SGE of the Yellow Fever mosquito, *Ae. aegypti*, was shown to cause cell death in mouse splenocytes (Wanasen *et al.*, 2004; Wasserman *et al.*, 2004) and apoptosis in human peripheral blood mononuclear cells (H. Wasserman, unpublished PhD dissertation). Mosquitoes and black flies belong to the same infraorder, Culicomorpha (Beckenbach and Borkent, 2003; Fallon and Li, 2007; Miller *et al.*, 1997; Yeates and Wiegmann, 1999), and it has been proposed that the Culicomorpha share a common blood-feeding ancestor (Grimaldi and Engel, 2005). Indeed, several families of salivary proteins are shared between mosquitoes and black flies (Andersen *et al.*, 2009), which suggested that the immunomodulatory mechanisms may also be shared. Black fly SGE-induced caspase 3 activation and reduction in cell size was observed, but only in CD4⁺ and CD8⁺ splenocytes and not in the cells without CD4 and CD8 expression (Figure 2.5(a)-2.5(b)). These observations suggest that black fly SGE induces apoptosis specifically in mouse spleen-derived CD4⁺ and CD8⁺ cells, which are likely to be T lymphocytes. These results strongly suggest that black fly saliva inhibits

T-cell activation by inducing apoptosis in the vicinity of the bite site, perhaps in both naive and black fly bite sensitized hosts. However, even the highest concentration of SGE tested (6 pairs/well) did not induce caspase 3 activation in the entire CD4⁺ or CD8⁺ population (89.5% and 82.8% maximum, respectively), although almost no cell division was observed at lower concentrations of SGE (Figure 2.2(a) and 2.2(b)), indicating that there may be other mechanisms contributing to inhibition of T-cell proliferation. The concentration of saliva to which cells are exposed will be very high in the immediate vicinity of the bite, and hence the pro-apoptotic effect of saliva at 3 and 6 pairs/well is likely to reflect conditions at the bite site.

Blood-feeding insects in general appear to have the ability to modulate host immune responses, in addition to haemostatic responses, through the action of salivary components. In addition to the effect of *Ae. aegypti* saliva already discussed, an immunomodulatory function has been described for saliva of phlebotomine sandflies, *Culicoides sonorensis* (Bishop *et al.*, 2006), *Rhodnius prolixus* (Kalvachova *et al.*, 1999), and the stable fly *Stomoxys calcitrans* (Swist *et al.*, 2002). The specific immunomodulatory component is known for sand flies in the genus *Lutzomyia*, where the activity is due to the peptide maxadilan, which interacts with PAC1 receptors on macrophages to modulate secretion of proinflammatory cytokines and the synthesis of nitric oxide. *Aedes aegypti* saliva contains a 30 kDa protein, SAAG-4, that biases T cells towards the production of Th2 cytokines (Boppana *et al.*, 2009). At least some of the activity of *R. prolixus* saliva may be due to lysophosphatidylcholine, which inhibits TNF- α secretion (Golodne *et al.*, 2003). Additional undescribed immunomodulatory components are present in *Ae. aegypti* saliva, as SAAG4 does not account for the inhibition of T- and B-cell proliferation, and the active component in *C. sonorensis* saliva is an as yet unidentified 68 kDa glycoprotein (Bishop *et al.*, 2006). In the case of the *S. vittatum* immunomodulatory activity described here,

despite the recent publication of the salivary gland transcriptome, no obvious candidate cDNAs encoding proteins with homology to known immunomodulatory or pro-apoptotic proteins were identified. Identification of the specific salivary component responsible for the activity will require further experimentation.

Overall, this study showed that black fly SGE has effects that modify mouse immune responses. In particular, this study showed for the first time that vector SGE induces apoptosis specifically in mouse T lymphocytes. This effect of black fly SGE might be expected to suppress induction of adaptive immune responses in the host, particularly at the bite site, leading to efficient pathogen transmission. Nevertheless, there may be additional mechanisms that are responsible for inhibition of T-cell proliferation because not all the T cells were induced to become apoptotic. Moreover, the cells that do not express CD4 or CD8 were unaffected by the apoptosis-inducing activity of black fly SGE. Such cells include B lymphocytes, macrophages, dendritic cells and other leucocytes. Study of such cells, especially cell types more abundant at the actual bite site (e.g. dendritic cells), may reveal other immunomodulatory functions of black fly SGE or saliva. The presence of multiple immunomodulatory functions in the saliva of a single species of vector is well known in ixodid ticks, which possesses anticomplement, suppression of T-cell activation and dendritic cell-modifying activities (Garg *et al.*, 2006; Hovius *et al.*, 2008; Sa-Nunes *et al.*, 2007; Tyson *et al.*, 2007; Valenzuela *et al.*, 2000). The modulation of immune cells by black fly SGE shown in this study implies that black fly saliva has a role in efficient transmission of pathogens. Further study of this activity may provide novel approaches to prevent the transmission of pathogens by these vectors.

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References

- Abebe, M., Cupp, M.S., Champagne, D., Cupp, E.W., 1995. Simulidin: a black fly (*Simulium vittatum*) salivary gland protein with anti-thrombin activity. *Journal of Insect Physiology* 41, 1001-1006.
- Abebe, M., Ribeiro, J.M.C., Cupp, M.S., Cupp, E.W., 1996. Novel anticoagulant from salivary glands of *Simulium vittatum* (Diptera: Simuliidae) inhibits activity of coagulation factor V. *Journal of Medical Entomology* 33, 173-176.
- Adler, P.H., McCreadie, J.W., 2002. Black Flies (*Simuliidae*), in: Mullen, G., Durden, L. (Eds.), *Medical and Veterinary Entomology*. Elsevier Science, San Diego, CA, pp. 185-202.
- Al-Nasiry, S., Geusens, N., Hanssens, M., Luyten, C., Pijnenborg, R., 2007. The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. *Hum Reprod* 22, 1304-1309.
- Andersen, J.F., Pham, V.M., Meng, Z., Champagne, D.E., Ribeiro, J.M., 2009. Insight into the Sialome of the Black Fly, *Simulium vittatum*. *J Proteome Res*.
- Beckenbach, A.T., Borkent, A., 2003. Molecular analysis of the biting midges (Diptera: Ceratopogonidae), based on mitochondrial cytochrome oxidase subunit 2. *Mol Phylogenet Evol* 27, 21-35.
- Bernardo, M.J., Cupp, E.W., 1986. Rearing black flies (Diptera: Simuliidae) in the laboratory: mass-scale *in vitro* membrane feeding and its application to collection of saliva and to parasitological and repellent studies. *J Med Entomol* 23, 666-679.
- Bishop, J.V., Mejia, J.S., Perez de Leon, A.A., Tabachnick, W.J., Titus, R.G., 2006. Salivary gland extracts of *Culicoides sonorensis* inhibit murine lymphocyte proliferation and no production by macrophages. *Am J Trop Med Hyg* 75, 532-536.
- Boppana, V.D., Thangamani, S., Adler, A.J., Wikel, S.K., 2009. SAAG-4 is a novel mosquito salivary protein that programmes host CD4 T cells to express IL-4. *Parasite Immunol* 31, 287-295.
- Cross, M.L., Cupp, E.W., Enriquez, F.J., 1994. Modulation of murine cellular immune responses and cytokines by salivary gland extract of the black fly *Simulium vittatum*. *Trop Med Parasitol* 45, 119-124.
- Cross, M.L., Cupp, M.S., Cupp, E.W., Galloway, A.L., Enriquez, F.J., 1993. Modulation of murine immunological responses by salivary gland extract of *Simulium vittatum* (Diptera: Simuliidae). *J Med Entomol* 30, 928-935.
- Crosskey, R.W., 1993. Blackflies (*Simuliidae*), in: Lane, R.P., Crosskey, R.W. (Eds.), *Medical Insects and Arachnids*, 1 ed, New York, NY, pp. 241-287.

- Cupp, E.W., 1996. Black flies and the agents they transmit, in: Beaty, B.J., Marquardt, W.C. (Eds.), *The biology of disease vectors*, 1 ed. University Press of Colorado, Niwot, CO, pp. 98-109.
- Cupp, M.S., Cupp, E.W., Ramberg, F.B., 1993. Salivary Gland Apyrase in Black Flies (*Simulium vittatum*). *Journal of Insect Physiology* 39, 817-821.
- Cupp, M.S., Ribeiro, J.M., Champagne, D.E., Cupp, E.W., 1998. Analyses of cDNA and recombinant protein for a potent vasoactive protein in saliva of a blood-feeding black fly, *Simulium vittatum*. *J Exp Biol* 201, 1553-1561.
- Cupp, M.S., Ribeiro, J.M., Cupp, E.W., 1994. Vasodilative activity in black fly salivary glands. *Am J Trop Med Hyg* 50, 241-246.
- Fallon, A.M., Li, L., 2007. The C-terminal extension that characterizes mosquito (Diptera: Culicidae) ribosomal protein S6 is widespread among the Culicomorpha. *J Med Entomol* 44, 608-616.
- Garg, R., Juncadella, I.J., Ramamoorthi, N., Ashish, Ananthanarayanan, S.K., Thomas, V., Rincon, M., Krueger, J.K., Fikrig, E., Yengo, C.M., Anguita, J., 2006. Cutting edge: CD4 is the receptor for the tick saliva immunosuppressor, Salp15. *J Immunol* 177, 6579-6583.
- Golodne, D.M., Monteiro, R.Q., Graca-Souza, A.V., Silva-Neto, M.A., Atella, G.C., 2003. Lysophosphatidylcholine acts as an anti-hemostatic molecule in the saliva of the blood-sucking bug *Rhodnius prolixus*. *J Biol Chem* 278, 27766-27771.
- Gray, E.W., Noblet, R., 1999. Laboratory Rearing of Black Flies, in: Maramorosch, K., Mahmood, F. (Eds.), *Maintenance of Human, Animal and Plant Pathogen Vectors*. Oxford & IBH Publishing Co. PVT. LTD., New Delhi and Calcutta.
- Grimaldi, D., Engel, M.S., 2005. *Evolution of the Insects*. Cambridge University Press, New York.
- Hovius, J.W., de Jong, M.A., den Dunnen, J., Litjens, M., Fikrig, E., van der Poll, T., Gringhuis, S.I., Geijtenbeek, T.B., 2008. Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization. *PLoS Pathog* 4, e31.
- Jacobs, J.W., Cupp, E.W., Sardana, M., Friedman, P.A., 1990. Isolation and characterization of a coagulation factor Xa inhibitor from black fly salivary glands. *Thromb Haemost* 64, 235-238.
- Kalvachova, P., Hribalova, V., Kodym, P., Volf, P., 1999. Modulation of murine lymphocyte responsiveness by the saliva of *Rhodnius prolixus* (Hemiptera: Reduviidae). *J Med Entomol* 36, 341-344.
- Kwack, K., Lynch, R.G., 2000. A new non-radioactive method for IL-2 bioassay. *Mol Cells* 10, 575-578.

- Larson, E.M., Doughman, D.J., Gregerson, D.S., Obritsch, W.F., 1997. A new, simple, nonradioactive, nontoxic *in vitro* assay to monitor corneal endothelial cell viability. *Invest Ophthalmol Vis Sci* 38, 1929-1933.
- Mead, D.G., Howerth, E.W., Murphy, M.D., Gray, E.W., Noblet, R., Stallknecht, D.E., 2004. Black fly involvement in the epidemic transmission of vesicular stomatitis New Jersey virus (Rhabdoviridae: Vesiculovirus). *Vector Borne Zoonotic Dis* 4, 351-359.
- Mead, D.G., Ramberg, F.B., Besselsen, D.G., Mare, C.J., 2000. Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice. *Science* 287, 485-487.
- Miller, B.R., Crabtree, M.B., Savage, H.M., 1997. Phylogenetic relationships of the Culicomorpha inferred from 18S and 5.8S ribosomal DNA sequences. (Diptera:Nematocera). *Insect Mol Biol* 6, 105-114.
- Ribeiro, J.M., Charlab, R., Rowton, E.D., Cupp, E.W., 2000. *Simulium vittatum* (Diptera: Simuliidae) and *Lutzomyia longipalpis* (Diptera: Psychodidae) salivary gland hyaluronidase activity. *J Med Entomol* 37, 743-747.
- Sa-Nunes, A., Bafica, A., Lucas, D.A., Conrads, T.P., Veenstra, T.D., Andersen, J.F., Mather, T.N., Ribeiro, J.M., Francischetti, I.M., 2007. Prostaglandin E2 is a major inhibitor of dendritic cell maturation and function in *Ixodes scapularis* saliva. *J Immunol* 179, 1497-1505.
- Swist, S.L., Wilkerson, M.J., Wyatt, C.R., Broce, A.B., Kanost, M.R., 2002. Modulation of bovine lymphocyte response by salivary gland extracts of the stable fly, *Stomoxys calcitrans* (Diptera: Muscidae). *J Med Entomol* 39, 900-907.
- Tyson, K., Elkins, C., Patterson, H., Fikrig, E., de Silva, A., 2007. Biochemical and functional characterization of Salp20, an *Ixodes scapularis* tick salivary protein that inhibits the complement pathway. *Insect Mol Biol* 16, 469-479.
- Valenzuela, J.G., Charlab, R., Mather, T.N., Ribeiro, J.M., 2000. Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, *Ixodes scapularis*. *J Biol Chem* 275, 18717-18723.
- Volfova, V., Hostomska, J., Cerny, M., Votypka, J., Volf, P., 2008. Hyaluronidase of bloodsucking insects and its enhancing effect on leishmania infection in mice. *PLoS Negl Trop Dis* 2, e294.
- Wanasen, N., Nussenzveig, R.H., Champagne, D.E., Soong, L., Higgs, S., 2004. Differential modulation of murine host immune response by salivary gland extracts from the mosquitoes *Aedes aegypti* and *Culex quinquefasciatus*. *Med Vet Entomol* 18, 191-199.
- Wasserman, H.A., Singh, S., Champagne, D.E., 2004. Saliva of the Yellow Fever mosquito, *Aedes aegypti*, modulates murine lymphocyte function. *Parasite Immunol* 26, 295-306.

Yeates, D.K., Wiegmann, B.M., 1999. Congruence and controversy: toward a higher-level phylogeny of Diptera. *Annu Rev Entomol* 44, 397-428.

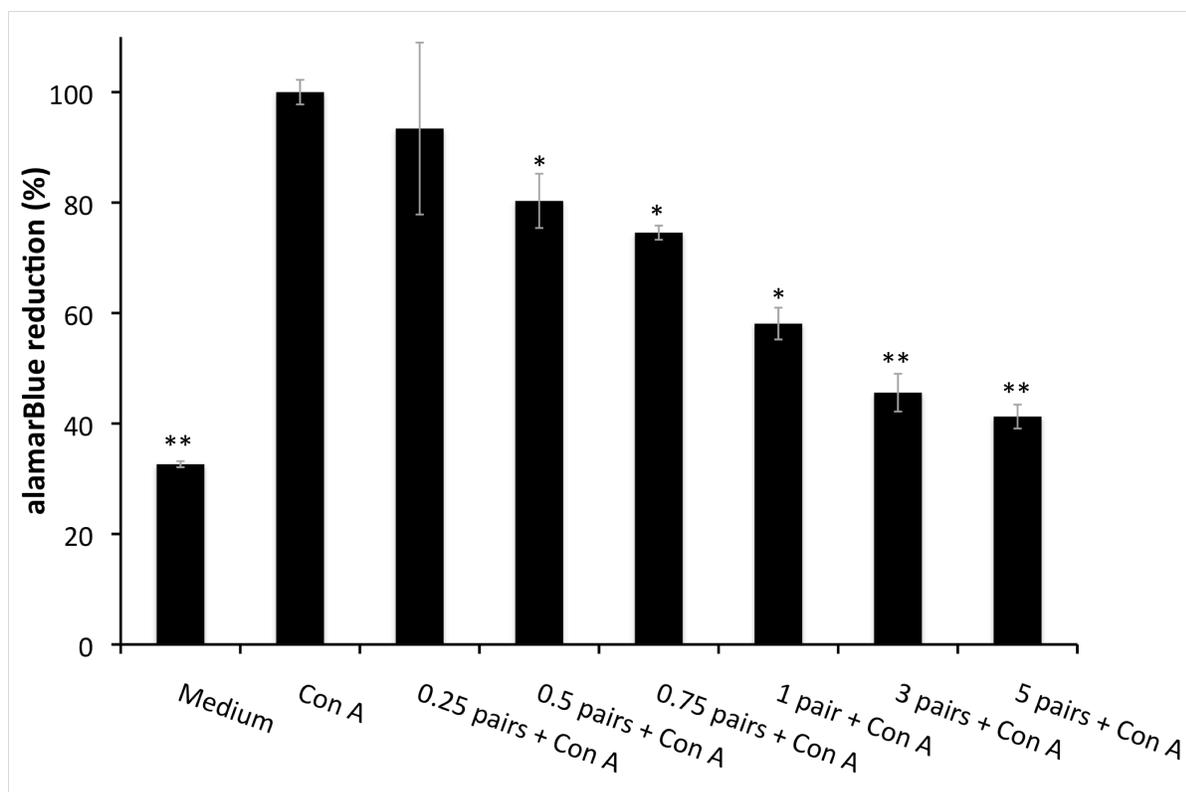


Figure 2.1: *Simulium vittatum* SGE inhibits proliferation of Con A-stimulated mouse splenocytes (BALB/c). Splenocytes were stimulated with Con A in the presence of SGE at the designated concentrations for 56 h before addition of alamarBlue for the detection of proliferation. Reduction in alamarBlue was measured after 48 h. Positive control: Con A without SGE, negative control: medium without Con A. The graph is shown as mean \pm SD. * and ** indicate statistical significance relative to positive control at $P < 0.05$ and $P < 0.01$, respectively, by two-sided t-test. Data are representative of three or more independent experiments, with samples duplicated within each experiment.

(a)

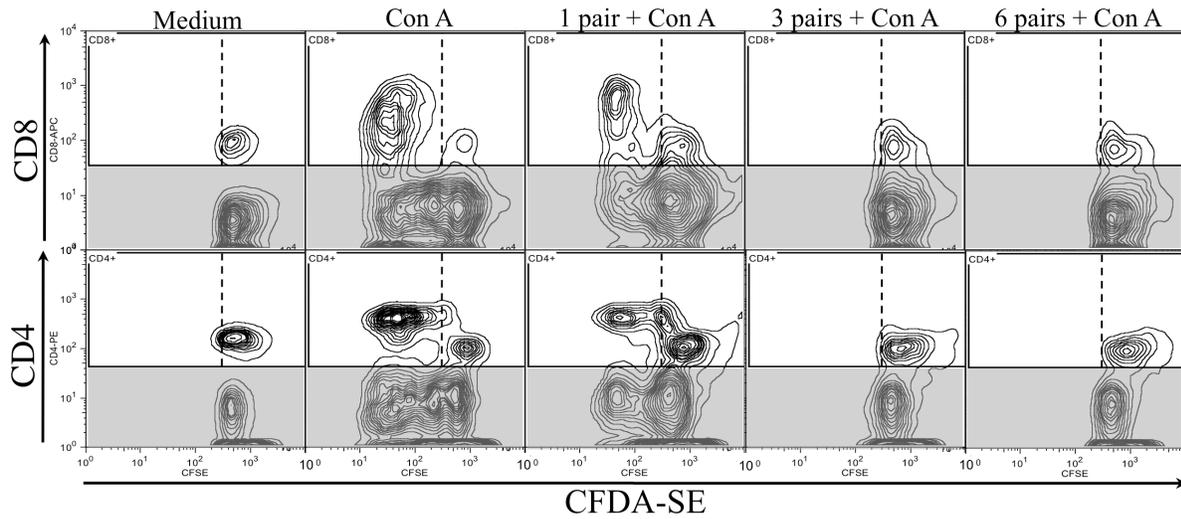


Figure 2.2(a): *Simulium vittatum* SGE inhibits cell division of Con A-stimulated mouse splenocytes (BALB/c). Splenocytes were stained with CFDA-SE (5 μ M), followed by stimulation with Con A in the presence of SGE at the indicated concentrations for 96 h. The cells were harvested, stained with anti-CD4-PE and anti-CD8-APC antibodies and analysed by flow cytometry. Vertical axis of top panels is plotted for CD8 and vertical axis of bottom panels is plotted for CD4. Events positive for either CD4 or CD8 were gated in a rectangle. Positive control: Con A without SGE, negative control: medium without Con A. Data are representative of three independent experiments.

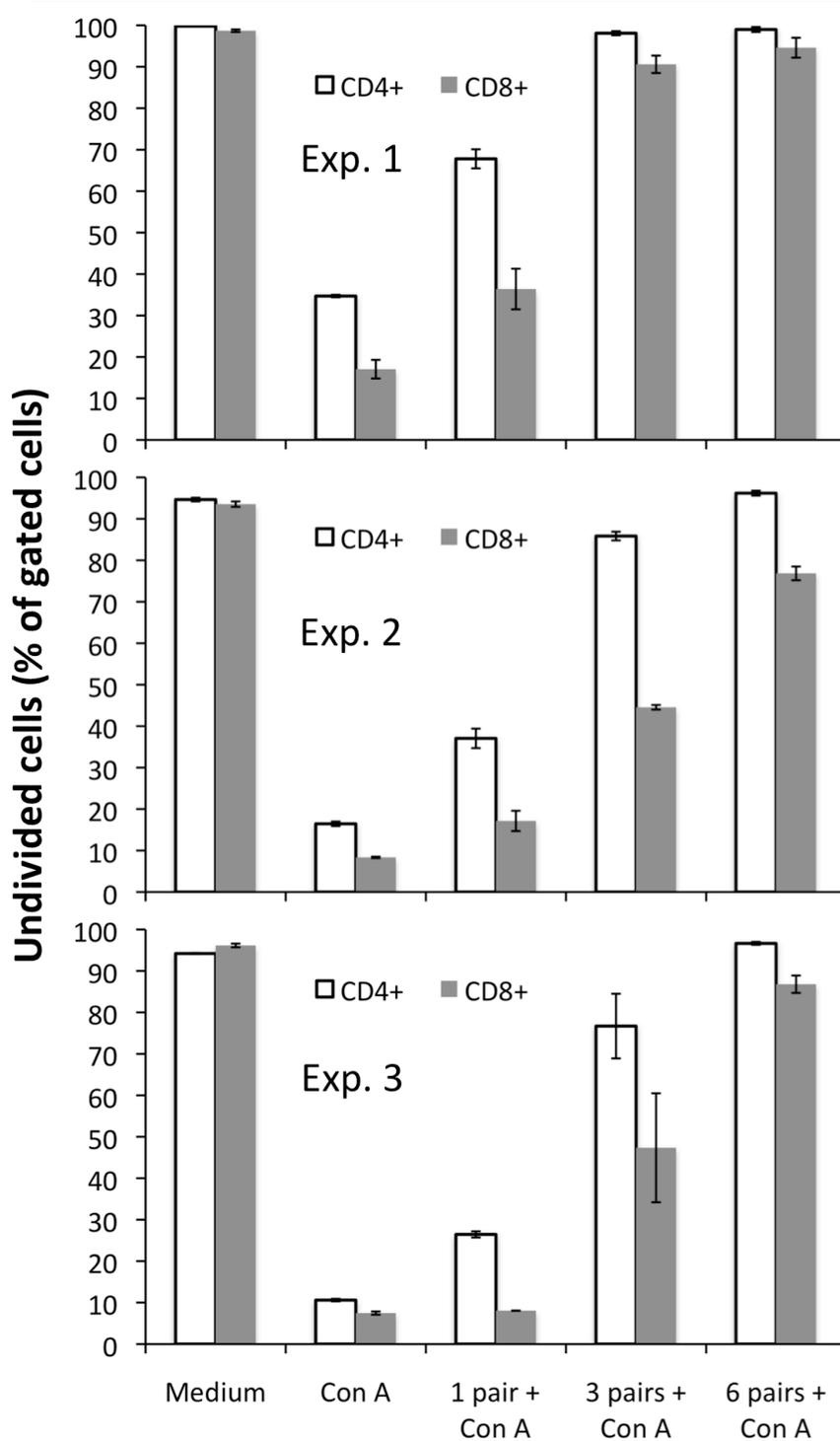


Figure 2.2(b): Per cent of undivided cells from CFDA-SE assay. Percentages of undivided cells (population in the right end of the plots in (a)) for CD4⁺ or CD8⁺ populations were plotted. White bars indicate the CD4⁺ population and grey bars the CD8⁺ population. The graphs show mean \pm SD of two wells for each treatment. Results of three independent experiments are shown.

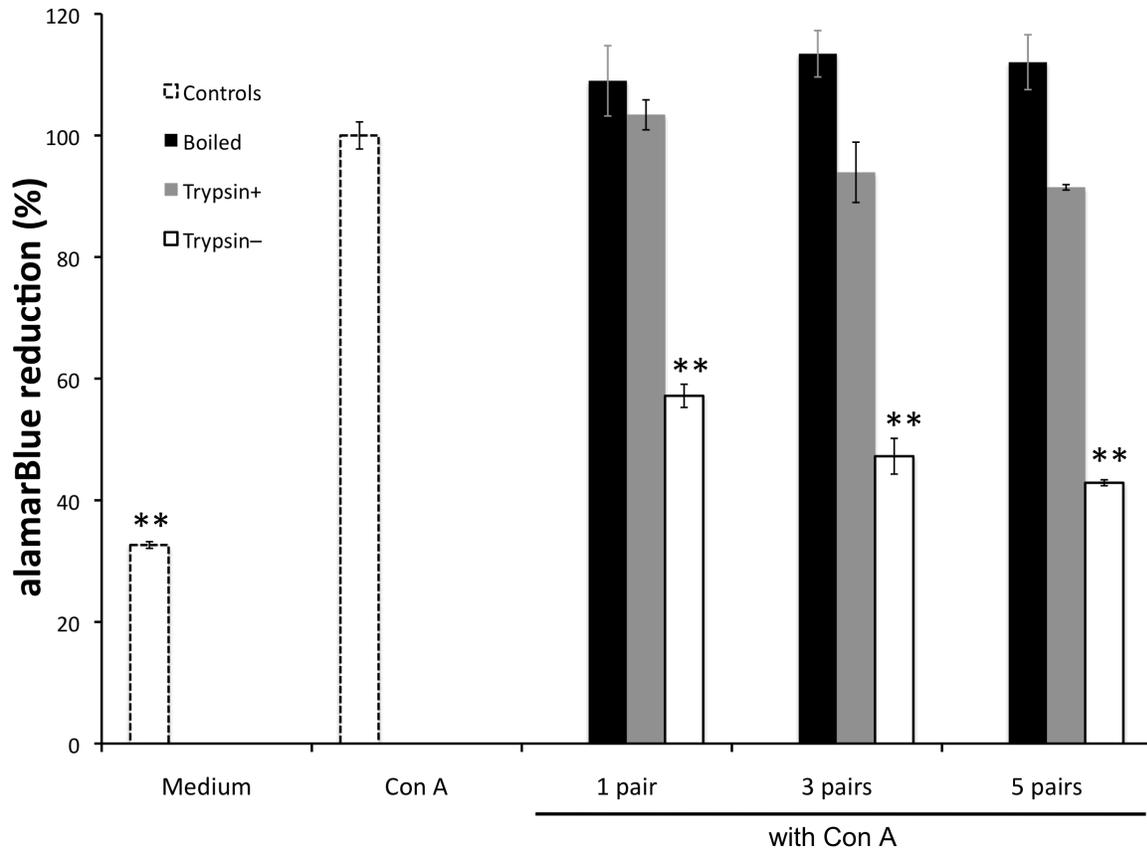


Figure 2.3: Inhibitory activity of SGE was abolished by boiling and trypsin treatment. Inhibitory activity of boiled (5 min) (black bars) and trypsin-treated (400 $\mu\text{g}/\text{mL}$ for 2 h; grey bars) *Simulium vittatum* SGE was assessed. White bars with solid outline are the no-trypsin control. White bars with dotted-outline are positive (with Con A) and negative (medium without Con A) controls. The graph shows mean \pm SD. **Indicates statistical significance relative to positive control at $P < 0.01$. A representative of two independent experiments.

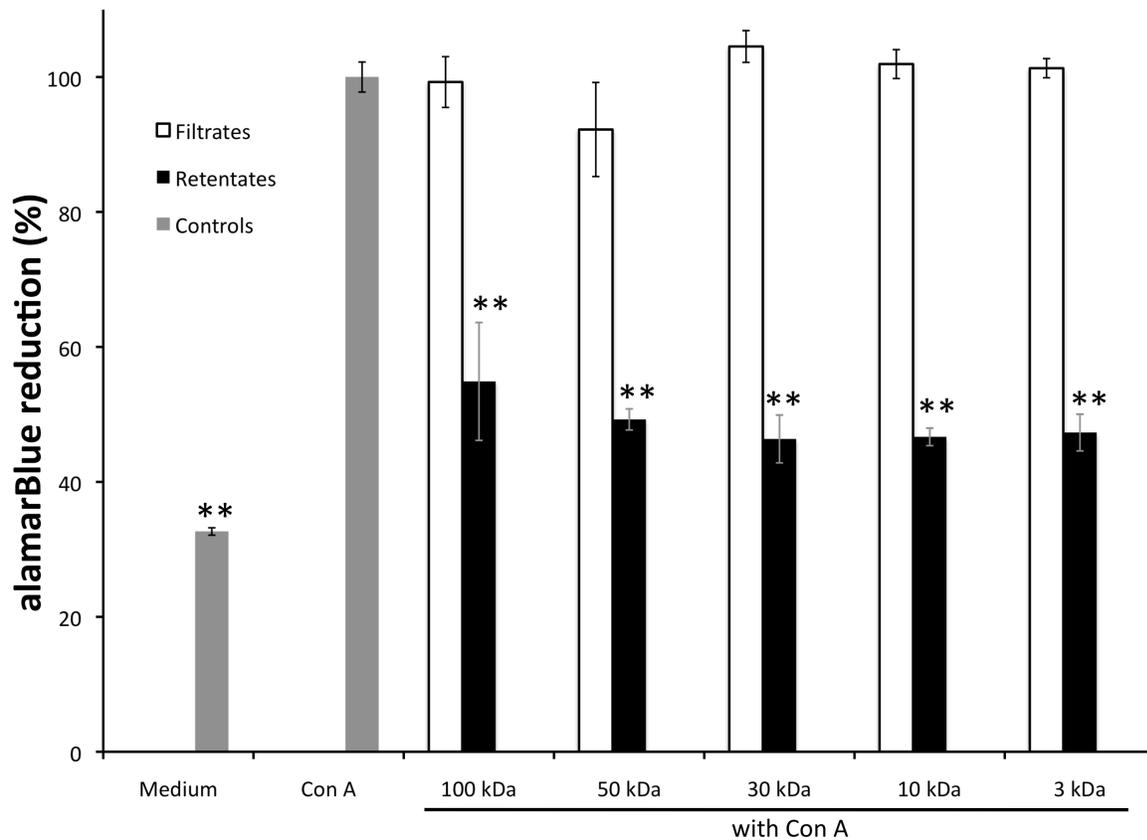


Figure 2.4(a): Size estimation of the inhibitory component. Inhibitory activity of size fractionated (100, 50, 30, 10 and 3 kDa) *Simulium vittatum* SGE was assessed. Four pairs of gland equivalent SGE per well was used for each treatment. White bars are filtrates of designated size filters and black bars are retentates. Grey bars are positive (with Con A) and negative (medium without Con A) controls. The graph shows mean \pm SD. **Indicates statistical significance relative to positive control at $P < 0.01$. A representative of three independent experiments.

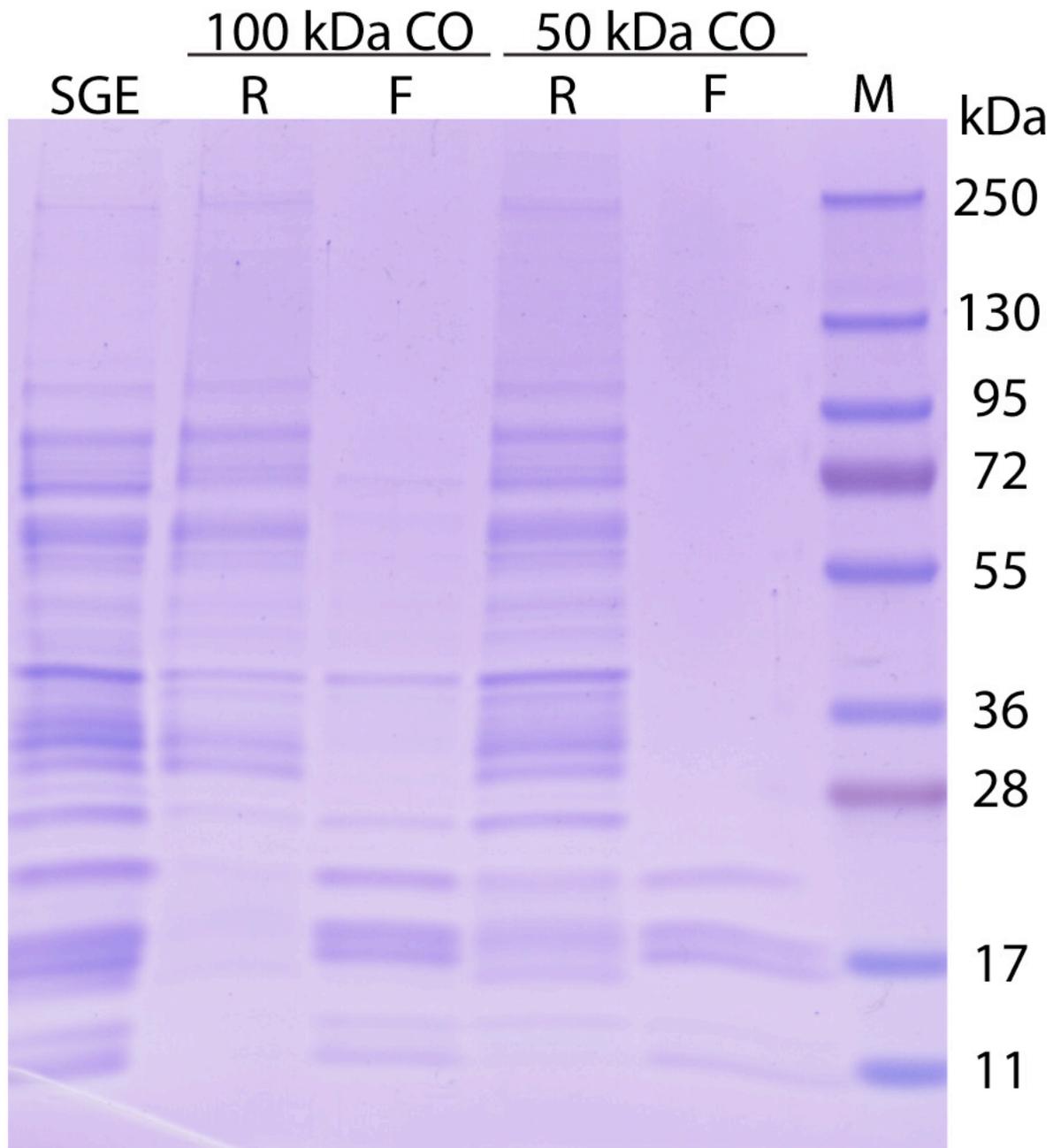


Figure 2.4(b): The efficiency of fractionation of SGE was examined by SDS-PAGE using a 4-20% gradient gel. SGE, unfractionated *S. vittatum* SGE; R, retentates; F, filtrates; M: molecular size marker (Fermentas); 100 kDa CO: 100 kDa cutoff filter; 50 kDa CO: 50 kDa cutoff filter. Each lane contains the equivalent of 12.5 salivary gland pairs. The sizes of the molecular size marker are indicated on the right.

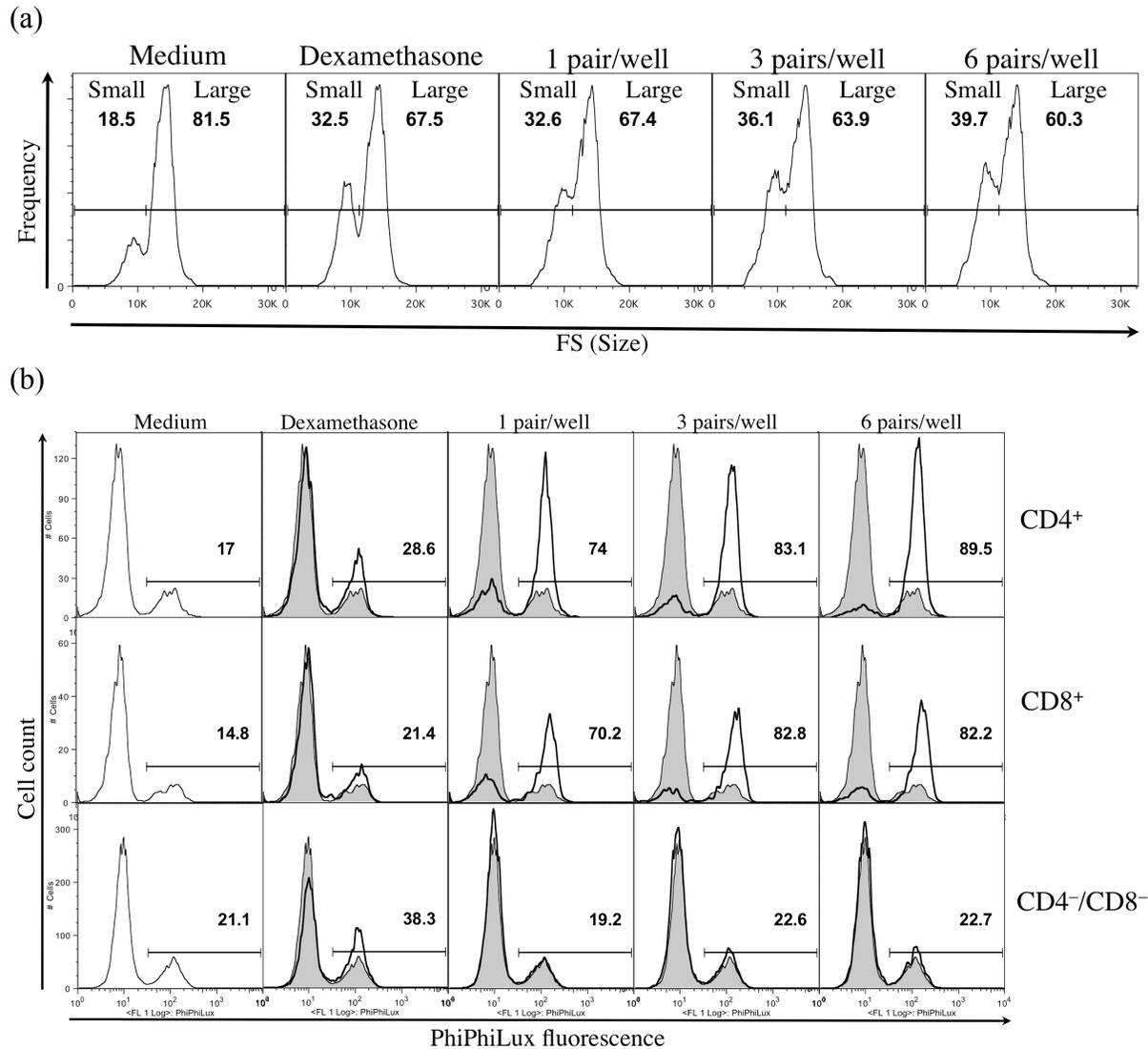


Figure 2.5: Morphological change and caspase 3 activation indicate apoptosis in T lymphocytes following exposure to black fly SGE. Mouse splenocytes were incubated in the presence of indicated amounts of black fly SGE for 5 h. Then cells were pulsed with PhiPhiLux G₁D₂ substrate, stained with anti-CD4 and anti-CD8 antibodies, and analysed by flow cytometry. The cells were also stained with propidium iodide (PI) to discriminate dead cells. (a) Size change in black fly SGE-treated mouse lymphocytes. Forward-scatter (FS: horizontal axis) vs. cell count was plotted for acquired samples. Numbers indicate percentages of the lymphocytes that are classified as ‘large’ (within the right bracket gate) or ‘small’. This figure shows typical results of a single experiment. (b) Caspase 3 is activated in CD4⁺, and CD8⁺, but not CD4⁻/CD8⁻ gated cells. These cells were also gated to exclude the PI⁺ cell population. PhiPhiLux fluorescence intensity is plotted in the horizontal axis and the height indicates the number of the cells with a particular fluorescence intensity. Bracket gate for positive population and the percentage in the designated populations (CD4⁺, CD8⁺ and CD4⁻/CD8⁻) are also shown. Results of the negative control (medium only) are shown as the shaded trace in each plot.

CHAPTER 3

MODULATION OF MURINE LYMPHOCYTE PROLIFERATION AND NITRIC OXIDE
PRODUCTION BY MACROPHAGES BY SALIVARY GLAND EXTRACT OF THE BLACK
FLY, *SIMULIUM VITTATUM*²

² To be submitted to “American Journal of Tropical Medicine and Hygiene.”

Abstract

Black flies not only transmit pathogens such as *Onchocerca* and vesicular stomatitis virus, but also cause death of livestock by the toxic effect of their bites. Salivary glands of black flies have been known to contain anti-hemostatic factors and immunomodulatory activity. Previous studies showed that black fly salivary gland extract (SGE) inhibits lymphocyte proliferation and modulates cytokine secretion *in vitro* using BALB/c mice (Cross *et al.*, 1994; Cross *et al.*, 1993). The present study extends these results in a different mouse strain, C3H, which shows an immune response more similar to humans in responding to infection of pathogens (Sacks and Noben-Trauth, 2002). The SGE inhibited T-cell proliferation as was seen previously with BALB/c-derived T cells. SGE also inhibited B-cell proliferation, although to a lesser degree. Moreover, the black fly SGE enhanced the production of nitric oxide (NO) in LPS-stimulated macrophages at low concentrations and inhibited NO production at a high concentration. This report is one of only two observations of arthropod saliva enhancing NO production by macrophages. The relevance of this phenomenon to pathogen transmission and inflammatory pathogenesis is discussed.

Introduction

Black flies (Diptera: Simuliidae) are small, stout-bodied flies, which are closely related to mosquitoes and biting midges within the order Diptera (Bertone *et al.*, 2008; Currie and Adler, 2008). Simuliidae, consisting of 2000 nominal species worldwide, spend the larval and pupal stages in running water. Immature stages are important for stream water ecosystems as larvae may occur in huge numbers under optimal conditions, and serve as a food source of predatory animals (Currie and Adler, 2008). On the other hand, massive emergence of adult flies under such conditions causes nuisance and sometimes death in livestock by the toxic effects of their

salivary injections (simuliotoxicosis) (Currie and Adler, 2008). Black flies are also known to transmit pathogens such as filarial parasites, *Onchocerca* spp. (Crosskey, 1993) and vesicular stomatitis virus (VSV) (Mead *et al.*, 2004a; Mead *et al.*, 2004b; Mead *et al.*, 2000; Smith *et al.*, 2009), among which *Onchocerca volvulus* causes human onchocerciasis (river blindness) in Africa and South America. The pathogens are transmitted by co-injection with saliva in the blood feeding process or through wounds created by the bites.

As blood feeders, female black flies evolved to contain a variety of anti-hemostatic factors in the salivary glands (Cupp and Cupp, 1997). The salivary glands have been shown to contain a platelet aggregation inhibitor, apyrase (Cupp *et al.*, 1993), a potent vasodilator (Cupp *et al.*, 1998), and at least three different anti-coagulants (Abebe *et al.*, 1995; Abebe *et al.*, 1996; Jacobs *et al.*, 1990). Apyrase is an enzyme that hydrolyzes an agonist of platelet aggregation, ADP (and ATP), into AMP and orthophosphates (Cupp *et al.*, 1993). The vasodilator has been determined to be a novel peptide, called *S. vittatum* erythema protein (SVEP) (Cupp *et al.*, 1998). SVEP is one of the most potent vasodilators known to date, and seems to act on the ATP-dependant K⁺ channel (Cupp *et al.*, 1998). Each of the three anti-coagulants specifically inhibits either thrombin, activated factor X (FXa) or FV (Abebe *et al.*, 1995; Abebe *et al.*, 1996; Jacobs *et al.*, 1990). However, only the anti-thrombin has been studied to the genetic level (Abebe *et al.*, 1995).

Previous studies showed that the salivary glands of black flies also contain an immunomodulatory activity using a BALB/c mouse model (Cross *et al.*, 1994; Cross *et al.*, 1993). The authors injected SGE or sham protein (fowl gamma globulin) 48 hours prior to the preparation of cells from lymphoid organs (spleen and lymph nodes) and the skin (Cross *et al.*, 1993). The I-A expression was reduced only in the splenocytes from the SGE-injected mice in

comparison to the splenocytes from the sham-injected mice without *in vitro* stimulation (Cross *et al.*, 1993). However, this difference disappeared when the cells were incubated in the presence of SGE or sham protein for 48 hours, and no difference was observed in the cells obtained from lymph nodes or epidermis with or without *in vitro* stimulation (Cross *et al.*, 1993). The same study also showed that the SGE suppressed *in vitro* proliferation of splenocytes stimulated either by the T-cell mitogen, concanavalin A (Con A) or by the B-cell mitogen, lipopolysaccharide (LPS) (Cross *et al.*, 1993). They further observed that SGE injection resulted in an increase in antibody titer and antibody-secreting cells against sheep red blood cells (Cross *et al.*, 1993). Another study used BALB/c mice repeatedly injected with SGE (Cross *et al.*, 1994). The authors described reduced IL-5 and IL-10 secretion by *ex vivo* splenocytes from SGE-injected mice, compared with those from saline-injected mice. The same study further described that pre-exposure of splenocytes from naive mice to black fly SGE reduced *ex vivo* responsiveness to IL-2 and IL-4, and lower IL-4 utilization (indicated by higher residual IL-4 in IL-4-stimulated culture supernatant).

In the previous study, we demonstrated using naive BALB/c mice that *S. vittatum* SGE inhibits Con A-stimulated splenocyte proliferation and characterized the factor(s) responsible for this inhibition as a protein of a size larger than 50 kDa (Tsujiimoto *et al.*, 2010). We further described a potential mechanism of this inhibition as induction of apoptosis, specifically in CD4⁺ and CD8⁺ T cells, but the induction was not observed in CD4⁻/CD8⁻ cells (Tsujiimoto *et al.*, 2010). The present study was conducted to evaluate whether the inhibitory effect of SGE on cell proliferation holds true using a different mouse strain, C3H/HeN, which shows more natural immune responses than BALB/c mice, which are known to show a Th2-biased immune response (Handman *et al.*, 1979; Nacy *et al.*, 1983; Sacks and Noben-Trauth, 2002). In the skin, the actual

bite site and the entry site for pathogens, resident innate immune cells such as macrophages are more abundant than lymphocytes, so this study also investigated the effect of SGE on the macrophages. In this paper, we report the inhibitory effect of the black fly SGE on C3H-derived T and B cells and concentration-dependent modification of NO production by macrophages.

Materials and Methods

Mice

Adult C3H/NeH mice 8-10 weeks of age were purchased from the National Cancer Institute (NCI) (Frederick, MD). In this paper, this strain is called “C3H.” Mice were maintained in the University of Georgia animal care facility. All work described in this study was performed in accordance with protocols approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC).

***Simulium vittatum* and salivary gland extract**

Adult female *Simulium vittatum* were obtained from the black fly colony of the Department of Entomology, the University of Georgia.

S. vittatum is an autogenous species that does not require blood meal for the first gonotrophic cycle, and it has been shown that total protein level in the salivary glands is low, and the level of apyrase activity does not increase, until after 3 days following adult emergence. Therefore, adult female *S. vittatum* which have oviposited, at which point the salivary glands are ready for blood feeding, were used for salivary gland dissection. The black flies were kept at 4 °C until dissection, when they were chilled on ice, and salivary glands were dissected under a stereomicroscope in sterile HEPES saline (HS) (10 mM HEPES pH 7.0, 150 mM NaCl). Salivary glands were collected in 1.6 mL microcentrifuge tubes with 20 µL HS and stored at -70 °C. To isolate salivary gland extract (SGE), the salivary glands in HS were thawed, sonicated

(Branson tissue sonicator) at 40% duty cycle with power setting at the microtip limit while the tube was submerged in ice water, and centrifuged at 4 °C at 14000× g for 10 minutes. The supernatant containing soluble components was transferred to a sterile tube. Total soluble protein content was assessed by the bicinchoninic acid (BCA) method using MicroBCA kit (Pierce, Rockford, IL) modified for a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE); an average of about 1 µg of protein was present per pair of salivary glands. After quantification of protein concentration, aliquots of SGE were made in order to use uniform quality (one thawing after SGE isolation) and stored at -70 °C until use. Sterility of SGE was assessed by streaking the SGE onto LB agar plate without antibiotics, and no colony growth was observed after 24 hours of culture at 37 °C.

Splenocyte (T and B cells) proliferation assay

Optimal concentrations for concanavalin A (Con A) and lipopolysaccharide (LPS), incubation and [³H]-thymidine ([³H]-TdR) pulsing times were determined. The spleen was dissected from an euthanized mouse (per experiment) and ground between frosted microscope slides to obtain a single-cell suspension. Erythrocytes were lysed by treating the cells in RBC lysis solution (154 mM NH₄Cl, 10 mM KHCO₃, 0.082 mM EDTA). After stopping RBC lysis by addition of 1/2 volume of 2× PBS and centrifugation at 600× g for 5 min, cells were resuspended with RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta biologicals, Lawrenceville, GA), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) and 1 µg/mL gentamycin (BioWhittaker, Basel, Switzerland) (complete RPMI, subsequently referred to as cRPMI) and counted by the trypan blue exclusion method. The cell density was adjusted to 6.25×10^6 cells/mL and 80 µL were transferred to each well of 96-well plates (BD Biosciences, San Jose, CA) so that each well contained 5×10^5 cells. For treatment, 20 µL of cRPMI

containing various concentrations of SGE or without SGE was added to each well and incubated for 2 hours in a CO₂ incubator (at 37 °C, 5% CO₂ in humidified condition) before addition of 100 µL of cRPMI containing the T cell mitogen, Con A (1 µg/mL final concentration) or the B cell mitogen, LPS (10 or 30 µg/mL final concentrations). 24 or 48 hours after addition of LPS or Con A, respectively, 20 µL of cRPMI containing 1 µCi of [³H]-TdR (MP Biomedicals, Solon, OH) was added to each well and incubated for 24 hours. [³H]-TdR incorporation was determined by harvesting the cells onto glass fiber filters (Wallac, Turku, Finland) with a cell harvester (Type 7000 cell harvester, Skatron, Inc., Sterling, VA), and radioactivity was measured by scintillation counting (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, Fullerton, CA).

Peritoneal macrophage NO assay

Peritoneal exudate cells (PECs) were obtained from a single C3H mouse per experiment. Briefly, a mouse was euthanized by CO₂, and 10 mL of ice-cold cRPMI was injected into the peritoneal cavity by penetrating the peritoneal membrane with a 25G needle attached to a syringe without damaging internal organs. After 5 min with occasional gentle agitation, the injected cRPMI was withdrawn by the syringe with the same 25G needle. The same procedure was repeated again with 7-8 mL fresh cRPMI. This peritoneal exudate was centrifuged at 600× g to collect cellular components, and the cell pellet was resuspended with 1 mL cRMPI. After counting the cells by the trypan blue exclusion method, the cell suspension was adjusted to 1.0×10^6 cells/mL. 100 µL of the cell suspension was transferred to each well of a 96-well plate and incubated in a CO₂ incubator for 24 hours to allow macrophages to adhere to the bottom of wells. After non-adherent cells were removed with culture medium by repeated gentle pipetting, 100 µL of cRPMI with or without various amounts of SGE was added to each well followed by 2 hours of incubation before LPS addition (10 ng/mL). At 24 hours following LPS addition, 50 µL

of culture supernatant was transferred to a 96-well plate to measure NO produced. The 96-well plate also contained serial concentrations (0-120 μM) of NaNO_2 in cRPMI for standards. NO production was measured by Griess reaction (according to Promega Griess Reagent System, reagents made in-house): 50 μL of 1% sulfanilamide in 5% phosphoric acid was added to each well and incubated at room temperature for 15 min, protected from light. Next, 50 μL of 0.1% N-1-naphthylethylenediamine dihydrochloride (NED) in water was added to each well and incubated at room temperature for 15 min, protected from light. Then absorbance was measured using a microtiter plate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA) at 540 nm. The standard curve was constructed by absorbance against NO_2 concentrations of the standards, and concentration of NO_2 in each sample was extrapolated based on the standard curve.

Statistical analyses

Data were analyzed by Student's T test, and differences were considered significant at $p < 0.05$. Statistical software "R" (<http://www.r-project.org/>) was used for the analysis.

Results

Black fly SGE inhibits Con A- and LPS-stimulated C3H splenocytes

As shown in Figure 3.1(a), *S. vittatum* SGE strongly inhibited proliferation of Con A-stimulated C3H splenocytes. The proliferation was reduced to about 39% in comparison to positive control at 0.25 μg /well of SGE, which is equivalent to 1/4 pairs of the salivary gland, and the proliferation was almost completely inhibited at 2 μg /well of SGE. In addition, SGE alone did not have a significant effect on splenocyte proliferation (Figure 3.1(a)).

Next the effect of SGE on LPS-stimulated C3H splenocytes was examined. With our experimental conditions, stimulation with either 10 $\mu\text{g}/\text{mL}$ or 30 $\mu\text{g}/\text{mL}$ LPS did not make much difference in proliferation. SGE reduced the proliferation of the splenocytes to lesser degrees in

comparison to Con A-stimulated splenocytes, although the differences are statistically significant (Figure 3.1(b)). 50% proliferation reduction was attained at 1 $\mu\text{g}/\text{well}$ of SGE, but complete inhibition of proliferation was not observed in the tested range of SGE concentrations.

Black fly SGE enhances macrophage NO production at low concentrations, while inhibits it at a high concentration

Next, we investigated the effects of black fly SGE on mouse peritoneal macrophages. Unstimulated peritoneal macrophages were treated with SGE (0.5-8.0 $\mu\text{g}/\text{well}$) for two hours prior to stimulation by 10 ng/mL of LPS. NO production was assessed at 24 hours after addition of LPS. SGE in the range of 0.5-2.0 $\mu\text{g}/\text{well}$, exhibited a trend of enhanced NO production, with more than a two-fold increase at 0.5 $\mu\text{g}/\text{well}$ (Figure 3.2(a)). Inhibition of NO production was seen only at the highest SGE concentration tested, 8.0 $\mu\text{g}/\text{well}$, which reduced NO to the level produced in the presence of medium alone without LPS. The stimulatory and inhibitory effects appeared to balance at 4 $\mu\text{g}/\text{well}$, as NO production did not differ from controls treated with LPS alone. SGE itself had no effect on NO production in the absence of LPS.

To scrutinize the NO-enhancing effect of SGE, SGE concentrations of 0.05 to 1.0 $\mu\text{g}/\text{well}$ have been used to perform the same assay (10 ng/mL LPS stimulation for 24 hours). Lower concentrations of SGE still enhanced NO production from LPS-stimulated macrophages (Figure 3.2(b)). SGE as low as 0.1 $\mu\text{g}/\text{well}$ significantly enhanced production of NO (relative to LPS-only control). To investigate the effect of high concentration of SGE on the macrophage NO production, the high SGE concentration (8.0 $\mu\text{g}/\text{well}$) with varying LPS concentrations have been tested, where significant reduction of NO production was expected based on our earlier result. However, a slight enhancing trend was observed with LPS concentrations lower than 10 ng/mL in the cells treated with 8 $\mu\text{g}/\text{well}$ of SGE (Figure 3.2(c)). Reduction in comparison to the

corresponding LPS-only control was seen only in the samples stimulated with 10 ng/mL.

Discussion

In the present study, C3H mice were used to investigate the immunomodulatory functions of the black fly SGE, as this strain is not biased towards a Th2 response and so provides a different immunological context than the BALB/c mice used previously (Tsujimoto *et al.*, 2010). The black fly SGE significantly inhibited proliferation of Con A-stimulated C3H splenocytes (T lymphocytes) (Figure 3.1(a)), with > 50% inhibition at 0.25 µg/well SGE (approximately 0.25 pairs of salivary glands per well) and > 90% inhibition with 1 µg/well SGE. The inhibition of T-cell proliferation could be partly due to induction of apoptosis, as was demonstrated with BALB/c T cells in the previous study (Tsujimoto *et al.*, 2010). It seems that SGE affects to a similar degree T cells from both C3H and BALB/c strains, as ~2.1 µg/well in the previous study almost completely suppressed the division of the Con A-stimulated BALB/c splenocytes (Tsujimoto *et al.*, 2010), but a caution should be taken to compare results directly as different methodologies (alamarBlue reduction vs. [³H]-TdR incorporation) were used in the two studies.

Black fly SGE also inhibited proliferation of LPS-stimulated splenocytes (B lymphocytes), although the effect was less pronounced than the effect of SGE on T cells (about a 50% inhibition with 1 µg SGE) (figure 3.1(b)). The inhibition of B cells may not be due to apoptosis as the previous study showed that the SGE did not induce apoptosis in CD4⁺/CD8⁻ cells (Tsujimoto *et al.*, 2010). Since induction of apoptosis in the BALB/c T cells did not reach 100% at the SGE concentration which inhibited Con A-stimulated splenocyte proliferation almost completely, additional mechanisms were suggested for the total inhibitory activity, which may also be responsible for inhibition of B-cell proliferation. This could explain why SGE inhibited B cell proliferation less strongly than it did T-cell proliferation; SGE inhibits T cells both by

inducing apoptosis and by additional non-apoptotic mechanisms, and B cells may be affected only by the non-apoptotic mechanisms. T and B cells play major roles in adaptive immune responses such as secreting cytokines and antibodies, respectively, to clear pathogen infections. Black fly SGE enhances infection with VSV when low titers of virus (10^2 - 10^3 pfu) are inoculated in cattle (Dr. D. Mead, personal communication), and suppressing proliferation of T and B lymphocytes might have a role in this process. However, more studies are needed to elucidate the additional mechanisms for the inhibition of splenocyte proliferation and the role of saliva in VSV infection.

The black fly SGE markedly enhanced NO production by LPS-stimulated peritoneal macrophages. Enhancement of NO production could be a unique activity in black fly SGE. The effect of arthropod SGE or saliva has been previously investigated for sand flies, ticks and a biting midge. SGE of the sand fly, *Phlebotomus papatasi* reduced NO production by C57BL/6 (B6) peritoneal exudate cells (PEC) (Hall and Titus, 1995), and maxadilan, the vasodilator and immunomodulator peptide in the salivary gland of the sand fly, *Lutzomyia longipalpis*, reduced NO production by a RAW264.7 macrophage-like cell line and B6 PECs (Brodie *et al.*, 2007). SGE of the tick, *Rhipicephalus appendiculatus*, reduced NO production from a JA-4 macrophage-like cell line (Gwakisa *et al.*, 2001). SGE of *Ixodes ricinus* also reduced NO production by BALB/c PECs (Kuthejlva *et al.*, 2001). SGE of the biting midge (*Culicoides sonorensis*) inhibited NO production by B6 PEC (Bishop *et al.*, 2006). A trend in these studies is that SGE inhibits NO production from macrophages, which may correlate with a reduction of the ability of macrophages to kill internalized parasites (*Borrelia afzelii* or *Leishmania major*) (Hall and Titus, 1995; Kuthejlva *et al.*, 2001). However, there is a report of an exception to this trend from the same group as the Kuthejlva *et al.* (2001) study, in which *I. ricinus* SGE enhanced NO

production from PECs, and saliva reduced it (Kyckova and Kopecky, 2006). As the authors suggested, this contradicts their previous observation, and might be due to the presence of non-secreted cellular proteins from the salivary glands in SGE (Kyckova and Kopecky, 2006). We also cannot exclude the possibility that our observation is due to non-secreted cellular proteins.

The enhancement did not happen in the absence of LPS, which indicates that the NO-enhancing effect is synergistic and not just the consequence of an additive effect of LPS and SGE. This study does not address the mechanism of the enhancement, but a hypothesis may be suggested. In classically activated macrophages, LPS is bound by lipopolysaccharide binding protein (LBP), and the LPS/LBP complex then binds to membrane-bound CD14, which interacts with the LPS recognition complex (including TLR4 and MD-2) to transduce the activation signal to the macrophage nucleus. Black fly SGE may contain a factor that enhances the efficiency of this signaling system, perhaps by strengthening the interaction of LPS with CD14 or TLR4/MD-2 complex, for example modifying the structure of LPS (for example, CD14-dependent “smooth” LPS and CD14-independent “rough” LPS) (Jiang *et al.*, 2005) or by mimicking LBP.

The suppression of NO production might be more relevant where the injection of saliva is limited to a small volume, thus high concentration. Cupp *et al.* (1993) described that 1.4 µg of total protein per pair of salivary gland was reduced by 31% after artificial membrane feeding on bovine blood, which implies ~430 ng of protein was injected in the feeding process. If the volume of the actual injection site is in the low microliter range, the concentration of salivary protein will be well above 80 µg/mL, which is in the concentration range to suppress NO production. However, the suppression did not happen with lower concentrations of LPS. LPS, a component of gram-negative bacteria, is unlikely to be present in the fly’s mouthparts or saliva, and will be present in the blood stream only in unusual circumstances such as sepsis. Therefore,

the physiological relevance of this phenomenon, if any, remains to be elucidated.

The enhanced NO production may have a negative impact on *Onchocerca* worms transmitted by the black flies, but the parasitic nematodes are also known to have defense mechanisms against nitrogen and oxygen radicals (Selkirk *et al.*, 1998). Indeed, cystatins secreted by filarial nematodes, including *O. volvulus*, have been demonstrated to increase NO production by macrophages (Hartmann *et al.*, 2002), which suggests that the nematodes themselves could enhance NO production *in vivo*. Moreover, NO-producing macrophages have an inhibitory activity on proliferation of Con A-stimulated T cells (Denham and Rowland, 1992; Kawabe *et al.*, 1992). This suppressive activity appeared to require cell-to-cell interaction between the macrophages and the T cells (Denham and Rowland, 1992; Kawabe *et al.*, 1992). Therefore, black fly saliva could act to suppress T cells in an indirect way by making macrophages NO producers, which would potentially inhibit T cell activation, at the bite site where pathogens also gain entry. Taken together with our previous report that the black fly SGE induces apoptosis in CD4⁺ and CD8⁺ lymphocytes, this effect implies the possibility that the black fly saliva may have specialized to inhibit T-cell functions *in vivo*. However, more studies must be done to test this hypothesis.

In addition, the enhancing effect of NO might also have some link to inflammatory pathology of diseases such as ocular onchocerciasis (river blindness) and simuliotoxicosis. The inflammatory pathology of river blindness has been demonstrated to be due to the inflammatory response to components of an endosymbiotic bacterium, *Wolbachia*, which is released from dead larval *Onchocerca* (Hise *et al.*, 2007; Hise *et al.*, 2004). It has been considered that the inflammation depends on a Toll-like receptor 4 (TLR4: receptor for LPS) mediated reaction (Hise *et al.*, 2003, 2004), but more recent study shows TLR4 is not involved in the reaction,

which is instead mediated by TLR2 and TLR6 (Hise *et al.*, 2007). The present study investigated the effect of SGE only on LPS-stimulated macrophages (activation through TLR4), and the effect of SGE on macrophages with other stimuli such as interferon- γ remains unknown. Although involvement of NO in the case of ocular onchocerciasis is unknown, and black flies apparently deposit saliva in a limited region of the skin, a systemic effect of the saliva might contribute to the pathology. This phenomenon might also explain toxic effect of black fly bites that sometimes cause a lethal outcome (simuliotoxicosis) in cattle. Moreover, NO is a potent vasodilator and has an important role in loss of vascular tone in the pathophysiology of sepsis (Parratt, 1998). Injection of saliva by large numbers of black flies could account for an increased systemic NO production which leads to loss of vascular tone and loss of responsiveness to endogenous regulators of vascular tone due to the damage in endothelial cells by NO, resulting in shock and death (simuliotoxicosis) (Currie and Adler, 2008; Parratt, 1998).

References:

- Abebe, M., Cupp, M.S., Champagne, D., Cupp, E.W., 1995. Simulidin: a black fly (*Simulium vittatum*) salivary gland protein with anti-thrombin activity. *Journal of Insect Physiology* 41, 1001-1006.
- Abebe, M., Ribeiro, J.M.C., Cupp, M.S., Cupp, E.W., 1996. Novel anticoagulant from salivary glands of *Simulium vittatum* (Diptera: Simuliidae) inhibits activity of coagulation factor V. *Journal of Medical Entomology* 33, 173-176.
- Bertone, M.A., Courtney, G.W., Wiegmann, B.M., 2008. Phylogenetics and temporal diversification of the earliest true flies (Insecta: Diptera) based on multiple nuclear genes. *Syst Entomol* 33, 668-687.
- Bishop, J.V., Mejia, J.S., Perez de Leon, A.A., Tabachnick, W.J., Titus, R.G., 2006. Salivary gland extracts of *Culicoides sonorensis* inhibit murine lymphocyte proliferation and no production by macrophages. *Am J Trop Med Hyg* 75, 532-536.
- Brodie, T.M., Smith, M.C., Morris, R.V., Titus, R.G., 2007. Immunomodulatory effects of the *Lutzomyia longipalpis* salivary gland protein maxadilan on mouse macrophages. *Infect Immun* 75, 2359-2365.
- Cross, M.L., Cupp, E.W., Enriquez, F.J., 1994. Modulation of murine cellular immune responses and cytokines by salivary gland extract of the black fly *Simulium vittatum*. *Trop Med Parasitol* 45, 119-124.
- Cross, M.L., Cupp, M.S., Cupp, E.W., Galloway, A.L., Enriquez, F.J., 1993. Modulation of murine immunological responses by salivary gland extract of *Simulium vittatum* (Diptera: Simuliidae). *J Med Entomol* 30, 928-935.
- Crosskey, R.W., 1993. Blackflies (*Simuliidae*), in: Lane, R.P., Crosskey, R.W. (Eds.), *Medical Insects and Arachnids*, 1 ed, New York, NY, pp. 241-287.
- Cupp, E.W., Cupp, M.S., 1997. Black fly (Diptera: Simuliidae) salivary secretions: importance in vector competence and disease. *J Med Entomol* 34, 87-94.
- Cupp, M.S., Cupp, E.W., Ramberg, F.B., 1993. Salivary Gland Apyrase in Black Flies (*Simulium vittatum*). *Journal of Insect Physiology* 39, 817-821.
- Cupp, M.S., Ribeiro, J.M., Champagne, D.E., Cupp, E.W., 1998. Analyses of cDNA and recombinant protein for a potent vasoactive protein in saliva of a blood-feeding black fly, *Simulium vittatum*. *J Exp Biol* 201, 1553-1561.
- Currie, D.C., Adler, P.H., 2008. Global diversity of black flies (Diptera : Simuliidae) in freshwater. *Hydrobiologia* 595, 469-475.
- Denham, S., Rowland, I.J., 1992. Inhibition of the reactive proliferation of lymphocytes by activated macrophages: the role of nitric oxide. *Clin Exp Immunol* 87, 157-162.

- Gwakisa, P., Yoshihara, K., Long To, T., Gotoh, H., Amano, F., Momotani, E., 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.
- Hall, L.R., Titus, R.G., 1995. Sand fly vector saliva selectively modulates macrophage functions that inhibit killing of *Leishmania major* and nitric oxide production. *J Immunol* 155, 3501-3506.
- Handman, E., Ceredig, R., Mitchell, G.F., 1979. Murine cutaneous leishmaniasis: disease patterns in intact and nude mice of various genotypes and examination of some differences between normal and infected macrophages. *Aust J Exp Biol Med Sci* 57, 9-29.
- Hartmann, S., Schonemeyer, A., Sonnenburg, B., Vray, B., Lucius, R., 2002. Cystatins of filarial nematodes up-regulate the nitric oxide production of interferon-gamma-activated murine macrophages. *Parasite Immunol* 24, 253-262.
- Hise, A.G., Daehnel, K., Gillette-Ferguson, I., Cho, E., McGarry, H.F., Taylor, M.J., Golenbock, D.T., Fitzgerald, K.A., Kazura, J.W., Pearlman, E., 2007. Innate immune responses to endosymbiotic *Wolbachia* bacteria in *Brugia malayi* and *Onchocerca volvulus* are dependent on TLR2, TLR6, MyD88, and Mal, but not TLR4, TRIF, or TRAM. *J Immunol* 178, 1068-1076.
- Hise, A.G., Gillette-Ferguson, I., Pearlman, E., 2003. Immunopathogenesis of *Onchocerca volvulus* keratitis (river blindness): a novel role for TLR4 and endosymbiotic *Wolbachia* bacteria. *J Endotoxin Res* 9, 390-394.
- Hise, A.G., Gillette-Ferguson, I., Pearlman, E., 2004. The role of endosymbiotic *Wolbachia* bacteria in filarial disease. *Cell Microbiol* 6, 97-104.
- Jacobs, J.W., Cupp, E.W., Sardana, M., Friedman, P.A., 1990. Isolation and characterization of a coagulation factor Xa inhibitor from black fly salivary glands. *Thromb Haemost* 64, 235-238.
- Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M., Beutler, B., 2005. CD14 is required for MyD88-independent LPS signaling. *Nat Immunol* 6, 565-570.
- Kawabe, T., Isobe, K.I., Hasegawa, Y., Nakashima, I., Shimokata, K., 1992. Immunosuppressive activity induced by nitric oxide in culture supernatant of activated rat alveolar macrophages. *Immunology* 76, 72-78.
- Kuthejlova, M., Kopecky, J., Stepanova, G., Macela, A., 2001. Tick salivary gland extract inhibits killing of *Borrelia afzelii* spirochetes by mouse macrophages. *Infect Immun* 69, 575-578.
- Kyckova, K., Kopecky, J., 2006. Effect of tick saliva on mechanisms of innate immune response against *Borrelia afzelii*. *J Med Entomol* 43, 1208-1214.
- Mead, D.G., Gray, E.W., Noblet, R., Murphy, M.D., Howerth, E.W., Stallknecht, D.E., 2004a. Biological transmission of vesicular stomatitis virus (New Jersey serotype) by *Simulium vittatum* (Diptera: Simuliidae) to domestic swine (*Sus scrofa*). *J Med Entomol* 41, 78-82.

- Mead, D.G., Howerth, E.W., Murphy, M.D., Gray, E.W., Noblet, R., Stallknecht, D.E., 2004b. Black fly involvement in the epidemic transmission of vesicular stomatitis New Jersey virus (Rhabdoviridae: Vesiculovirus). *Vector Borne Zoonotic Dis* 4, 351-359.
- Mead, D.G., Ramberg, F.B., Besselsen, D.G., Mare, C.J., 2000. Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice. *Science* 287, 485-487.
- Nacy, C.A., Fortier, A.H., Pappas, M.G., Henry, R.R., 1983. Susceptibility of inbred mice to *Leishmania tropica* infection: correlation of susceptibility with *in vitro* defective macrophage microbicidal activities. *Cell Immunol* 77, 298-307.
- Parratt, J.R., 1998. Nitric oxide in sepsis and endotoxaemia. *J Antimicrob Chemother* 41 Suppl A, 31-39.
- Sacks, D., Noben-Trauth, N., 2002. The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol* 2, 845-858.
- Selkirk, M.E., Smith, V.P., Thomas, G.R., Gounaris, K., 1998. Resistance of filarial nematode parasites to oxidative stress. *Int J Parasitol* 28, 1315-1332.
- Smith, P.F., Howerth, E.W., Carter, D., Gray, E.W., Noblet, R., Mead, D.G., 2009. Mechanical transmission of vesicular stomatitis New Jersey virus by *Simulium vittatum* (Diptera: Simuliidae) to domestic swine (*Sus scrofa*). *J Med Entomol* 46, 1537-1540.
- Tsujimoto, H., Gray, E.W., Champagne, D.E., 2010. Black fly salivary gland extract inhibits proliferation and induces apoptosis in murine splenocytes. *Parasite Immunol* 32, 275-284.

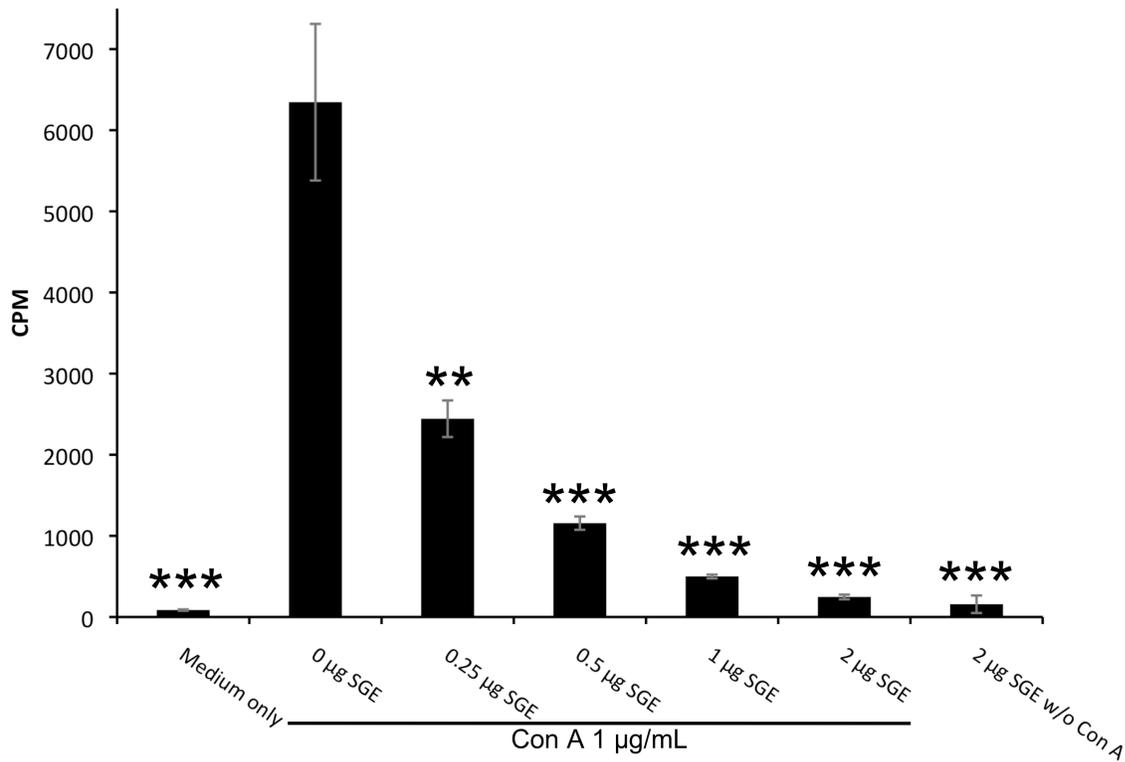


Figure 3.1(a): *S. vittatum* SGE inhibits proliferation of Con A-stimulated C3H mouse splenocytes. Splenocytes were preincubated with various concentrations of SGE for 2 hrs followed by stimulation with Con for 24 hrs before addition of [³H]-TdR. The cells were harvested 24 hrs later and incorporation of [³H]-TdR was quantified by scintillation counting. Medium only and 0 µg SGE served as negative and positive controls, respectively. SGE amounts are in µg protein per well (1 µg of protein in a pair of salivary glands). Histograms show mean ± SD. ** and *** indicate statistical significance relative to positive control at $p < 0.02$ and $p < 0.01$, respectively. Figure is representative of three independent experiments with treatments triplicated per experiment.

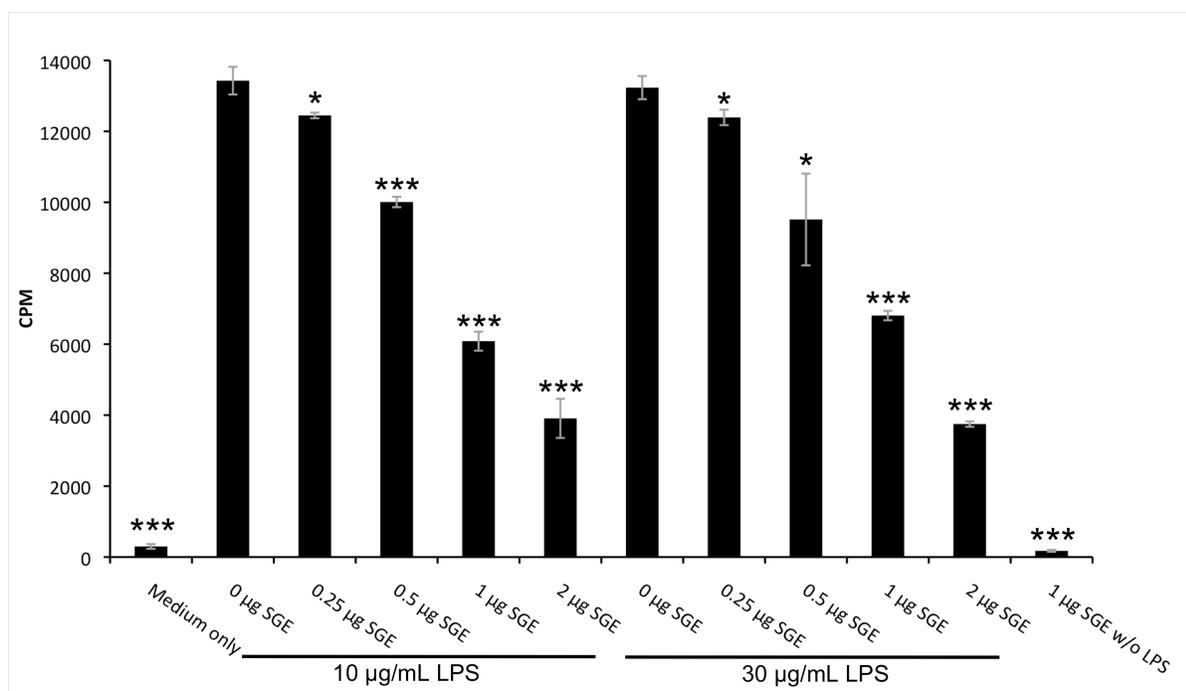


Figure 3.1(b): *S. vittatum* SGE inhibits proliferation of LPS-stimulated C3H mouse splenocytes. Splenocytes were preincubated with various concentrations of SGE for 2 hrs followed by stimulation with LPS for 48 hrs before addition of [³H]-TdR. The cells were harvested 24 hrs later and incorporation of [³H]-TdR was quantified by scintillation counting. Medium only served as negative control. In LPS-stimulated sample groups, 0 µg SGE samples served as positive controls. SGE amounts are in µg protein per well. Histograms show mean ± SD. * and *** indicates statistical significance relative to positive control at $p < 0.05$ and $p < 0.01$, respectively. Figure is representative of three independent experiments with treatments triplicated per experiment.

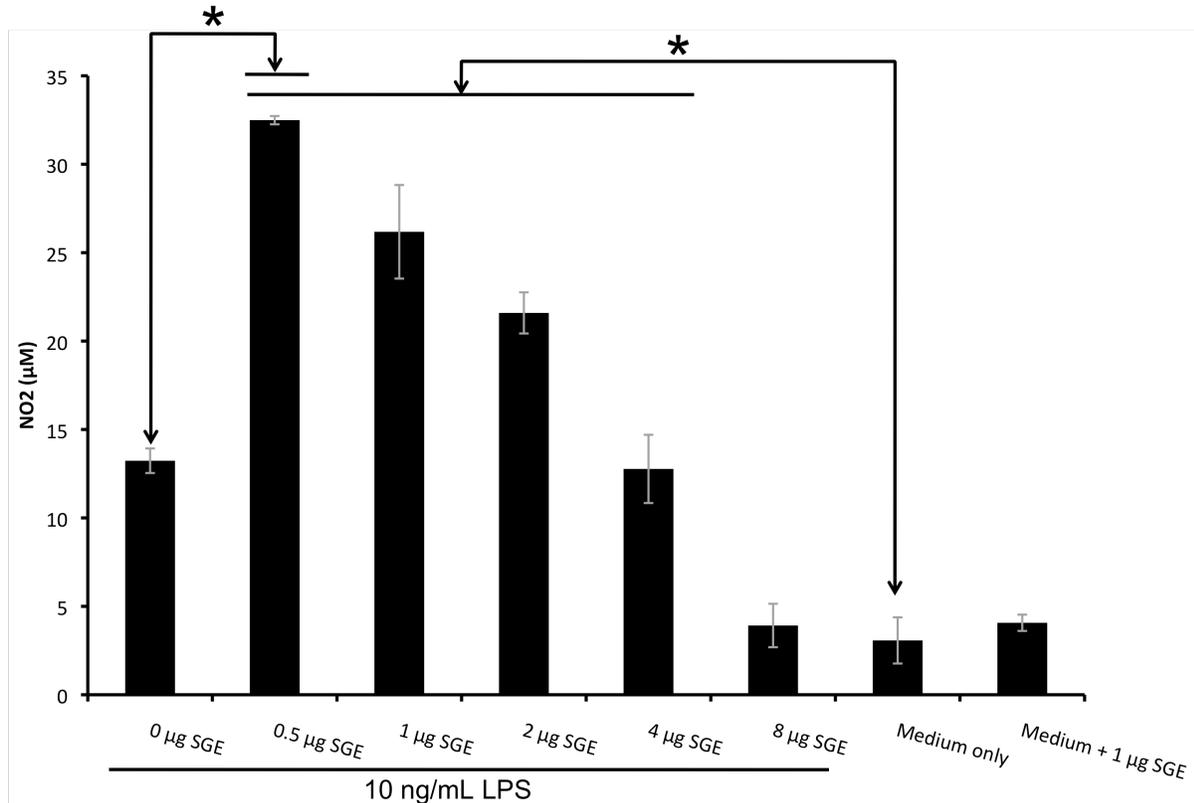


Figure 3.2(a): Low concentrations of *S. vittatum* SGE enhance NO production, while a high concentration inhibits NO production. C3H peritoneal macrophages were treated with various concentrations of SGE for 2 H before LPS stimulation (10 ng/mL). After 24 H of culture, 50 µL of supernatant from each well was analyzed by the Griess method. * indicates statistical significance at $p < 0.05$ by T test between arrowed samples. Figure is representative of three independent experiments with treatments duplicated per experiment.

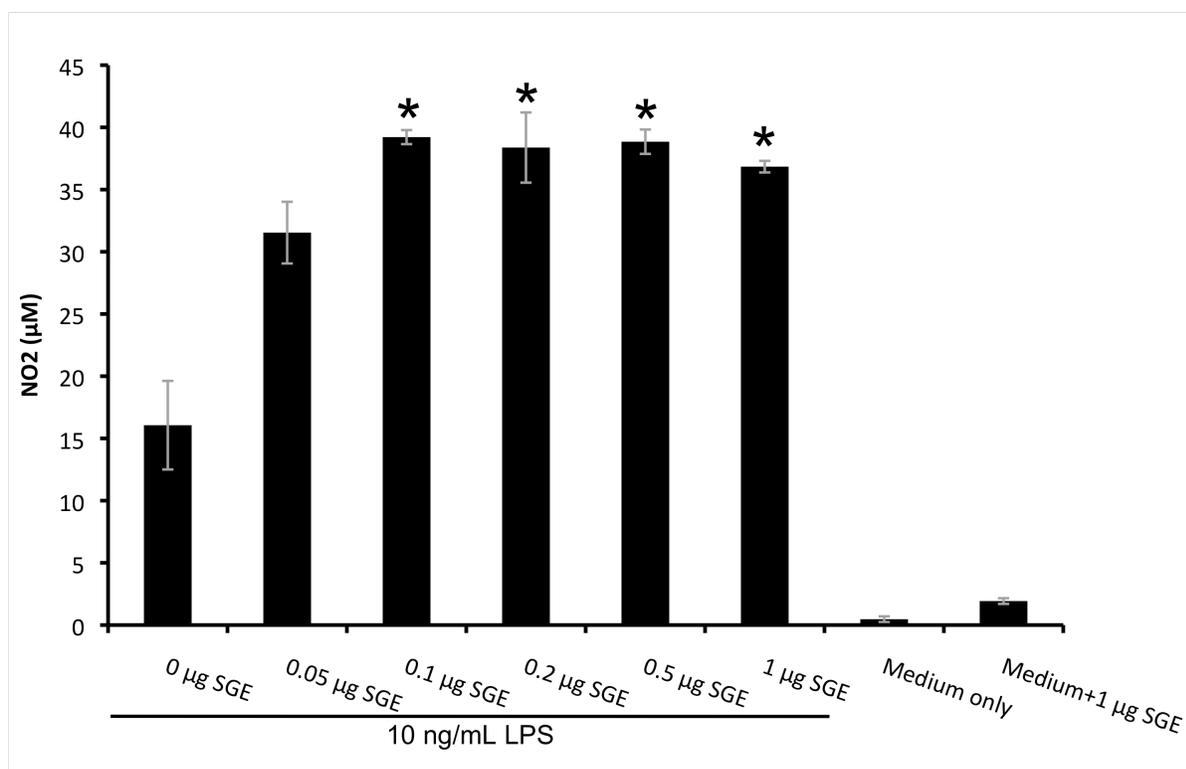


Figure 3.2(b): Lower concentrations of SGE enhance NO production by peritoneal macrophages. C3H peritoneal macrophages were treated with SGE in a concentration range between 0.05 and 1 µg/well for 2 hrs before LPS stimulation (10 ng/mL). After 24 hrs of culture, 50 µL of supernatant from each well was analyzed by the Griess method. * indicates statistical significance at $p < 0.05$ by T test in comparison to LPS-only control (the bar at the left end). Figure is representative of three independent experiments with treatments triplicated per experiment.

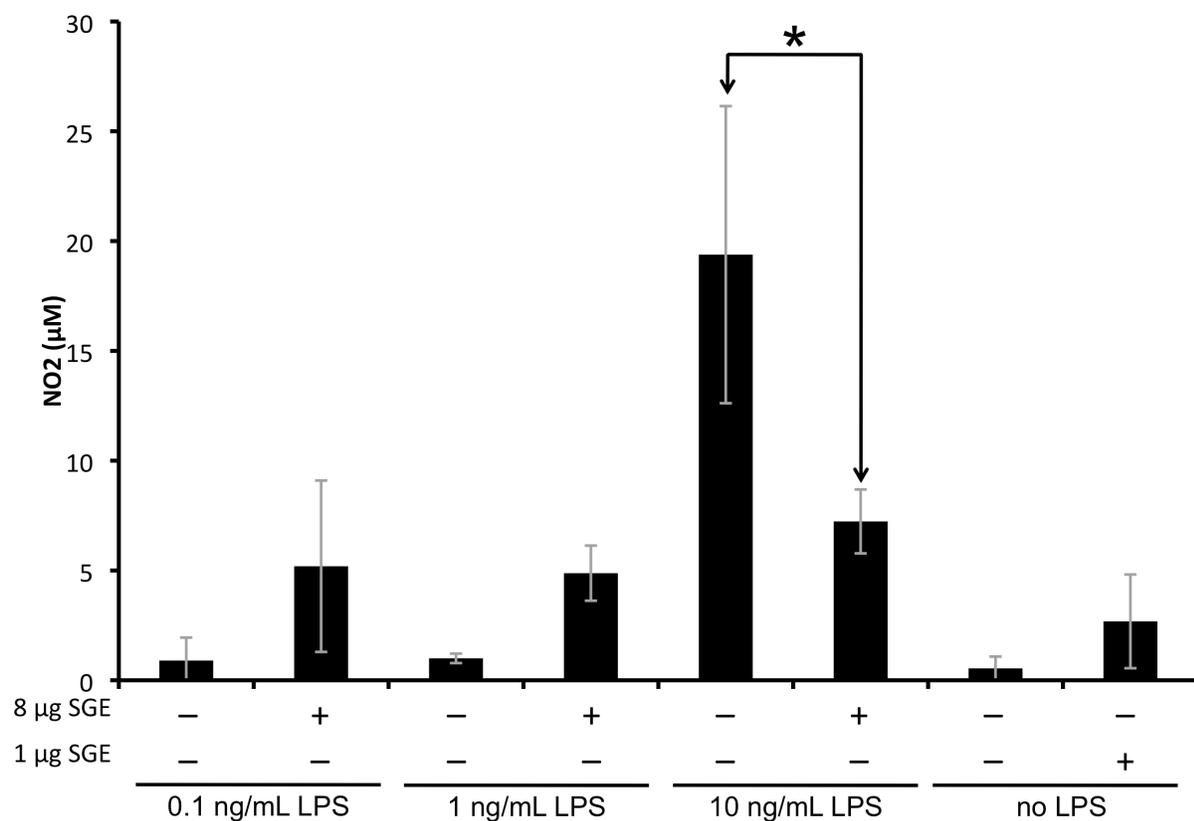


Figure 3.2(c): High concentration of SGE enhance NO production by peritoneal macrophages when stimulated with lower than 10 ng/mL of LPS. C3H peritoneal macrophages were treated with 8 µg/well of SGE for 2 hrs before LPS stimulation (0.1, 1 or 10 ng/mL). After 24 hrs of culture, 50 µL of supernatant from each well was analyzed by the Griess method. * indicates statistical significance at $p < 0.05$ by T test in comparison to each control without SGE. Figure is representative of three independent experiments with treatments triplicated per experiment.

CHAPTER 4

CLONING, EXPRESSION AND FUNCTIONAL ANALYSIS OF KUNITZ FAMILY PROTEINS FROM THE BLACK FLY (*SIMULIUM VITTATUM*) SALIVARY GLAND³

³ To be submitted to “Thrombosis and Haemostasis.”

Abstract

The Kunitz family proteins are found in diverse organisms and primarily function as inhibitors of serine proteases. Blood-feeding arthropods, specifically ticks, produce many Kunitz family proteins to inhibit host hemostasis so as to facilitate blood feeding. The tick salivary gland Kunitz family proteins include inhibitors of coagulation, inflammation and platelet aggregation, among which most of them are inhibitors of coagulation factors that are serine proteases. Amongst lower Diptera a few salivary Kunitz family proteins have been identified at the transcript level in the black fly and biting midges, but functional characterization has not been undertaken. Here, we characterize the functions of Kunitz family proteins expressed in the salivary glands of the black fly, *Simulium vittatum*, in recombinant forms. One of the two recombinant proteins, SV-66, exhibited anti-coagulation activity and inhibitory activity against a broad range of serine proteases. Our results suggest this Kunitz protein in the black fly salivary gland may function as an anti-coagulant and a modulator of local inflammation. Mutational analysis indicates that SV-66 behaves as a canonical BPTI-like protease inhibitor.

Introduction

Kunitz family protease inhibitors have been found in a variety of organisms, not only from the animal kingdom, but also from other eukaryotes and even prokaryotes, which indicates an ancient origin (Ascenzi *et al.*, 2003; Liener, 1986). The most conserved function of the Kunitz family proteins is inhibition of serine proteases (Ascenzi *et al.*, 2003). A single Kunitz domain is quite small (~60 aa) and forms a compact globular fold typically containing three disulfide bonds. Based on the structure of bovine pancreatic trypsin inhibitor (BPTI), a typical Kunitz domain contains cysteine residues at positions 5, 14, 30, 38, 51 and 55 in the mature peptide, which form three disulfide bonds C5-C55, C14-C38 and C38-C51 (Ascenzi *et al.*, 2003). The

Kunitz domain may exist singly, or as multiple domains within a single polypeptide (Corral-Rodriguez *et al.*, 2009). Many Kunitz domains act as protease inhibitors through their scissile bond between positions 15 (P₁) and 16 (P₁'), which typically consists of a basic residue (K or R) and A/G, respectively, and interacts with the active site of the proteases (Ascenzi *et al.*, 2003). Different modes of action have also been characterized. For example, the deer tick, *Ixodes scapularis* produces the anticoagulant protein, ixolaris, in its saliva. Ixolaris contains an N-terminal Kunitz domain which interacts with the heparin-binding exosite (HBE) of activated coagulation factor X (FXa) (Corral-Rodriguez *et al.*, 2009). Kunitz family proteins that have weak anti-protease activity have been found from snake venoms (Harvey, 2001). These proteins are called dendrotoxins and primarily act as blockers of neuronal K⁺ channels (Harvey, 2001).

Blood coagulation is a physiological response that involves complex activation of a cascade of enzymes regulating formation of a fibrin clot in mammals. Most of the components of this cascade are serine proteases. As an enzymatic process, coagulation is negatively regulated by protease inhibitors, which include a Kunitz family protein, tissue factor pathway inhibitor (TFPI) (Crawley and Lane, 2008). TFPI inhibits FXa generation through binding to the FVIIa-Tissue factor-FXa complex (Corral-Rodriguez *et al.*, 2009; Crawley and Lane, 2008).

Blood-feeding arthropods require vertebrate blood for egg maturation, development or both. Many blood-feeding arthropods produce anti-hemostatic factors in the saliva which facilitate blood feeding by interfering with the hosts' hemostatic responses (Ribeiro and Francischetti, 2003). Over the course of 30 years of study, a variety of anti-coagulation factors have been found from salivary glands of blood-feeding arthropods. Kunitz family proteins are a common component of the salivary secretion of ticks, with several mechanisms as inhibitors of coagulation (Corral-Rodriguez *et al.*, 2009; Francischetti *et al.*, 2009). In addition to ixolaris

mentioned above, the saliva of *I. scapularis* contains another type of Kunitz family coagulation inhibitor, penthalaris, which contains five Kunitz domains and inhibits tissue factor pathway in a manner similar to ixolaris (Francischetti *et al.*, 2004). Many other Kunitz family proteins have been described from ticks, and their functions vary from anti-thrombin and anti-FXa to anti-kallikrein and anti-platelet aggregation (Corral-Rodriguez *et al.*, 2009; Maritz-Olivier *et al.*, 2007).

Black flies (Diptera: Simuliidae) are small, stout-bodied, and females of most species require blood meal for egg maturation. Black flies are not only a nuisance for humans and livestock, but also vector several pathogens, including *Onchocerca* spp. (causative agents of onchocerciasis, such as *Onchocerca volvulus* which causes “river blindness”) and vesicular stomatitis virus, which occasionally impacts on livestock. As blood feeders, black flies also produce anti-hemostatic factors in their salivary glands. Black fly bites cause a very pronounced and persistent erythema (Cupp *et al.*, 1994). One of the most potent vasodilators known to date, *Simulium vittatum* erythema protein (SVEP) was described from the black fly salivary glands (Cupp *et al.*, 1998). *S. vittatum* saliva also contains at least three different anti-coagulation factors, each of which exhibit activity against thrombin, FXa, or FV (Abebe *et al.*, 1995; Abebe *et al.*, 1994; Abebe *et al.*, 1996; Jacobs *et al.*, 1990).

A recent publication on the combined transcriptome and proteome (collectively called “the sialome”) of *S. vittatum* salivary glands detected many transcripts and corresponding peptide fragments, which potentially include the previously described anti-coagulation factors (Andersen *et al.*, 2009). These include two Kunitz family proteins, designated as SV-66 and SV-170. Here we cloned SV-66 and SV-170 and assessed whether the recombinant proteins exhibited anti-coagulant activity. Our results indicated that SV-66 exhibits anti-coagulant

activity and inhibits several serine proteases.

Materials and Methods

***Simulium vittatum* and salivary gland dissection**

Adult female *Simulium vittatum* were reared at the University of Georgia, as cultured by the Department of Entomology according to the previously described conditions (Bernardo and Cupp, 1986; Gray and Noblet, 1999).

For RNA isolation, salivary glands were dissected from 2-3 days old *S. vittatum* adult female. The black flies were kept at 4 °C until dissection, when they were chilled on ice, and salivary glands were dissected under a stereomicroscope in sterile HEPES saline (HS) (10 mM HEPES pH 7.0, 150 mM NaCl). 50 pairs of salivary glands were collected in a 1.6 mL microcentrifuge tube containing 20 µL HS mixed with 20 µL *RNAlater* (Ambion, Foster City, CA) and stored at -70 °C until RNA isolation.

Salivary gland cDNA synthesis

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. After isolation of total RNA, removal of remaining genomic DNA was performed by treatment with RNase-free DNase (Turbo DNase, Ambion, Foster City, CA). First strand cDNA was reverse-transcribed by SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo d(T) primer (5'-(T)₂₅-VN-3'). Reverse transcription was performed at 50 °C for 60 min, and the reverse transcriptase was inactivated at 70 °C for 15 min. Resultant cDNA was stored at -20 °C until use.

Bioinformatic analyses

The SignalP server (Emanuelsson *et al.*, 2007) was used to predict the signal sequences in order to exclude the signal sequence for expression in the bacterial system (see next section for

details in expression procedure). Amino-acid sequences of SV-66 and SV-170 were submitted to NetOGlyc server (Julenius *et al.*, 2005) for potential glycosylation site prediction.

We aligned SV-66 and SV-170 to other Kunitz domains, from typical and relevant Kunitz inhibitors including bovine pancreatic trypsin inhibitor (BPTI) from *Bos taurus* (single Kunitz inhibitor), tissue factor pathway inhibitor (TFPI) from human (consisting of 3 Kunitz domains), a thrombin inhibitor amblin from the tick *Amblyomma hebraeum* (2 Kunitz domains) and a prothrombinase inhibitor boophilin from the tick *Rhipicephalus (Boophilus) microplus* (2 Kunitz domains). Amino acid sequences were retrieved from GenBank, and the accession numbers are: BPTI: AAI49369; TFPI: P10646; amblin: AAR97367; boophilin: CAC82583. Individual Kunitz domains of multi-domain proteins were separated manually for sequence comparison, and all the sequences were submitted to ClustalW2 server for alignment (Larkin *et al.*, 2007).

Cloning and expression of Kunitz family proteins

To express recombinant proteins, primers were designed to amplify full-length cDNA sequences encoding SV-66 and SV-170 that contain a signal sequence indicative of secretion. The reference sequences were obtained from supplemental table 1 of Andersen *et al.* (2009) (GenBank accession numbers EU930300 and EU930227, respectively, but these sequences do not contain 5'- and 3' UTRs). Primer sequences were: for SV-66 (5' primer (SV66UA): TGAATTGGATCGAAATGAATATACTTCCA; 3' primer (SV66DA): TTAGTTTGAATGTCCTTTTGTAGTCCAACGA); for SV-170 (5' primer (SV170UA): CACCTGAGAGAATCTTCTGCGTCAAA; 3' primer (SV170DA): CGGTCAATACATTTTTATCCTCTTGTGCT). Polymerase chain reactions (PCR) were performed to amplify the full-length sequences using Platinum HIFI Taq DNA polymerase (Invitrogen, Carlsbad, CA) in the following reaction mixture: 2.5 μ L of 10 \times reaction buffer, 200

nM of each primer, 2 mM of MgCl₂, 0.5 μL of cDNA template, 200 μM of dNTPs, 0.5 U of Taq polymerase in a final volume of 25 μL reaction. PCR was performed by Mastercycler ep gradient (Eppendorf, Hauppauge, NY) with initial 2 min at 94 °C, followed by 35 PCR cycles (30 sec at 94 °C, 30 sec at 57 °C 30 sec at 68 °C) and the final 5 min at 68 °C. The presence of correctly-sized products was assessed by agarose gel (1.5%) electrophoresis.

Primary cloning: The amplified full-length sequences were treated with 1 U of AmpliTaq (Applied biosystems, Foster City, CA) in the presence of 0.5 mM dATP 15 min at 72 °C to add 3' A-overhangs for TOPO cloning. The resultant products were cloned into the pCR4-TOPO vector (TOPO TA cloning kit, Invitrogen). The target-inserted vectors were transformed into *E. coli* TOP10 competent cells (Invitrogen). Insertion-positive clones were cultured, and plasmids were purified using the Wizard *Plus* minipreps DNA purification system (Promega, Madison, WI). Concentrations of the purified plasmid were quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Plasmids were submitted for sequencing (Macrogen, Rockville, MD), and the inserts were confirmed to correspond to the reference sequences from Andersen *et al.* (2009).

Secondary cloning: In order to make inserts for secondary cloning into the expression vector pET-30 Ek/LIC (Novagen, San Diego, CA), primers containing 5' LIC (ligation independent cloning) adapter sequences were designed. Primer sequences were (the adapter sequences underlined): for SV-66 (5' primer (SV66UB):

GACGACGACAAGATGCAAGAGAACGTTTGCAATCTTC; 3' primer (1) (SV66DB):

GAGGAGAAGCCCGGTTAGTCCAACGAAATAATTGGTATC; 3' primer (2) (SV66DC):

GAGGAGAAGCCCGGTGCGTCCAACGAAATAATTGGTATCG); for SV-170 (5' primer

(SV170UB): GACGACGACAAGATGAAGTCAGCTGACATCTGCAGA; 3' primer (2)

(SV170DC): GAGGAGAAGCCCGGTGCCATACACTTGGCTTTACATTCTTG); where 3' primer (1) (SV66DB) is for the expression of single His-tagged (on the N-terminus) protein containing the original stop codon (UAA in mRNA: shown in bold), and 3' primers (2) (SV66DC and SV170DC) are for the expression of double His-tagged (on both the N-terminus and C-terminus) proteins containing an Ala read-through (GCA in mRNA: shown in bold) instead of the stop codon. These primers amplified sequences encoding proteins without signal peptide for expression in bacterial cells. The DNA was amplified by PCR (using Platinum HIFI DNA polymerase (Invitrogen)) and purified plasmids from primary cloning as templates with the following conditions: initial 2 min at 94 °C, followed by 30 PCR cycles (30 sec at 94 °C, 30 sec at 57 °C and 30 sec at 68 °C) and the final 5 min at 68 °C. The PCR products were purified from an excised band of agarose gel (1.5%) electrophoresis with Zymoclean Gel DNA Recovery kit (Zymo, Orange, CA). Gel-purified products were quantified using a NanoDrop. Approximately 10 ng of the products were treated with 1 U of T4 DNA polymerase (Invitrogen) for 30 min at 37 °C followed by inactivation for 20 min at 75 °C in order to expose 5' LIC overhangs. The T4-treated DNA was inserted into the pET-30 Ek/LIC vector (Novagen), and the target-inserted vector was transformed into TOP10 competent cells (Invitrogen). Insertion-positive clones were cultured, and plasmid was purified as described above. After quantification of purified plasmid, we confirmed that the inserts were without mutation by sequencing. The purified plasmid was then transformed into BL21(DE3) competent cells (Novagen).

Expression: BL21(DE3) cells were inoculated in 5 mL of LB medium containing kanamycin (50 µg/mL) and incubated at 37 °C overnight with constant shaking, and the overnight culture was inoculated into SOC medium containing kanamycin (50 µg/mL) at a volume ratio of 1:100. After incubation for 2.5 hours at 37 °C with constant shaking, isopropyl

β -D-1-thiogalactopyranoside (IPTG) (0.1 mM final) was added to induce protein production. The induction culture was incubated for 17-24 hours at 20 °C with constant shaking, and the cells were collected by centrifugation for 10 min at 4500 \times g. The collected cells were resuspended in lysis solution (50 mM NaH₂PO₄, 10 mM imidazole, 300 mM NaCl, pH 8.0) containing 1 mg/mL lysozyme and incubated overnight at 4 °C with constant shaking. Then the suspended cells were lysed by two freeze-thaw cycles between -80 °C and room temperature followed by sonication for ~300 cycles (Branson sonifier 450, Danbury, CT: Duty cycle 50%, output 1). The lysate was centrifuged for 10 min at 13800 \times g, and supernatant containing the recombinant protein was transferred to a clean tube followed by addition of 0.3-0.5 mL of PerfectPro Ni-NTA Superflow Ni²⁺-charged resin (5 PRIME, Gaithersburg, MD). After incubation for 3 hours at 4 °C to allow His-tagged protein to bind to the resin, the resin was washed with 2 resin volumes of 60-140 mM imidazole solution (containing 300 mM NaCl and 50 mM NaH₂PO₄ pH 8.0) and 140-220 mM imidazole solution followed by protein elution with 3 resin volumes of 420-460 mM imidazole solution. To remove imidazole, NaCl and NaH₂PO₄, eluent was filtered through Amicon Ultra-4 3000 MWCO (Millipore, Billerica, MA) by centrifugation at 3500 \times g, and the protein was resuspended with 1-1.5 mL HPLC grade H₂O (J.T. Baker, Phillipsburg, NJ). The size and yield of the protein was checked by SDS-PAGE. Briefly, the protein solution was mixed with SDS sample buffer and boiled for 5 min before loading onto a 4-20% gradient polyacrylamide gel (Lonza, Basel, Switzerland). After electrophoresis, the gel was stained with Coomassie Brilliant blue (CBB).

Generation of point mutants: The Quick Change II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used to construct point mutants at the putative active residues determined by comparison to the active site of bovine pancreatic trypsin inhibitor in double His-

tagged SV-66. Briefly, primers containing a mutated codon were designed. The sequence of the primers were: for V13A mutation (V13A-UA: CCGGTGGACGAAGGTGCATGTAGAGCGTTATTC, V13A-DA: GAATAACGCTCTACATGCACCTTCGTCCACCGG), for C14A mutation (C14A-UA: AATCTTCCGGTGGACGAAGGTGTAGCTAGAGCGTTATTCA, C14A-DA: TGAATAACGCTCTAGCTACACCTTCGTCCACCGGAAGATT), for R15A mutation (R15A-UA: CTTCCGGTGGACGAAGGTGTATGTGCAGCGTTATTCAAGC, R15A-DA: GCTTGAATAACGCTGCACATACACCTTCGTCCACCGGAAG), and K19A mutation (K19A-UA: GGTGTATGTAGAGCGTTATTCGCGCGTTTTTACTACGAACCC, K19A-DA: GGGTTCGTAGTAAAAACGCGCGAATAACGCTCTACATACACC). Mutations were generated by PCR using each pair of primers and the purified plasmid from secondary cloning described above as a template with following thermal profile: 30 sec at 95 °C, 16 cycles of 30 sec at 95 °C, 1 min at 55 °C and 6 min at 68 °C. The PCR products were treated with *Dpn* I for 1 hour at 37 °C to destroy methylated template plasmid, and the resultant plasmids containing the mutation were transformed into XL-II Blue Supercompetent cells (Stratagene). After transformation-positive cells were recovered, the plasmid was purified and the mutation was confirmed by sequencing. Then, the plasmid was transformed into BL21(DE3) competent cells. Expression of mutant recombinant proteins was performed as described above.

HPLC purification of the recombinant proteins

The recombinant proteins were purified further by reversed-phase high-performance liquid chromatography (RP-HPLC). Five hundred μ L of the protein solution was separated with a Jupiter C4 column (5 μ m particle size, 300 Å pore size, 250 mm length \times 2.00 mm ID) (Phenomenex, Torrance, CA) by linear gradient between the stationary phase (95% H₂O, 5%

acetonitrile (ACN), 0.05% trifluoroacetic acid (TFA)) and the mobile phase (95% ACN, 5% H₂O, 0.03% TFA) monitored at 220 nm. Fractions at the elution peak were collected every minute and lyophilized to remove ACN and TFA. Lyophilized fractions were resuspended in 20 mM Tris pH 8.0 and quantified by the BCA method described above. Purity of the proteins was assessed by SDS-PAGE and immunoblotting. SDS-PAGE was performed as described above. The separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio Rad, Hercules, CA). After the transfer, the membrane was incubated in blocking buffer (1× PBS containing 0.05% Tween-20 and 2% no-fat skim milk) for 1 hour at room temperature followed by incubation with an anti-His primary antibody (rabbit IgG) (1:5000). The membrane was washed three times with washing buffer (1× PBS containing 0.05% Tween-20), incubated with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:10000) and visualized by chemiluminescence using the ECL Plus Western Blotting system (GE Healthcare, Piscataway, NJ).

Recalcification time assay

Recalcification time assays were performed to examine anti-coagulation activity of the recombinant proteins as described by Valenzuela *et al.* (1996). Briefly, 10 µL of various concentrations of recombinant proteins, 40 µL of 0.15 M NaCl, 10 mM HEPES pH7.4 and 50 µL of citrated human plasma (TriniCHECK Level 1, Trinity Biotech, Co Wicklow, Ireland) were mixed in a flat-bottomed 96-well plate and prewarmed at 37 °C for 15 min. To initiate plasma clotting, 50 µL of 25 mM CaCl₂ prewarmed at 37 °C was added in the 96-well plate. Immediately after the addition of CaCl₂ absorbance was taken at 650 nm at 10-sec intervals by microtiter plate reader at 37 °C (SpectraMax 340, Molecular Devices, Sunnyvale, CA). Absorbance at 650 nm increases in a sigmoidal manner as plasma clots, and clotting onset time

was set at the time when the absorbance reached an optical density (OD) of 0.04, where the increase in the absorbance is linear. This value was used as the “Clotting Time”. A t-test was used for the statistical analysis of the observed clotting time, and when $p < 0.05$, the difference was considered as statistically significant.

Serine protease inhibition assays (this part was performed in collaboration with Michail Kotsyfakis, Institute of Parasitology, Czech Republic)

For the initial screen, 500 nM of the protein was pre-incubated with each enzyme for 10 minutes before the addition of the corresponding substrate. After incubation for 5 min at 30 °C, substrate hydrolysis rate was followed in a Spectramax Gemini XPS 96 well plate fluorescence reader (Molecular Devices, Sunnyvale, CA) using 365 nm excitation and 450 nm emission wavelength with a cutoff at 435 nm for 20 min at 30 °C. The wells containing only the inhibitor and the substrate were also used to monitor spontaneous substrate hydrolysis and protease contamination in the inhibitor preparation. A t-test was used for the statistical analysis of the observed inhibition in the presence of 500 nM SV-66 and when $p < 0.05$, it was considered as statistically significant. Apparent inhibition constants of sialostatin L for various proteases were obtained as described by (Kotsyfakis *et al.*, 2007) by measuring the loss of enzymatic activity at increasing concentrations of inhibitor in the presence of a fluorogenic substrate in large excess.

All enzymes used were of human origin, purified or recombinant. Thrombin, α -chymotrypsin, plasmin and chymase were purchased from Sigma (St. Louis, MO), β -tryptase was purchased from Promega (Madison, WI), activated coagulation factor X (FXa) was purchased from EMD Biosciences (La Jolla, CA), FXIIa was purchased from Haematologic Technologies Inc. (Essex Junction, VT), kallikrein was purchased from Fitzgerald Industries International (Concord, MA), elastase was purchased from Elastin Products (Owensville, MO),

FXIa, urokinase-type plasminogen activator (u-PA) and tissue plasminogen activator (t-PA) from Molecular Innovations (Southfield, MI), matriptase from R&D Systems (Minneapolis, MN), cathepsin G from Enzo Life Sciences (Plymouth Meeting, PA) and sequencing grade trypsin was purchased from Roche (Chicago, IL). The amount of enzyme used in each assay is shown in the Table 4.1.

The assay buffers were: 1) for elastase and chymase, 50 mM HEPES buffer pH 7.4, 100 mM NaCl, 0.01% Triton X-100; 2) for trypsin, α -chymotrypsin, factor XIa, factor XIIa and thrombin, 50 mM Tris buffer pH 8.0, 150 mM NaCl, 20 mM CaCl₂, 0.01% Triton X-100; 3) for β -tryptase, 50 mM Tris pH 8.0, 50 mM NaCl, 0.05% Triton X-100; 4) for kallikrein, matriptase and plasmin, 20 mM Tris buffer pH 8.5, 150 mM NaCl, 0.02% triton X-100; 5) for factor Xa, 20 mM Tris buffer pH 8.0, 200 mM NaCl, 5 mM CaCl₂, 0.1%BSA; 6) for u-PA and t-PA, 20 mM Tris buffer pH 8.5, 0.05% Triton X-100; 7) for cathepsin G, 50 mM Tris buffer pH 7.4, 150 mM NaCl, 0.01% Triton X-100.

The substrates used were Suc-Ala-Ala-Pro-Val-AMC for elastase, Boc-Asp-Pro-Arg-AMC for thrombin and plasmin, Boc-Gln-Ala-Arg-AMC for trypsin, factor XIa and u-PA (Sigma, St. Louis, MO), Boc-Phe-Ser-Arg-AMC for β -tryptase, Suc-Leu-Leu-Val-Tyr-AMC for chymase (Bachem, King of Prussia, PA), Suc-Ala-Ala-Pro-Val-AMC for α -chymotrypsin and chymase (EMD Biosciences, La Jolla, CA), methylsulfonyl-D-cyclohexylalanyl-Gly-Arg-AMC acetate for factor Xa, factor XIIa, t-PA, matriptase and kallikrein (American Diagnostica Inc., Stamford, CT). All substrates were used in 250 μ M final concentration in all the assays.

3-dimensional (3D) structures by homology modeling

Homology modeling of SV-66 was performed using the SWISS-MODEL server (<http://swissmodel.expasy.org/>). Crystal structure of bovine pancreatic trypsin inhibitor (BPTI),

the most extensively-studied Kunitz type serine protease inhibitor was used as a template (PDB ID: 4PTI). Resulting 3D structures were visualized using PDB viewer software DeepView (ver. 4.0: <http://spdbv.vital-it.ch/>).

Results

SV-66 and SV-170 encode conserved Kunitz proteins

Coding sequences for SV-66 and SV-170 consist of 309 and 237 nucleotides, respectively, and the translated polypeptides consist of 102 and 78 amino acid residues (Figure 4.1). SignalP predicted signal sequences for SV-66 and SV-170 of 19 and 22 amino acids. We therefore assigned residue numbers based on the predicted mature proteins and indicated signal sequence residues as negative numbers (Figure 4.1). Alignment to selected other Kunitz-domain containing proteins indicated that SV-66 and SV-170 exhibited six conserved cysteine residues and other conserved residues (Figure 4.2). SV-66 has a conserved basic (Arg) P₁ residue at position 15 from the N-terminal end of the putative mature peptide, while SV-170 has Thr at the P₁ position that suggested a lack of a canonical inhibitory activity against trypsin-like serine proteases similar to the C-terminal Kunitz domain of boophilin (Macedo-Ribeiro *et al.*, 2008).

Cloning and expression of SV-66 and SV-170

We expressed SV-66 and SV-170 in a thioredoxin (Trx)-conjugated form (using pET-32 Ek/LIC vector (Novagen)) in a preliminary study. Trx-rSV-66 inhibited coagulation, while Trx-rSV-170 did not. When we attempted to cleave the N-terminal extension containing a Trx domain and a His tag using enterokinase, the enzyme could not cleave the N-terminal domain from the Trx-rSV-66, probably because Trx-rSV-66 has inhibitory activity against a broad range of serine proteases (see below). On the other hand, enterokinase not only cleaved the N-terminal extension of Trx-rSV-170, but also further degraded the protein. Therefore, we expressed SV-66

and SV-170 without a Trx domain (using pET-30 Ek/LIC vector (Novagen)) to see whether these proteins exhibit anti-coagulation activity without the Trx domain. Two-step purification by Ni²⁺ resin and RP-HPLC yielded relatively pure proteins (Figure 4.3). Therefore, we used recombinant proteins without Trx domain throughout this study; these proteins are referred to as rSV-66 and rSV-170.

rSV-66 inhibits plasma clotting, while rSV-170 does not

rSV-66 exhibited anti-coagulation activity in a dose-dependent manner (Figure 4.4). rSV-66 prolonged coagulation at concentrations as low as 12.5 nM, whereas rSV-170 did not exhibit anti-coagulation activity even at 400 nM (Figure 4.4). These results suggest that the recombinant Kunitz proteins could fold properly when expressed in a bacterial system, and that a trace amount of bacterial components remaining after purification did not interfere with the coagulation assays.

Residues in the reactive site loop (RSL) are important for rSV-66 anti-coagulation activity

Since canonical Kunitz inhibitors interact with target proteases using the reactive site loop (RSL), we asked whether introducing mutations, especially at the RSL, affects the inhibitory activity. We expressed the mutant forms, each of which contained a point mutation at residue 13 (Val to Ala), 14 (Cys to Ala), 15 (Arg to Ala) or 19 (Lys to Ala). We analyzed the expressed and purified recombinant proteins including wild type (WT) by immunoblot after the two-step purification (Figure 4.5). The WT and mutant forms made in double-His tag form exhibited double-bands in SDS-PAGE analysis after RP-HPLC purification. Tryptic digestion and mass spectrometry analysis verified that both bands contained intact recombinant SV-66 proteins (not shown). The low molecular-weight band appears to be the result of loss one of the tags, which should not affect the RSL or activity. The relative proportion of the “small” and

“large” bands was also consistent across all the expressed variants of the protein. Thus, we used microgram notations instead of molar concentrations for the coagulation assay as calculation in molar concentrations was impossible. Mutation in the RSL reduced anti-coagulation activity significantly; in particular the C14A and R15A mutants almost completely lacked anti-coagulation activity (Figure 4.6). The V13A mutant exhibited weak, but statistically significant anti-coagulation activity. In contrast, a mutation outside the RSL (K19A) had little effect on anti-coagulation activity as the coagulation time did not differ significantly from WT (Figure 4.6).

rSV-66 inhibits activity of multiple proteases including coagulation factors

As rSV-66 inhibited enterokinase as well as coagulation, rSV-66 likely targets multiple serine proteases. We therefore examined its inhibitory activity against 15 different serine proteases, and 10 proteases were inhibited with statistical significance (Figure 4.7). In the coagulation cascade rSV-66 significantly inhibited FXa and FXIa, but not thrombin or FXIIa.

We further investigated the IC_{50} s of rSV-66 to the strongly inhibited proteases to determine the affinity of rSV-66 to the targets (Table 4.1). Table 4.1 shows the IC_{50} s and ratios of IC_{50} s to the concentrations of the enzymes used. The smallest ratio was 27.2 obtained for elastase, which indicates that more than 27-fold excess rSV-66 was needed to attain 50% inhibition of elastase, which indicates that rSV-66 was not a tight-binding inhibitor. The results suggest that rSV-66 was not a specifically tight-binding inhibitor of any of the proteases tested.

Discussion

We investigated the functions of *S. vittatum* salivary gland Kunitz family proteins by expressing the proteins in recombinant forms. We tested the recombinant proteins (rSV-66 and rSV-170) for their anti-coagulation activity by recalcification time assay. rSV-66 significantly delayed coagulation, but rSV-170 did not even at 400 nM (Figure 4.4).

The *in vitro* anti-clotting activity of SV-66 may account for the anti-FXa activity of the black fly SGE described previously (Jacobs *et al.*, 1990). However, SV-66 might not be the major component of the anti-FXa activity of SGE since the estimated size of the FXa inhibitor was 18000 Da, which does not match the native size of SV-66 (9627.22 Da, without signal peptide). Although SV-66 has a putative O-glycosylation site in the N-terminal region, 1D SDS-PAGE gel band from which SV-66 fragments were recovered migrated between 14.1 and 6 kDa markers (Andersen *et al.*, 2009), which indicates that the native size of SV-66 does not match the previously found anti-FXa (Jacobs *et al.*, 1990). Nevertheless, SV-66 could act as an anti-FXa in the black fly saliva as well as an inhibitor of other proteases (see below), whose presence was not detected previously.

SV-66 is a BPTI-like Kunitz serine protease inhibitor

Since SV-66 has conserved residues in the RSL, especially at the P₁ position (basic Arg residue: R15), we generated point mutants to examine the effect of mutation on the inhibition of coagulation. R15A mutation almost completely abolished the inhibition of coagulation, as did the C14A mutation, while K19A mutation, which is not in the RSL, had almost no effect on the activity (Figure 4.6). This result suggests that SV-66 acts like BPTI, whose Lys15 residue is critical for inhibition of trypsin and chymotrypsin (Tschesche *et al.*, 1987). R15A mutation could have significantly reduced access to the catalytic active site of FXa and FXIa (the target coagulation factors), and C14A mutation could have disrupted the stable structure of the RSL by eliminating the disulfide bond, resulting in a significantly reduced inhibitory activity.

We performed homology modeling to visualize the structural change induced by the mutations using the SWISS-MODEL server (<http://swissmodel.expasy.org/>) with the crystal structure of BPTI (PDB ID: 4TPI) as a template. Figure 4.8 shows the homology-modeled 3D

structures of WT and mutant SV-66. The models show the side chain of mutated residues in magenta with red label (Figure 4.8). R15A mutation could have changed the shape significantly, specifically in the RSL, which limited the access of RSL to the active site cleft of coagulation factors. Moreover, in BPTI, the basic residue Lys at P₁ site is important in stabilizing docking into the S₁ specificity subsite of trypsin by forming polar interactions with a negatively charged Asp189 side chain (Ascenzi *et al.*, 2003). Therefore, replacement of the basic side chain in the P₁ site of SV-66 could reduce the stable interaction of the inhibitor with the enzyme, specifically at the enzyme's specificity subsite. In addition, the change may be quite small in the V13A mutation, but it reduced the inhibition of clotting, which suggests that even a slight change in RSL can alter the effect (specificity/affinity) of the inhibitor as the RSL interacts directly with the catalytic cleft.

The K19A mutation could have also changed the shape to a similar degree to R15A, but the change was not in the RSL, and the effect in clotting inhibition was negligible due to a minimal effect in interaction between the target factors and rSV-66. Considering all the results and modeling studies, it is highly likely that SV-66 uses its RSL in inhibition of coagulation factors in a similar fashion to BPTI acting on trypsin and trypsin-like enzymes (Ascenzi *et al.*, 2003).

Screening of rSV-66 against 15 serine proteases revealed that SV-66 inhibits a wide range of proteases, including multiple factors of the coagulation cascade (FXa, FXIa and kallikrein), the fibrinolytic enzyme, plasmin, the major components of neutrophil azurophil granules (elastase and cathepsin G), the proteases in mast cells (tryptase, chymase and cathepsin G) and the digestive enzymes (trypsin and chymotrypsin). Except for the digestive enzymes, all the enzymes inhibited by SV-66 regulate immune responses, in many cases in addition to their

major functions (Levi and van der Poll, 2010; Pham, 2006, 2008; Trivedi and Caughey, 2010). The regulation of immune responses by these enzymes is quite complex. To mention a few examples, some protease-activated receptors (PARs) can be activated by FXa, which results in upregulation of inflammatory responses (activation of macrophages, neutrophil infiltration, and expression of proinflammatory cytokines (TNF- α and IL-1 β)) (Levi and van der Poll, 2010). Fibrinogen and fibrin, the latter being the substrate of plasmin, directly influence the production of proinflammatory cytokines including TNF- α and IL-1 β , which suggests that the balance between fibrinogen and fibrin may be an important factor, hence the level of plasmin activity may also be involved in this regulation (Levi and van der Poll, 2010). The major function of elastase and cathepsin G is killing internalized microbes in neutrophils. Exocytosis of the azurophil granules is limited, but extracellular release of these enzymes has critical roles in regulation of immune responses. Cathepsin G cleaves the N-terminal region of chemokines CXCL5 and CCL15, leading to enhanced chemotactic potency toward neutrophils and monocytes, respectively, while cathepsin G digests CCL5 (RANTES), and elastase and cathepsin G digest CCL3 and CXCL12 (and its cognate receptor CXCR4) leading to decreased chemotactic activity (Pham, 2006, 2008). These conflicting immune effects of azurophil granule proteases indicate that inhibition of these proteases may either inhibit or promote leukocyte recruitment to the bite site depending on local conditions (Pham, 2006, 2008). The azurophil granule proteases including elastase and cathepsin G also regulate functions of cytokines, cell surface receptors and adhesion molecules either positively or negatively (Pham, 2006, 2008). Mast cell-derived proteases also have some functions in inflammation (Trivedi and Caughey, 2010). Tryptase may be involved in some inflammatory pathogenesis such as arthritis and asthma (Trivedi and Caughey, 2010), and chymase and cathepsin G degrade IL-6 and IL-13

released from mast cells (Zhao *et al.*, 2005). The functions of these proteases may be relevant in the actual bite site, the skin, since mast cells are the resident cells in the skin, and neutrophils are recruited to the bite site during the inflammatory response. Modulation of immune response in the skin by SV-66 in black fly saliva could happen in order to modify the microenvironment in favor of their blood feeding. However, which direction (i.e., pro-inflammatory, anti-inflammatory or more specific manner) SV-66 modifies the response and the physiological relevance *in vivo* remain to be uncovered.

We demonstrated that rSV-66 not only has anticoagulation activity, but also has inhibitory activity against a wide-range of serine proteases, while rSV-66 has been determined to bind to the targeted enzymes with moderate affinity. These observations suggest that SV-66 might have evolved to be a wide-range inhibitor of proteases, at the expense of being a tight binder of any specific serine protease. This property could confer to SV-66 the functions to inhibit coagulation and to inhibit or promote inflammation caused by the cells containing targeted enzymes (neutrophils, mast cells, and basophils).

In addition, the Kunitz protein, BPTI is the one of the most extensively studied globular proteins. Studies have elucidated the interaction of BPTI with different proteases by creating many mutant forms to assess affinity and molecular interaction (Ascenzi *et al.*, 2003). SV-66 might also be useful to study molecular interaction between enzymes and the inhibitor, especially using the enzymes inhibited in the present study.

Speculations regarding SV-170

In the sialome, SV-170 is more abundantly expressed than SV-66 in the transcript level (9 sequences vs 4 sequences per contig). The proteomic analysis also identified a peptide fragment whose mass matched a portion of SV-170 (Andersen *et al.*, 2009). These results

suggest that SV-170 is highly likely to be expressed in the salivary glands. Although absence of anti-coagulation activity may be due to the presence of His tags, it may be more likely due to the lack of conserved residues in the RSL as rSV-66 with His tags is active. SV-170 may have a different function rather than anti-coagulation or inhibition of proteases, analogous to disagregin found from the salivary glands of the soft tick, *Ornithodoros moubata* or dendrotoxins in snake venom (Harvey, 2001; Karczewski and Connolly, 1997; Karczewski *et al.*, 1994). Disagregin inhibits platelet aggregation by binding to the platelet fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa: integrin α_{IIb}/β_3) (Karczewski and Connolly, 1997; Karczewski *et al.*, 1994). Dendrotoxins are blockers of neuronal K^+ channels (Harvey, 2001). However, the amino-acid sequence identity of SV-170 either to disagregin or to dendrotoxins is quite low, and the function of SV-170 remains to be elucidated.

References

- Abebe, M., Cupp, M.S., Champagne, D., Cupp, E.W., 1995. Simulidin: a black fly (*Simulium vittatum*) salivary gland protein with anti-thrombin activity. *Journal of Insect Physiology* 41, 1001-1006.
- Abebe, M., Cupp, M.S., Ramberg, F.B., Cupp, E.W., 1994. Anticoagulant Activity in Salivary-Gland Extracts of Black Flies (Diptera, Simuliidae). *Journal of Medical Entomology* 31, 908-911.
- Abebe, M., Ribeiro, J.M.C., Cupp, M.S., Cupp, E.W., 1996. Novel anticoagulant from salivary glands of *Simulium vittatum* (Diptera: Simuliidae) inhibits activity of coagulation factor V. *Journal of Medical Entomology* 33, 173-176.
- Andersen, J.F., Pham, V.M., Meng, Z., Champagne, D.E., Ribeiro, J.M., 2009. Insight into the Sialome of the Black Fly, *Simulium vittatum*. *J Proteome Res.*
- Ascenzi, P., Bocedi, A., Bolognesi, M., Spallarossa, A., Coletta, M., De Cristofaro, R., Menegatti, E., 2003. The bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor): a milestone protein. *Curr Protein Pept Sci* 4, 231-251.
- Bernardo, M.J., Cupp, E.W., 1986. Rearing black flies (Diptera: Simuliidae) in the laboratory: mass-scale *in vitro* membrane feeding and its application to collection of saliva and to parasitological and repellent studies. *J Med Entomol* 23, 666-679.
- Corral-Rodriguez, M.A., Macedo-Ribeiro, S., Barbosa Pereira, P.J., Fuentes-Prior, P., 2009. Tick-derived Kunitz-type inhibitors as antihemostatic factors. *Insect Biochem Mol Biol* 39, 579-595.
- Crawley, J.T., Lane, D.A., 2008. The haemostatic role of tissue factor pathway inhibitor. *Arterioscler Thromb Vasc Biol* 28, 233-242.
- Cupp, M.S., Ribeiro, J.M., Champagne, D.E., Cupp, E.W., 1998. Analyses of cDNA and recombinant protein for a potent vasoactive protein in saliva of a blood-feeding black fly, *Simulium vittatum*. *J Exp Biol* 201, 1553-1561.
- Cupp, M.S., Ribeiro, J.M., Cupp, E.W., 1994. Vasodilative activity in black fly salivary glands. *Am J Trop Med Hyg* 50, 241-246.
- Emanuelsson, O., Brunak, S., von Heijne, G., Nielsen, H., 2007. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* 2, 953-971.
- Francischetti, I.M., Mather, T.N., Ribeiro, J.M., 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, 886-898.
- Francischetti, I.M., Sa-Nunes, A., Mans, B.J., Santos, I.M., Ribeiro, J.M., 2009. The role of saliva in tick feeding. *Front Biosci* 14, 2051-2088.

Gray, E.W., Noblet, R., 1999. Laboratory Rearing of Black Flies, in: Maramorosch, K., Mahmood, F. (Eds.), Maintenance of Human, Animal and Plant Pathogen Vectors. Oxford & IBH Publishing Co. PVT. LTD., New Delhi and Calcutta.

Harvey, A.L., 2001. Twenty years of dendrotoxins. *Toxicon* 39, 15-26.

Jacobs, J.W., Cupp, E.W., Sardana, M., Friedman, P.A., 1990. Isolation and characterization of a coagulation factor Xa inhibitor from black fly salivary glands. *Thromb Haemost* 64, 235-238.

Julenius, K., Molgaard, A., Gupta, R., Brunak, S., 2005. Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites. *Glycobiology* 15, 153-164.

Karczewski, J., Connolly, T.M., 1997. The interaction of disagregin with the platelet fibrinogen receptor, glycoprotein IIb-IIIa. *Biochem Biophys Res Commun* 241, 744-748.

Karczewski, J., Endris, R., Connolly, T.M., 1994. Disagregin is a fibrinogen receptor antagonist lacking the Arg-Gly-Asp sequence from the tick, *Ornithodoros moubata*. *J Biol Chem* 269, 6702-6708.

Kotsyfakis, M., Karim, S., Andersen, J.F., Mather, T.N., Ribeiro, J.M., 2007. Selective cysteine protease inhibition contributes to blood-feeding success of the tick *Ixodes scapularis*. *J Biol Chem* 282, 29256-29263.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947-2948.

Levi, M., van der Poll, T., 2010. Inflammation and coagulation. *Crit Care Med* 38, S26-34.

Liener, I.E., 1986. Trypsin inhibitors: concern for human nutrition or not? *J Nutr* 116, 920-923.

Macedo-Ribeiro, S., Almeida, C., Calisto, B.M., Friedrich, T., Mentele, R., Sturzebecher, J., Fuentes-Prior, P., Pereira, P.J., 2008. Isolation, cloning and structural characterisation of boophilin, a multifunctional Kunitz-type proteinase inhibitor from the cattle tick. *PLoS One* 3, e1624.

Maritz-Olivier, C., Stutzer, C., Jongejan, F., Neitz, A.W., Gaspar, A.R., 2007. Tick anti-hemostatics: targets for future vaccines and therapeutics. *Trends Parasitol* 23, 397-407.

Pham, C.T., 2006. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol* 6, 541-550.

Pham, C.T., 2008. Neutrophil serine proteases fine-tune the inflammatory response. *Int J Biochem Cell Biol* 40, 1317-1333.

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

Trivedi, N.N., Caughey, G.H., 2010. Mast cell peptidases: chameleons of innate immunity and host defense. *Am J Respir Cell Mol Biol* 42, 257-267.

Tschesche, H., Beckmann, J., Mehlich, A., Schnabel, E., Truscheit, E., Wenzel, H.R., 1987. Semisynthetic Engineering of Proteinase-Inhibitor Homologs. *Biochimica Et Biophysica Acta* 913, 97-101.

Valenzuela, J.G., Guimaraes, J.A., Ribeiro, J.M., 1996. A novel inhibitor of factor X activation from the salivary glands of the bed bug *Cimex lectularius*. *Exp Parasitol* 83, 184-190.

Zhao, W., Oskeritzian, C.A., Pozez, A.L., Schwartz, L.B., 2005. Cytokine production by skin-derived mast cells: endogenous proteases are responsible for degradation of cytokines. *J Immunol* 175, 2635-2642.

SV-66

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TGAATTGGATCGAAATGCAATATACTTCCAATAAGTGCCTTCTTCCTGCTATATCTTGGCCATTCTTTGGCCCAA
            M N I L P I S A F F L L Y L G H S L A Q      
      -19                                     -10                                     1

GAGAACGTTTGCAATCTTCCGGTGGACGAAGGTGTATGTAGAGCGTTATTCAAGCGTTTTTACTACGAACCCGCA
E N V C N L P V D E G V C R A L F K R F Y Y E P A
      10                                     20

ACCGATAGTTGCAAAGAGTTCTACTATGGAGGTTGTGAGGGAAATGGGAACAGGTTCAAAAGTAAAAGGAATGC
T D S C K E F Y Y G G C E G N G N R F K S K K E C
      30                                     40                                     50

ATTCTCAAGTGTGAGAAGAATAAACAGCTCATAAAAAACAAGAAAACGAAACCAAAAAAGACAACCAAAACCCCGG
I L K C Q K N K Q L I K T R K R K P K K T T K P P
      60                                     70

ATACCAATTATTTGTTGGACTTAAAAAGGACATTCAAACCTAAGTTATAGACAAACATTTATATTTTACAATTACT
I P I I S L D -
      80

TGAAAAATAAAATCGAACTGTGAAAAATTTTAATTTGACCAGAAAAA
  
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SV-170

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GCATTAATCATTAGTACACCTGAGAGAATCTTCTGCGTCAAAGTATGTTGAAAACAATCATCCTCGGAACAATC
            M L K T I I L G T I      
      -22

GCCATACTGATATGTATGGCAAACAATTCTGAAGCAAAGTCAGCTGACATCTGCAGACTTCCAATGGATAAAGGT
      A I L I C M A N N S E A       K S A D I C R L P M D K G
      -10                                     1                                     10

ATTTGCACTCCAACAGAATGGCGTTATCATTTTTGACCCAGCGAAAAACAAGTGCCTTTATGTTTCCGTGGGGATGC
I C T P T E W R Y H F D P A K N K C F M F P W G C
      20                                     30

CTTGGGAATGCGAACAATTTCAAAACAGACAAGAATGTAAAGCCAAGTGTATGTTAAACAAGACAGCACAAGAGGA
L G N A N N F K T R Q E C K A K C M -
      40                                     50

TAAAAATGTATTGACCGATTGTTATTAATAAGGCATAACAATTTGATAGACAAAACCTGAAAAA
AAAAAA
  
```

Figure 4.1: Nucleotide sequences and translated polypeptide sequences of SV-66 and SV-170. Start and stop codons are in white with black shading. Numbers below the amino acid residues are designated based on putative mature protein. Signal sequences predicted by SignalP are underlined. Top: SV-66 encodes 102 amino-acid polypeptide, which includes a 19 amino-acid signal sequence on the N terminus. The mature SV-66 is predicted to consist of an 82 amino-acid residues, and theoretical mass and pI are 9627.22 Da and 9.93, respectively. SV-66 also contains a putative O-glycosylation site at position 81 (Ser). Bottom: SV-170 encodes a 78 amino-acid polypeptide, which includes a 22 amino-acid signal sequence on the N terminus. The mature SV-170 is predicted to consist of 56 amino-acid residues, and theoretical mass and pI are 6526.66 Da and 8.87, respectively.

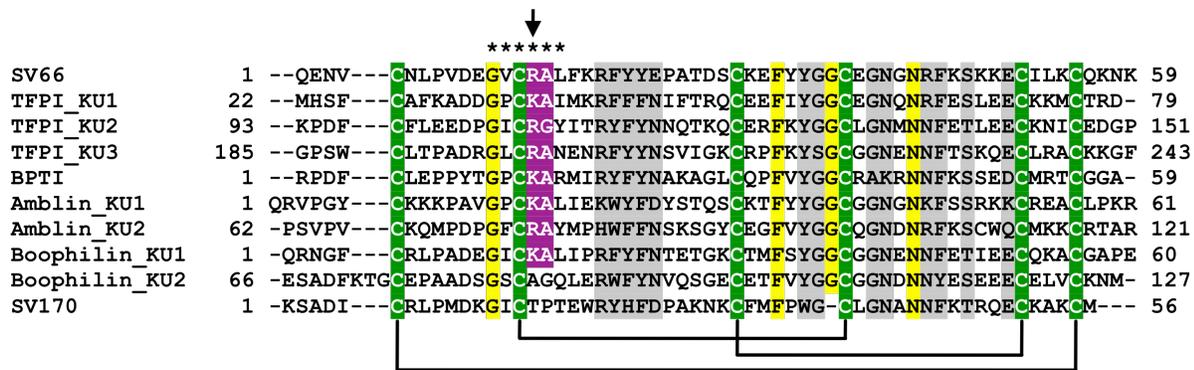


Figure 4.2: Alignment of Kunitz domain sequences from different animal species. Each Kunitz domain was separated from the original sequences for alignment (numbers denote amino-acid positions in the original mature peptides). All reference sequences were retrieved from GenBank. Accession numbers are: TFPI (human: 3 Kunitz), P10646; BPTI (*Bos taurus*: 1 Kunitz), AAI49369; Amblin (*Amblyomma hebraeum*: 2 Kunitz), AAR97367; Boophilin (*Rhipicephalus microplus*: 2 Kunitz), CAC82583. Strictly conserved cysteine residues are white with green shading, and typical disulfide bonds are shown in solid lines. The reactive site loop (RSL) for canonical binding inhibitors (P₄-P₂') are indicated by asterisks. P₁ residue is indicated with an arrow. Highly conserved P₁-P₁' (Arg/Lys-Ala/Gly) residues are white with purple shading. Other identical residues across the modules are shaded with yellow and conserved or semi-conserved residues are shaded with grey. Note that SV-170 has a Thr substitution instead of a basic residue at P₁ position.

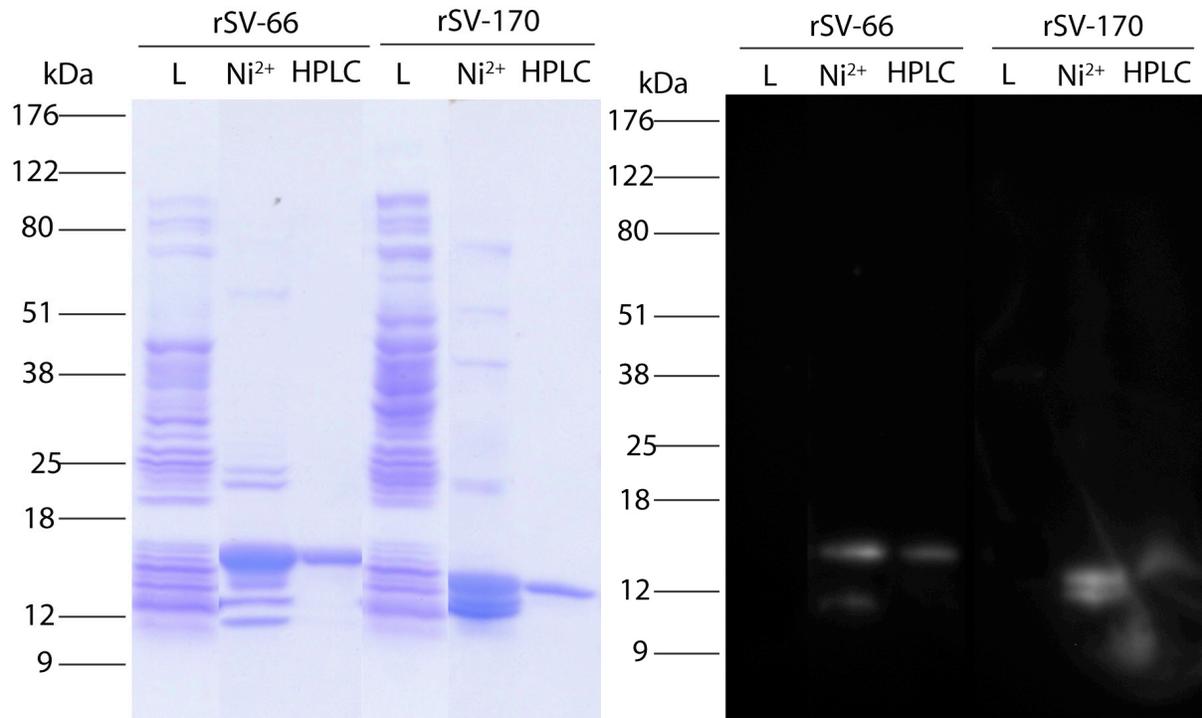


Figure 4.3: SDS-PAGE (left) and immunoblot (right) analyses of recombinant proteins. Bacterial lysate (L lanes) was purified by Ni²⁺ resin (Ni²⁺ lanes). Ni²⁺ resin-purified protein was further purified by RP-HPLC (HPLC lanes).

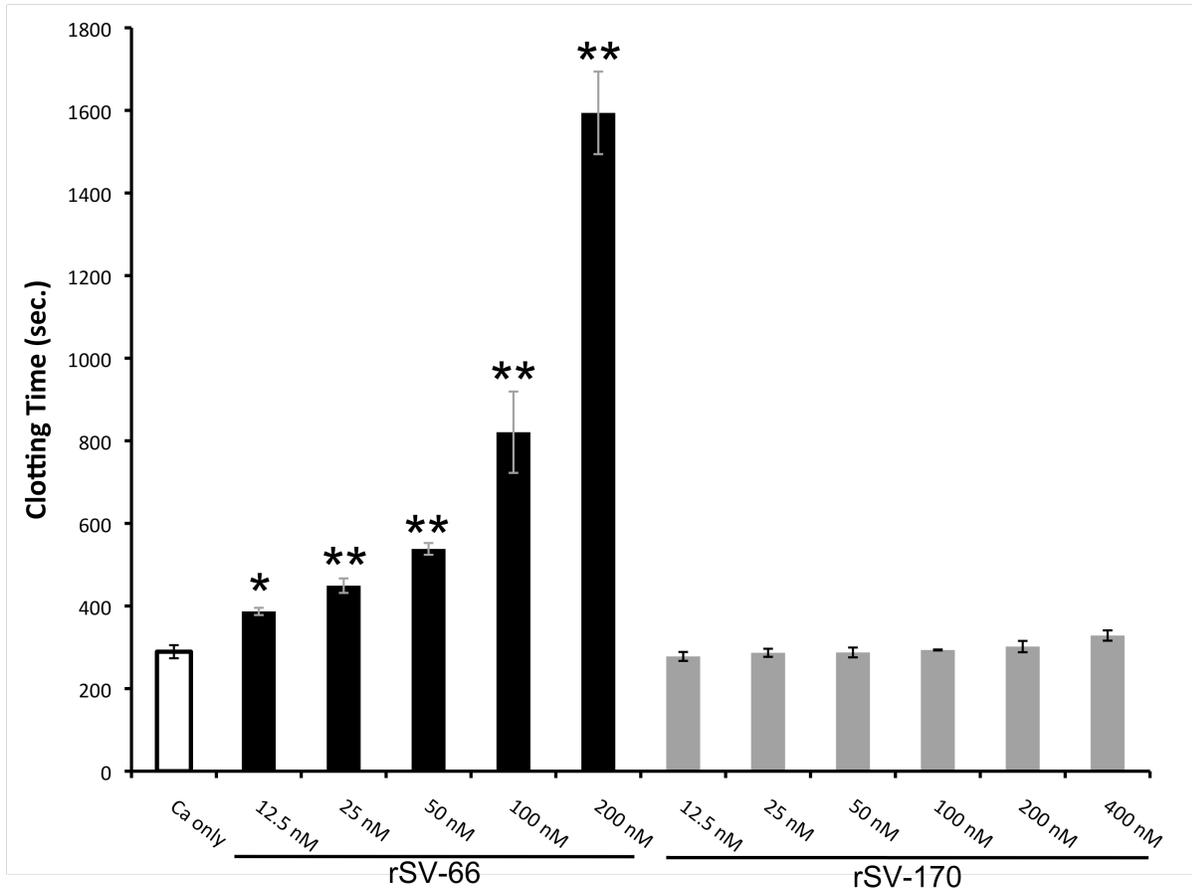


Figure 4.4: Inhibition of plasma clotting by recombinant black fly salivary Kunitz proteins. Various concentrations of rSV-66 and rSV-170 were tested by recalcification time assay. Plasma clotting was initiated by the addition of CaCl₂ (25 mM). Clotting time for rSV-66 and rSV-170 are shown in black bars and grey bars, respectively. White bar is Ca²⁺-only control. The graph shows mean \pm SD. * and ** indicate statistical significance at $p < 0.05$ and $p < 0.01$, respectively by one-sided t-test, where the alternative hypothesis is that Ca²⁺-only control is smaller than the sample. Representative of three independent experiments.

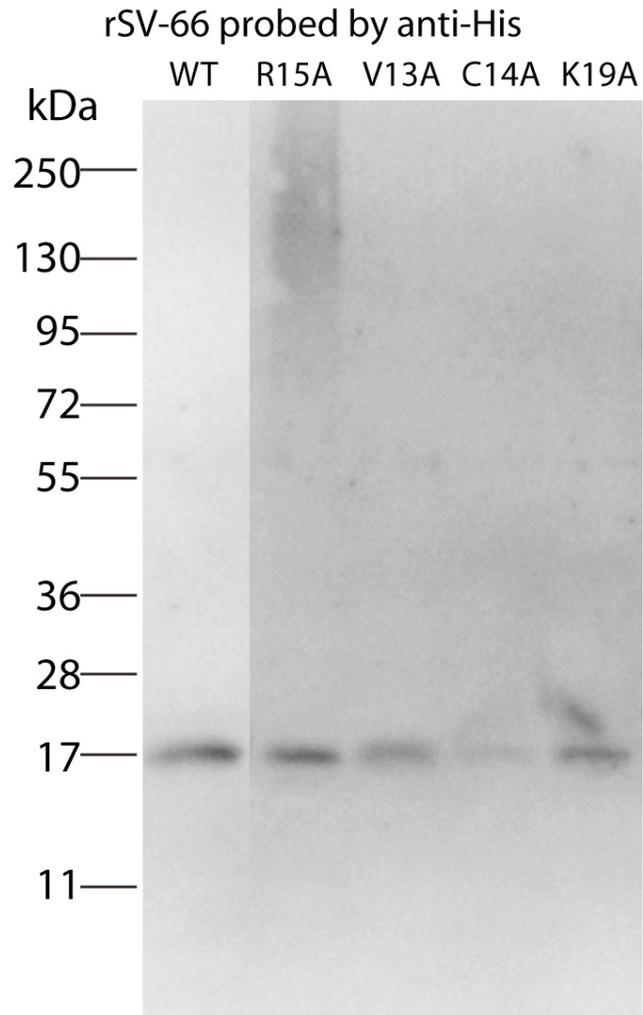


Figure 4.5: Wild type and mutant rSV-66 were analyzed by immunoblot using anti-His antibody as the primary antibody. RP-HPLC-purified recombinant proteins were used. Sizes are shown in kDa on the left.

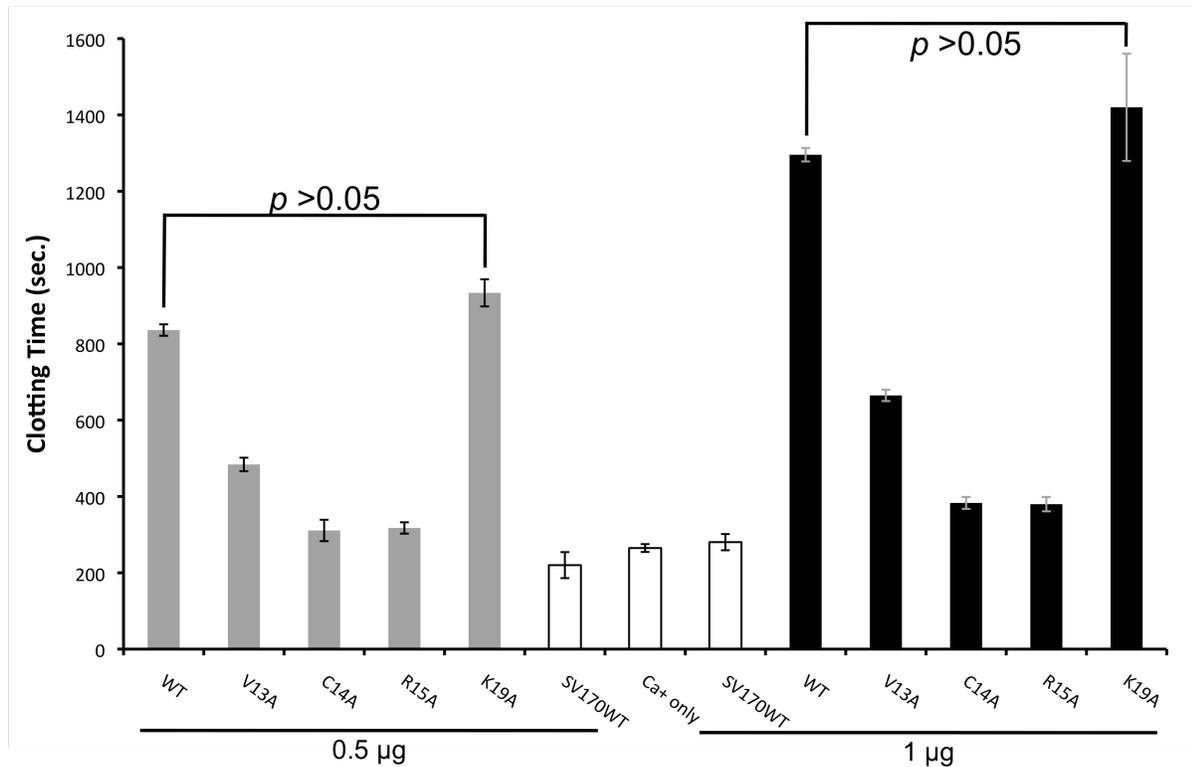


Figure 4.6: Recalcification time assay with rSV-66 mutants. 0.5 µg/well (grey bars) and 1 µg/well (black bars) were tested. Plasma was mixed with recombinant proteins and pre-warmed at 37 °C for 15 min before addition of 8.3 mM (final concentration) CaCl₂ (pre-warmed) to initiate clotting. White bars are Ca²⁺-only control and rSV-170. The graph shows mean ± SD. *p*-values for non-significant differences by one-sided t-test are shown, where the alternative hypothesis is that WT is greater than the sample compared. Representative of three independent experiments.

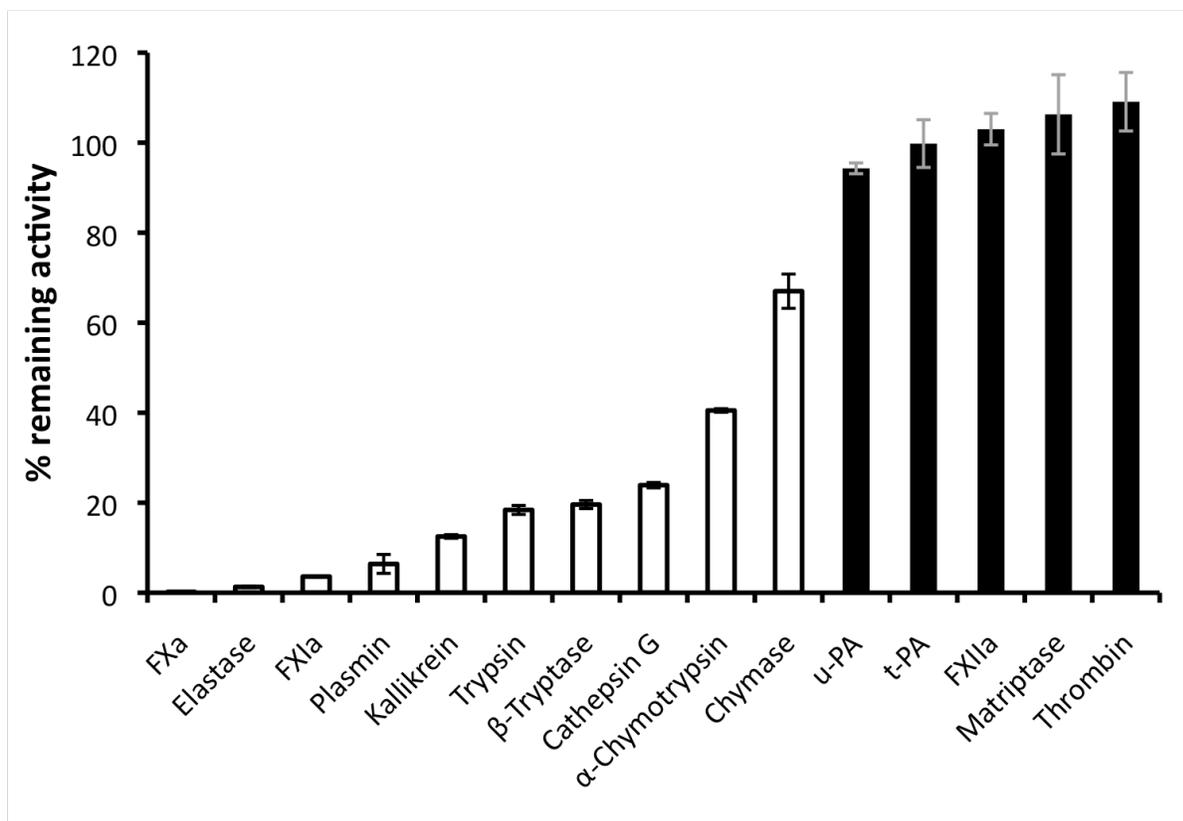


Figure 4.7: Inhibitory activity of rSV-66 against 15 serine proteases. Enzyme activity in the presence of 500 nM of rSV-66 is shown in the graph. Results with statistically significant differences compared to the controls are shown in white bars (all are at the level of $p < 0.01$). The graph shows mean \pm SEM. u-PA: urokinase-type plasminogen activator, t-PA: tissue plasminogen activator.

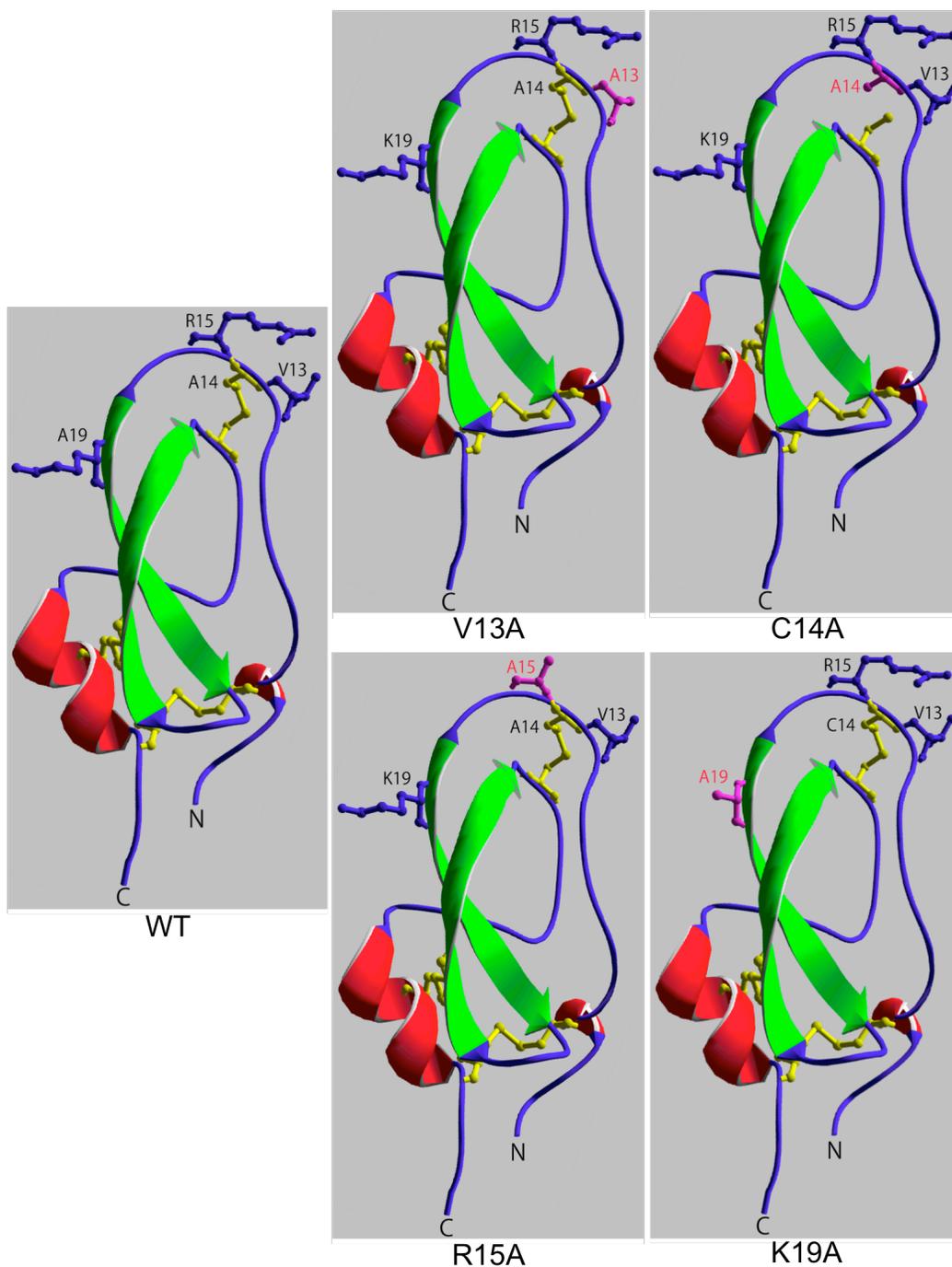


Figure 4.8: Homology-modeling 3D structures of SV-66 (WT and mutants). The 3D structures were generated by SWISS-MODEL server using BPTI (PDB ID: 4PTI) as a template. In the ribbon structures, α -helices are shown in red, β -sheets are shown in green, and loops are shown in blue. Side chains of mutated residues and Cys are shown. The Cys residues forming disulfide bonds are shown in yellow and mutated residues are shown in magenta with a label in red. Other residues are shown in blue with a label in black. Oxygen atoms in the backbone of the residues are not shown. N and C indicate N and C termini, respectively.

Table 4.1: IC₅₀ values for SV-66 in nM against various serine proteases. Titrated concentrations of SV-66 were tested with constant concentrations of enzymes (in Concentration column) to determine the concentrations of SV-66 that gave a 50% inhibition of the enzyme activity.

*Ratios of IC₅₀ to the used enzyme concentration are also shown as different concentrations of enzymes were necessary to obtain linear reaction rates.

Enzyme	Concentration (nM)	IC₅₀ (mean ± SEM in nM)	Ratio*
Elastase	0.18	4.9 ± 0.6	27.22
Factor Xa	0.1	5.2 ± 0.3	52.00
Plasmin	0.2	32.2 ± 5.2	161.00
Factor XIa	0.06	56.7 ± 13.1	945.00
β-Tryptase	0.01	66.8 ± 14.6	6680.00
Kallikrein	0.08	91.8 ± 6.3	1147.50
Cathepsin G	6.7	217.4 ± 5.7	32.45
Trypsin	0.1	379.3 ± 30.2	3793.00

CHAPTER 5

CONCLUSIONS

A number of studies have demonstrated that blood-feeding arthropods contain novel anti-hemostatic factors and immunomodulatory factors in the salivary glands (Ribeiro and Francischetti, 2003; Titus *et al.*, 2006). Despite the fact that black flies are important vectors of pathogens such as *Onchocerca* and vesicular stomatitis virus, only a few studies are available concerning the anti-hemostatic and immunomodulatory functions in the black fly salivary glands (Abebe *et al.*, 1995; Abebe *et al.*, 1994; Abebe *et al.*, 1996; Cross *et al.*, 1994; Cross *et al.*, 1993; Cupp and Cupp, 1998; Cupp *et al.*, 1993; Cupp *et al.*, 1998; Cupp *et al.*, 1994; Jacobs *et al.*, 1990). This dissertation follows up on these studies by characterizing the factor that inhibits murine lymphocyte proliferation and the potential mechanism of the inhibition. This dissertation also describes a putative anti-coagulation factor which is expressed in the black fly salivary glands.

In Chapter 2, I investigated the immunomodulatory effects of black fly salivary gland extract (SGE) using BALB/c mice. SGE significantly inhibited proliferation of concanavalin A (Con A)-stimulated splenocytes in a dose-dependent manner. Con A stimulates proliferation of T lymphocytes in an antigen-independent manner. The cell division analysis by flow cytometry using carboxyfluoresceine diacetate succinimidyl ester (CFDA-SE) coupled with antibodies against T cell markers CD4 and CD8 confirmed that SGE indeed inhibited cell division. As a potential mechanism of this inhibition, I examined SGE's ability to induce apoptosis in mouse

splenocytes. SGE strongly enhanced activation of caspase 3, the executioner enzyme in apoptotic pathway. The induction of apoptosis was also indicated by decrease in cell size. SGE induced apoptosis only in the cells positive for either CD4 or CD8, which suggests that SGE specifically targets T lymphocytes and inhibits their functions. The responsible factor for the inhibition was determined to be a protein of a size larger than 50 kDa.

Chapter 3 describes immunomodulatory effects of black fly SGE on splenocytes and peritoneal cells (PCs) using C3H/HeN (C3H) mice, which are considered to show more human-like immune responses than the BALB/c mice used in Chapter 2. SGE inhibited proliferation of C3H splenocytes stimulated with Con A to a similar degree to BALB/c splenocytes. SGE also inhibited proliferation of LPS-stimulated C3H splenocytes, albeit to a lesser degree compared to Con A-stimulated splenocytes. LPS stimulates the B-lymphocyte population in the splenocytes. The difference in inhibition could be explained by the observation that SGE induced apoptosis only in CD4⁺ and CD8⁺ cells in Chapter 2. The weak inhibition in B-cell proliferation may be due to non-apoptotic mechanisms because with BALB/c-derived splenocytes the induction of apoptosis was T-cell specific and did not reach 100% at the concentrations of SGE that inhibited T-cell proliferation almost completely, which suggests that there are additional mechanisms that inhibit cell proliferation.

I also examined the effect of SGE on peritoneal cells in Chapter 3. SGE exhibited a biphasic effect on nitric oxide (NO) production from LPS-stimulated peritoneal cells; low concentrations of SGE enhanced NO production, and a high concentration inhibited it. SGE enhanced NO production only in the presence of LPS, which suggested that SGE acts synergistically with LPS. Inhibition of NO production by a high concentration of SGE may be relevant at the vicinity of the bite site as the injection of the saliva is limited to a small volume of

the skin (therefore higher effective concentration). Pathogens like vesicular stomatitis virus which are injected together with saliva may potentially be benefitted by this inhibitory effect on the immune cells. Although enhancement of NO has rarely been observed with other arthropod SGE (Kyckova and Kopecky, 2006), a speculation may be made to explain this phenomenon. Massive attacks by black flies can sometimes cause death in livestock and wild animals (Currie and Adler, 2008), which is called simuliotoxicosis. Molecular mechanism of simuliotoxicosis is not well understood. Enhancement of NO production by SGE indicates hyperactivation of macrophages, which could lead to shock and death. Moreover, the product, NO, is a potent vasodilator, which may also be responsible for systemic vasodilation and shock. The biphasic effect of black fly SGE may be due to the presence of multiple components affecting the target cells (in this case, macrophages). At low concentrations, a factor or factors responsible for the activation enhances specifically LPS-mediated signal transduction, which leads to enhancement of NO production. The mechanism could be mimicking LPS-binding protein (LBP) or modifying the structure of LPS (for example, CD14-dependent “smooth” LPS and CD14-independent “rough” LPS) (Jiang *et al.*, 2005). At the high concentration, a factor or factors responsible for suppressing the NO production transduces the signal that dominates over activation signals. Alternatively, a salivary component could affect signal transduction by acting not on receptors, but on common mechanisms of signaling such as phosphorylation and de-phosphorylation.

Chapters 2 and 3 demonstrate that black fly SGE affects mouse lymphocytes derived from two different genetic backgrounds. Strong inhibition of T-cell proliferation and induction of apoptosis only in T cells by the SGE indicate that black fly SGE may target specifically T lymphocytes so as to prevent development of adaptive immune response. Furthermore, NO-producing macrophages are known to inhibit T-cell proliferation (Denham and Rowland, 1992;

Kawabe *et al.*, 1992), which implies that black fly SGE could indirectly inhibit T-cell proliferation by making macrophages NO producers. Overall, black fly SGE could specifically target T cells to inhibit development of adaptive immune responses, directly by inducing apoptosis and indirectly by modulating macrophages.

Previous studies described three distinct anti-coagulation factors from salivary glands of the black fly, *Simulium vittatum*, namely, anti-thrombin, anti-activated coagulation factor X (FXa) and anti-FV (Abebe *et al.*, 1995; Abebe *et al.*, 1996; Jacobs *et al.*, 1990). Among these, only the anti-thrombin has been characterized to the N-terminal amino-acid sequence (Jacobs *et al.*, 1990). Building on the recent release of the sialome (transcriptome combined with proteome of salivary glands) of *S. vittatum*, Chapter 4 characterizes the functions of the two Kunitz-family proteins, which are putative coagulation inhibitors. The two proteins were expressed in a bacterial system, and one of them (SV-66) exhibited anti-coagulation activity, while the other (SV-170) lacked anti-coagulation activity. Site-directed mutagenesis of SV-66 indicated that residues in the reactive site loop (RSL), especially the residue in the P₁ position (R15), are important in the inhibition of coagulation. Investigation of the specificity and estimation of affinity of the inhibitor against various serine proteases revealed that SV-66 is a wide-ranging inhibitor of serine proteases and not a tight-binding inhibitor of any of the tested proteases. SV-66 inhibited factors in the coagulation cascade (FXa, FXIa and plasmin) and other serine proteases found in neutrophils and mast cells as well as digestive enzymes, trypsin and chymotrypsin. The anti-FXa activity implied that SV-66 was the previously identified anti-FXa (Jacobs *et al.*, 1990), but the determined native sizes of two proteins do not match. Therefore we conclude that SV-66 is not the anti-FXa, but another anti-coagulant in the black fly salivary glands. Hence, *S. vittatum* has multiple factors that enforce the inhibition of coagulation. The

known anticoagulation activities including SV-66 in the black fly saliva are summarized in Figure 5.1. Except for the digestive enzymes, the target enzymes are known to have roles in regulation of inflammatory reactions (Levi and van der Poll, 2010; Pham, 2006, 2008; Trivedi and Caughey, 2010; Zhao *et al.*, 2005). Thus, SV-66 could play a role in the regulation of local inflammation.

SV-66 may also be useful in studying molecular interactions with the target enzymes. Such studies have been done extensively using the Kunitz protein, bovine pancreatic trypsin inhibitor (BPTI) (Ascenzi *et al.*, 2003).

Overall, this dissertation expands our knowledge of the effect of black fly SGE on vertebrate immune responses and anti-coagulation factors in the salivary glands. These findings may provide a foundation to the future research that will give further insights in the effects of black fly saliva in pathogen transmission, pathogenesis of the saliva and novel anti-hemostatic factors. For example, immunization against salivary gland components may reduce the efficacy of the immunomodulating activity, thus reducing the establishment of pathogen infection.

References:

- Abebe, M., Cupp, M.S., Champagne, D., Cupp, E.W., 1995. Simulidin: a black fly (*Simulium vittatum*) salivary gland protein with anti-thrombin activity. *Journal of Insect Physiology* 41, 1001-1006.
- Abebe, M., Cupp, M.S., Ramberg, F.B., Cupp, E.W., 1994. Anticoagulant Activity in Salivary-Gland Extracts of Black Flies (Diptera, Simuliidae). *Journal of Medical Entomology* 31, 908-911.
- Abebe, M., Ribeiro, J.M.C., Cupp, M.S., Cupp, E.W., 1996. Novel anticoagulant from salivary glands of *Simulium vittatum* (Diptera: Simuliidae) inhibits activity of coagulation factor V. *Journal of Medical Entomology* 33, 173-176.
- Ascenzi, P., Bocedi, A., Bolognesi, M., Spallarossa, A., Coletta, M., De Cristofaro, R., Menegatti, E., 2003. The bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor): a milestone protein. *Curr Protein Pept Sci* 4, 231-251.
- Cross, M.L., Cupp, E.W., Enriquez, F.J., 1994. Modulation of murine cellular immune responses and cytokines by salivary gland extract of the black fly *Simulium vittatum*. *Trop Med Parasitol* 45, 119-124.
- Cross, M.L., Cupp, M.S., Cupp, E.W., Galloway, A.L., Enriquez, F.J., 1993. Modulation of murine immunological responses by salivary gland extract of *Simulium vittatum* (Diptera: Simuliidae). *J Med Entomol* 30, 928-935.
- Cupp, M.S., Cupp, E.W., 1998. Antithrombin protein and DNA sequences from black fly, USA.
- Cupp, M.S., Cupp, E.W., Ramberg, F.B., 1993. Salivary Gland Apyrase in Black Flies (*Simulium vittatum*). *Journal of Insect Physiology* 39, 817-821.
- Cupp, M.S., Ribeiro, J.M., Champagne, D.E., Cupp, E.W., 1998. Analyses of cDNA and recombinant protein for a potent vasoactive protein in saliva of a blood-feeding black fly, *Simulium vittatum*. *J Exp Biol* 201, 1553-1561.
- Cupp, M.S., Ribeiro, J.M., Cupp, E.W., 1994. Vasodilative activity in black fly salivary glands. *Am J Trop Med Hyg* 50, 241-246.
- Currie, D.C., Adler, P.H., 2008. Global diversity of black flies (Diptera : Simuliidae) in freshwater. *Hydrobiologia* 595, 469-475.
- Denham, S., Rowland, I.J., 1992. Inhibition of the reactive proliferation of lymphocytes by activated macrophages: the role of nitric oxide. *Clin Exp Immunol* 87, 157-162.
- Jacobs, J.W., Cupp, E.W., Sardana, M., Friedman, P.A., 1990. Isolation and characterization of a coagulation factor Xa inhibitor from black fly salivary glands. *Thromb Haemost* 64, 235-238.

- Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M., Beutler, B., 2005. CD14 is required for MyD88-independent LPS signaling. *Nat Immunol* 6, 565-570.
- Kawabe, T., Isobe, K.I., Hasegawa, Y., Nakashima, I., Shimokata, K., 1992. Immunosuppressive activity induced by nitric oxide in culture supernatant of activated rat alveolar macrophages. *Immunology* 76, 72-78.
- Kyckova, K., Kopecky, J., 2006. Effect of tick saliva on mechanisms of innate immune response against *Borrelia afzelii*. *J Med Entomol* 43, 1208-1214.
- Levi, M., van der Poll, T., 2010. Inflammation and coagulation. *Crit Care Med* 38, S26-34.
- Pham, C.T., 2006. Neutrophil serine proteases: specific regulators of inflammation. *Nat Rev Immunol* 6, 541-550.
- Pham, C.T., 2008. Neutrophil serine proteases fine-tune the inflammatory response. *Int J Biochem Cell Biol* 40, 1317-1333.
- Ribeiro, J.M., Francischetti, I.M., 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu Rev Entomol* 48, 73-88.
- Titus, R.G., Bishop, J.V., Mejia, J.S., 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, 131-141.
- Trivedi, N.N., Caughey, G.H., 2010. Mast cell peptidases: chameleons of innate immunity and host defense. *Am J Respir Cell Mol Biol* 42, 257-267.
- Zhao, W., Oskeritzian, C.A., Pozez, A.L., Schwartz, L.B., 2005. Cytokine production by skin-derived mast cells: endogenous proteases are responsible for degradation of cytokines. *J Immunol* 175, 2635-2642.

