MODULATION OF MAMMALIAN IMMUNE EFFECTOR CELL FUNCTIONS BY SALIVA OF THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

by

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ABSTRACT

Immune responses to saliva of blood-feeding arthropods are important for both host and vector. Saliva contains factors that inhibit the host's hemostatic defenses, and these factors can be compromised by host immunity. In addition, transmission of many pathogens may be enhanced by vector saliva.

This dissertation focuses on the effects of saliva of the yellow fever mosquito, *Aedes aegypti*, on mouse and human immune responses *in vitro*. *A. aegypti* saliva decreased proliferation and secretion of Th1/Th2 cytokines by mitogen- and antigen-stimulated mouse splenocytes and T-cells. Inhibition of lymphocyte function involved modulation of viable T-cells at low salivary gland extract (SGE) concentrations, and decreased viability at higher concentrations. Secretion of Th1/Th2 cytokines was inhibited, but Th1 cytokines were inhibited at lower SGE concentrations than the Th2. Dendritic cells remained viable at high SGE concentrations. A single salivary immunomodulatory component, 387kDa protein, was partially purified by HPLC.

A. aegypti SGE inhibited several effector functions of stimulated mouse macrophages, including phagocytosis and MHC Class I/II expression. Macrophages

incubated with SGE decreased secretion of pro-inflammatory cytokines, but low doses did not modulate IL-10. Inducible nitric oxide synthase was not inhibited. Neither cAMP nor PGE_2-dependent signaling pathways were involved in immunomodulation.

Saliva decreased proliferation of human PBMCs in a dose-dependent manner. Low doses of saliva stimulated CD4+ and CD8+ T-cell division, but higher doses were inhibitory. Secretion of IL-12 by saliva-treated PBMCs was stimulated, IFN-γ and TNF-α were not affected, and IL-2 was decreased. Secretion of GM-CSF, and the Th2 cytokines IL-5 and IL-13, was decreased. IL-4 secretion was not affected by saliva, and secretion of IL-10 was stimulated. IL-10 secretion from monocytes followed a similar pattern, IL-6 was stimulated, and TNF-α secretion was not affected. In neutrophils, both TNF-α and IL-6 were stimulated; IL-10 secretion was strongly inhibited. In dendritic cells, TNF-α and IL-10 secretion was inhibited by saliva.

The results indicate that *A. aegypti* modulates a Th2 response in mice, and a more complex response with inflammatory and Th2 elements in humans. Characterization of these responses, and identification of the salivary components responsible, may be useful in the development of anti-arbovirus or transmission-blocking vaccines.

INDEX WORDS: *Aedes aegypti*; saliva; immunomodulation; Th1, Th2, CD4+, CD8+, neutrophil; macrophage; dendritic cell

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DEDICATION

This work is dedicated to my husband, Gene, whose support and love have allowed me to complete this dissertation.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Feeding on blood is complicated by a highly efficient set of responses that maintain hemostasis in vertebrates. Blood-feeding arthropods have evolved mechanisms to inhibit these hemostatic responses. These mechanisms involve factors that are present in the saliva and are injected into the host. Three main classifications exist for arthropod anti-hemostatic activities: platelet anti-aggregating factors, vasodilators, and anticoagulants (50). The complexity of salivary secretions is well illustrated by a recent study by Valenzuela and coworkers (99), who described an inventory of 31 secreted proteins in the saliva of *A. aegypti*, based on cDNA sequences of salivary gland specific proteins with signal peptides and on amino terminal sequences of proteins present in saliva. Other arthropods including ticks, triatomine bugs, blackflies, and sandflies also have these anti-hemostatic activities present in their saliva (75). In most cases the anti-hemostatic molecules are proteins, and as they are injected into the vertebrate host they are potential antigens.

Such a challenge may be expected to elicit an initial innate response, which in turn may stimulate an acquired immune response (89). Host inflammation and associated cytokine secretion is upregulated upon injection of these antigens. In addition to anti-hemostatic factors, saliva may also contain immunomodulatory components that may be secreted into the host during blood-feeding (104). Immunomodulators that manipulate the host's immune response and facilitate blood-

feeding are not surprising in ixodid ticks that remain attached to their hosts for up to 10 days or more, but this activity has also been described for insects that feed in only minutes, including phlebotomine sandflies, blackflies, and mosquitoes (36). The host does develop a response to salivary antigens, but in most cases the response is strongly Th2. For example, *Lutzomyia* sandflies and ixodid ticks have specific salivary components that have been shown to direct this bias towards a Th2 response (as defined on pg 7) (37). Each of these roles for saliva in the blood-feeding process are described in more detail below.

HEMOSTASIS

To better understand the various anti-hemostatic factors and their mechanisms, it is first necessary to explain the host's hemostatic response to a blood-feeding arthropod's bite. Initially, the blood-feeder will insert their mouth parts into the host's skin and probe for a blood vessel. The lanced blood vessel will then release several signals which are usually presented to cells in the circulation only in the context of an injury, including ADP, collagen, and thrombin. The host's hemostatic response is to induce platelet aggregation and adherence to the endothelium in response to these signals. The platelets then undergo degranulation and release the vasoconstrictor serotonin, the platelet aggregation agonist/vasoconstrictor thromboxane A_2, and additional ADP, which will recruit further platelets to the developing platelet plug (18). These responses should control blood loss from small peripheral vessels or capillaries (where the insects are feeding) within a matter of seconds. However, this is not the case, as blood-feeding

arthropods inhibit hemostasis by secreting anti-hemostatic molecules into the blood vessel injury site.

Some of these salivary anti-hemostatic compounds have been isolated and characterized. Most have been found to be proteins; examples include apyrase isolated from many insects and ticks (21), nitrophorins from triatomine bugs (84), and maxadilan from *Lutzomyia* sandflies (40, 53, 54). However, some are small molecules, including lipids such as the salivary prostaglandins PGE_2 and $PGF_{2\alpha}$ produced by the hard ticks (27), and the amine adenosine, found in saliva of *Phlebotomus* sandflies (43). The ability to feed on blood has evolved independently in several groups of arthropods, and the specific compounds and mechanisms vary between these hemotaphagous organisms. In several instances, non-homologous proteins have been used to provide the same anti-hemostatic activity, an interesting case of convergent evolution. An example of this is apyrase, which has been identified in *A. aegypti* and the bedbug *Cimex lectularius* (21, 96). Apyrases are platelet aggregation inhibitors, that hydrolyze ATP to ADP to AMP. *C. lectularius* apyrase, unlike *A. aegypti* apyrase, requires Ca2+ to be enzymatically active (23). Many of these salivary proteins have the potential to induce a host immune response, which will be reviewed in the next section.

Arthropod salivary anti-hemostatic components have been reviewed recently (18, 22, 75), the following comments focus on the mosquito anti-hemostatic molecules in greater detail than other known arthropod anti-hemostatic factors.

Vasodilators

To take a blood-meal quickly, the arthropod injects salivary anti-hemostatic molecules into the host (22). Release of salivary vasodilators into the bite area

enhances blood flow, thereby facilitating blood feeding and, by decreasing duration of contact with the host, decreases the possibility that the vector will be killed by the host. Vasodilators that have been isolated from insect saliva include nitrophorins from *Rhodnius prolixus* (19, 78), *Simulium vittatum* erythema protein from blackflies (26), and maxadilan from *Lutzomyia* saliva which is the most potent vasodilator known (53). Maxadilan also has immunomodulatory activity and will be discussed in greater detail in the next section.

Vasoconstriction by the host is also subverted by the mosquitoes *A. aegypti*, *Anopheles* (abbreviated *An.*) *albimanus*, and *An. gambiae*. These characterized vasodilators are tackykinin peptides from the culicine mosquito, *A. aegypti,* and catechol oxidase/peroxidase from the anophelene mosquitoes *An. albimanus* and *An. gambiae* (20, 79). The *A. aegypti* tackykinin is a decapeptide named sialokinin, which has similarities to the vertebrate Substance P and induces vasodilation by binding to an endothelial tachykinin receptor (6, 20). *A. triseriatus* saliva also contains a putative tackykinin. The catechol oxidase/ peroxidase of *An. gambiae* and *An. albimanus* oxidizes host-produced catecholamine and serotonin (80). Catecholamine and serotonin are important endogenous vasoconstrictors; their removal results in a slow but persistent vasodilation.

Platelet aggregation

A second important phase of hemostasis is host platelet aggregation. Injury to the vasculature results in release of ADP, generation of thrombin, and exposure of subendothelial collagen to circulating platelets. Platelet activation induces the release of additional ADP, which helps recruit further platelets to the developing platelet plug

(18). *A. aegypti* saliva contains apyrase, a peptide that inhibits ADP induced platelet aggregation by hydrolysis of ADP to AMP (82). The inhibition of host platelet aggregation shortens the time the insect requires to complete the blood meal (21, 82). Apyrase has been isolated from the saliva of the sandflies, *Lutzomyia longipalpis* and *Phlebotomus papatasi*, the mosquitoes *A. albopictus* and *An. albimanus* (20, 23, 58, 72, 73, 96) and the triatomid *Rhodnius prolixus* (35, 76, 87). Nitrophorins, isolated from *R. prolixus* (69) and *C. lectularius* (101) dissociate nitric oxide, which has platelet anti-aggregating as well as vasodilatory activity (79) (77, 84). A third salivary platelet inhibitor, Platelet Activating Factor (PAF) phosphorylcholine hydrolase, isolated from *Culex quinquefasciatus* saliva, also inhibits platelet aggregation (74). Interestingly, *A. aegypti* and *An. gambiae* salivary glands were also bioassayed and did not contain PAF activity (74). This is not surprising in *A. aegypti,* considering apyrase has been isolated as that mosquito's salivary anti-platelet aggregation molecule.

Anti-coagulants

The last phase of host hemostasis involves the formation of a fibrin blood clot, which involves numerous factors from the blood coagulation cascade. After tissue injury occurs, coagulation is accomplished through the intrinsic and extrinsic pathways, both of which result in cleavage of pro-thrombin to form thrombin, the linch pin of the blood coagulation cascade (22). Thrombin is a protease that cleaves fibrinogen to yield fibrin, a linear protein that interacts with receptors on the surface of platelets, linking them together and stabilizing the platelet plug into a clot. Blood-sucking arthropods have evolved a variety of salivary molecules to circumvent this final phase of host hemostasis. Anophelinae mosquito saliva contains inhibitors that bind to the active site

of thrombin, preventing the subsequent cleavage of fibrinogen (89). Saliva of culicine mosquitoes contains inhibitors of FXa, a coagulation factor common to both the intrinsic and extrinsic pathways, blocking the cleavage and activation of thrombin from pro-thrombin (92). Several anticoagulants have been cloned, including anophelin and americanin from the mosquito *A. albimanus* (32) and the tick *Amblyomma americanum* (109) respectively.

IMMUNE RESPONSE

When anti-hemostatic proteins are secreted into the host during vector feeding, the host's immune system responds to the proteins as antigens. Initially an innate response is elicited, often including inflammation, which then stimulates an acquired immune response (89). Two important components of the innate immune system are the complement system and phagocytic cells, which include neutrophils and macrophages. Initially, antigen is endocytosed by phagocytic cells, and/or opsonized by complement. Phagocytic cells detect, engulf, and destroy pathogens. Macrophages present peptide fragments complexed with major histocompatibility class (MHC) Class II- to CD4+ T-cells, which then up-regulates adaptive immunity. Additionally, Langerhan's cells as well as macrophages, present in the dermis, take up antigen and migrate to the lymph node. Once in the lymph nodes, the Langerhan's cells mature into dendritic cells and also present MHC Class II-bound antigen to naive T-cells.

The adaptive arm of the immune system contains two basic subsets: humoral and cell-mediated immunity (CMI). Humoral immunity involves the interaction of antibodies produced by B-cells with antigen, and CMI involves the recognition of cells

presenting antigen complexed with MHC Class II by CD4+ T-cells, or of cells presenting antigen complexed with MHC Class I by CD8+ T-cells. Humoral immunity acts to fight against many extracellular pathogens, while CMI's main role is for CD4+ and CD8+ T-cells to up-regulate effector functions of immune cells such as phagocytosis and production of anti-pathogen/parasite molecules such as nitric oxide (NO) and antibodies.

T-lymphocytes are produced in the bone marrow and mature in the thymus. The naive mature lymphocytes exit the thymus as either CD4+ or CD8+ T-cells and enter the blood and peripheral lymphoid tissue, which they inhabit until they are activated by a specific antigen presented by the MHC of antigen presenting cells (APCs). Naive CD4+ T-cells are referred to as T-helper cells or Th0. Th0 lymphocytes can be activated by cytokines to differentiate into Th1 or Th2 type lymphocytes.

The major inducer of Th1 cells is the cytokine interleukin (IL) -12, produced by dendritic cells and macrophages. Th1 cells are the major responders to pathogens and intracellular microbes, such as those that may infect or activate macrophages and natural killer (NK) cells. The Th2 response is activated by allergens and helminth infection; Th2 cells differentiate in the presence of IL-4. This induces chronic T-cell stimulation and little macrophage activation. Th1 cells are characterized by the production of the macrophage activating cytokine interferon gamma (IFN-γ) and Interleukin-2 (IL-2), which stimulates T-lymphocyte proliferation. Th2 cells are defined by the production of IL-4, IL-5, IL-6, IL-10, and IL-13; the latter two cytokines inhibit activation of macrophages and have anti-inflammatory activity (67). Th1 and Th2 cells have opposing roles in determining how the immune system will respond to an antigen

(67). The Th1/Th2 paradigm is an important issue when determining the effects of a pathogen on immune responses. Parasites and pathogens may induce either a Th1 or Th2 response; or a mixed response.

In my research, I am specifically interested in immunomodulation by mosquito saliva. It is well documented that, in many people, mosquito saliva induces an inflammatory, delayed type hypersensitivity response (DTH) (66). The specific immune response of an individual host to mosquito bites develops through 4 phases, related to duration and intensity of exposure: no reaction, observed in an individual that is naive for that specific antigen → delayed type hypersensitivity (DTH), in those individuals that have had some antigen exposure→ immediate type hypersensitivity, seen in those individuals that have had regular antigen exposure → desensitization, which may develop in those individuals that have chronic, consistent, antigen exposure (75). In a DTH response, there will be an initial accumulation of macrophages at the bite site. Langerhans cells and macrophages will process antigen, migrate to the lymph nodes, and drive clonal expansion of antigen-specific CD4+ and CD8+ T-cells, which will in turn infiltrate the bite site (in response to chemokines released from macrophages and/or neutrophils) within 24 hrs. These clonally expanded DTH-T cells produce pro-inflammatory cytokines, such as TNF-α and IL-1 (12, 75). However, there is evidence that the systemic acquired immune response that develops has a net Th2 character. For example, the major antibody isotypes are IgE and IgG2, and sensitized patients challenged with mosquito saliva had increased levels of the cytokines IL-4 and IL-10 in the circulation (65).

As discussed below, there is evidence that these responses are modulated by components of the saliva of mosquitoes. In fact, the ability to immunomodulate the host seems to be widespread amongst blood-feeding arthropods. In the following I will present a brief synopsis of immunomodulatory activity of arthropod saliva.

Arthropod Immunomodulators

Immunomodulators, which have been investigated in sandflies, blackflies, mosquitoes, and hard ticks, manipulate the host's immune response to antigenic anti-hemostatic proteins and facilitate blood-feeding (36). Immunomodulators may prevent the host from becoming sensitized to the anti-hemostatic proteins, and they may also modulate the response to one that has a greater fitness advantage for the blood-feeder. Immunomodulation may also enhance saliva-borne pathogen transmission (15, 104). Immunosuppressive saliva is directed against cells, complement molecules, and cytokines needed for the host's resistance to arthropod feeding (105).

Effects on Innate Immunity

Arthropod saliva has been reported to modulate host immunity during the initial innate immune response. For example, SGE (salivary gland extract) from the tick *Ixodes ricinus* suppresses murine complement hemolysis of sheep red blood cells (SRBC) (60). Saliva from another tick, *I. scapularis*, contains an inhibitor of the alternative pathway of complement activation (71). This protein, named Isac, was cloned, expressed, and sequenced, and the expressed protein was found to regulate anti-complement activity in mice (97).

Effects on Adaptive Immunity

Numerous salivary immunomodulators have been shown to directly or indirectly target T-cells. The early stages of CMI involve an expansion of populations of antigen-specific T-cells. This T-cell clonal expansion is triggered by activation of the T-cell receptor, a process that normally depends on presentation of the antigen as a complex with MHC Class I (for CD8+ cells) or MHC Class II (for CD4+ T-cells), but that may be mimicked by the mitogen Concanavalin A (Con A), a polyclonal stimulant of T-cell proliferation. Autocrine release of IL-2 is also necessary to sustain T-cell proliferation.

Ticks are very slow blood-feeders and spend an extensive length of time (as much as 10-14 days) on the host. In contrast, fast blood-feeders such as mosquitoes and sandflies complete the blood meal in 2-5 minutes. Therefore ticks are particularly vulnerable to the effector functions of CMI, which take longer to develop than the more immediate innate immune response and inflammation. Consequently, ticks have evolved a variety of salivary immunomodulators that stymie the host's immune response. Saliva from ixodid ticks has been shown to disrupt or modulate many aspects of T-cell function. Con A stimulated murine lymphocytes have decreased proliferation following incubation with saliva from *I. ricinus* (33, 34), *I. scapularis* (94), and *Dermacentor andersoni* (9, 10, 70). Salp 15, a feeding-inducible protein that inhibits CD4+ T-cell activation, has been isolated from *I. scapularis* saliva. Salp 15 represses calcium fluxes in CD4+ cells triggered by T-cell receptor (TCR) ligation, resulting in lower IL-2 production (3). A separate protein with IL-2 binding activity has also been isolated from *I. scapularis* saliva (36). These proteins work cooperatively, resulting in a profound inhibition of IL-2 dependent T-cell proliferation, as well as

inhibiting other effector cells that are responsive to IL-2 stimulation. In the case of *I. ricinus,* a novel salivary gland protein, Iris, has been shown to modulate a decrease in BALB/c mice T-lymphocyte proliferation (49). A 36 kDa immunomodulatory protein isolated from *D. andersoni* saliva (9) was cloned and expressed. The expressed protein suppressed murine T-lymphocyte proliferation *in vitro* in a dose-dependent manner (2).

In addition to inhibiting T-cell proliferation, saliva of blood-feeding arthropods may modulate cytokine secretion, changing the character of the resultant immune response. Tick saliva reduces the secretion of Th1 cytokines, and upregulates Th2 cytokines, in *D. andersoni* (70), *Rhipicephalus sanguineous* (30), *I. dammini* (94), *I. ricinus* (45), and *I. pacificus* (88). Iris, an *I. ricinus* protein mentioned above, induces a pro-Th2 environment and inhibits production of pro-inflammatory cytokines (52). Similarly, SALP15 and the IL-2 binding protein from *I. scapularis* drive a Th2 response by inhibiting IL-2-dependent cell functions.

Insects which feed in only minutes, including sandflies, blackflies, and mosquitoes, have also been shown to inhibit T-cell responses, including proliferation and cytokine secretion. Pre-exposure of mouse T-lymphocytes to salivary gland lysate of the sandfly *L. longipalpis* suppressed the proliferative response to sheep red blood cells *in vivo* and to (Con A) *in vitro*. The effects of mosquito saliva on host immune responses will be discussed below.

Tick salivary immunomodulation of human PBMC T-cell proliferation and cytokine secretion has also been investigated. Human T-cell proliferation was dose-dependently inhibited by *I. ricinus* saliva *in vivo*. However, the main difference in cytokine profiles observed between human peripheral blood mononuclear cells (PBMCs) and mouse

splenocytes treated with *I. ricinus* saliva was the downregulation of the anti-inflammatory IL-10 in mouse cells (47). On the other hand, *I. ricinus* SGE inhibited the secretion of Th1 cytokines and enhanced secretion of Th2 cytokines (47).

Studies of salivary effects on Con A-stimulated splenocytes do not distinguish between effects on CD4+ and CD8+ T-cells. However, these cell types play distinct roles in immunity, and information on the effects of saliva on each cell type would be desirable. To date, few studies have examined the specific effects of saliva on either cell type. *I. scapularis,* infected with the Lyme disease spirochete *Borrelia burgdorferi*, were fed on mice and modulated an increase in the Th2 cytokines IL-4 and a decrease in the Th1 cytokines IL-2 and IFN-γ. CD4+ splenocytes secreted IFN-γ and IL-10, but both CD4+ and CD8+ splenocytes produced IL-2 and IL-4 (107).

The Role of Macrophages in Salivary Immunomodulation

Macrophages are cells derived from bone marrow; their precursors are monocytes which circulate in the blood (16). Macrophages are found in various tissues, where they may be activated by LPS (lipopolysaccaride) and/or IFN-γ. These cells play key roles in both innate and adaptive immunity by phagocytizing microbes (or endocytosing foreign proteins), acting as APCs, and secreting cytokines that further stimulate T-cell effector functions as well as other inflammatory cells. Specifically, macrophages activated by LPS will secrete the pro-inflammatory cytokines IL-1, IL-6, TNF-α (13, 61). On the other hand, these cells can secrete the anti-inflammatory cytokine IL-10 in response to stimulation with IL-4. Macrophages can also secrete IL-12, which stimulates natural killer cells and T-cells to secrete IFN-γ, an activator of macrophages (16).

Effects of sandfly saliva on macrophages

Saliva from the sandfly *L. longipalpis* has been found to contain the vasodilator maxadilan . Maxadilan has immunosuppressive effects as well as anti-hemostatic properties. In particular, maxadilan has been found to inhibit macrophage production of nitric oxide, hydrogen peroxide, and tumor necrosis factor (TNF)-α (an inflammation inducer) in murine models of *Leishmaniasis*. Inhibition of these macrophage functions effectively inactivates the macrophage leaving it vulnerable to *Leishmania* invasion. Maxidilan is an agonist of the PACAP (pituitary cyclase activating peptide) type I receptor (62). Activation of this receptor up-regulates a negative feedback loop that controls pro-inflammatory responses by increasing secretion of IL-10 and IL-6, and decreasing secretion of TNF-α (14) (86). Elevated levels of IL-10 and decreased TNF-α suppress Th1 and favor development of a Th2 response. Further study of *L. longipalpis* maxadilan isolated from field strains and lab colonies has uncovered a 23% difference in amino acid sequence amongst the different isolates, an extremely high amount of variation for the products of a single gene. However, all the isolates increased IL-6 secretion. Therefore the amino acid residues needed for immunomodulation has been conserved (49). Saliva from the old world sandfly, *P. papatasi* has also been shown to modulate the immune response and potentiate infection with *L. major*. Saliva upregulated a Th2 and downregulated a Th1 response (49). Interestingly, this sandfly' saliva lacks maxadilan, and instead the salivary glands contain large amounts of AMP and adenosine. *P. papatasi* saliva does not alter the ability of macrophages to phagocytose *L. major* or express MHCII, but it does suppress IFN-γ activation and NO production by the macrophage (39). These effects are likely due to adenosine, as this

molecule has been shown to down regulate the nitric oxide synthase gene in activated macrophages (43).

Tick

Tick salivary immunomodulation of macrophages has also been studied. A macrophage inflammatory response by the host is an important defense factor against invading microbes as discussed above. Suppression of pro-inflammatory murine cytokines and nitric oxide activity by *R. appendiculatus* saliva has been observed. *R. appendiculatus* saliva modulates LPS-stimulated macrophages by suppressing IL-1α, TNF-α, and IL-10 (38). In addition to the decrease in IL-1 and TNF-α, Con A stimulated splenocytes incubated with saliva had a decrease in the levels of the Th1 cytokines IL-2 and IFN-γ (70). Following treatment with *I. scapularis* saliva, macrophages and dendritic cells, which are targets for spirochete infection, have decreased intracellular nitric oxide production due to the down-regulation of a Th1 response (31, 107).

Effect of Saliva on Dendritic Cell Effector functions

Dendritic cells, usually the primary APCs, and macrophages are most likely to initiate a T-cell mediated DTH response. *R. sanguineus* saliva also affects dendritic cell function by decreasing differentiation of immature dendritic cells to mature dendritic cells, increasing levels of MHC Class II, and decreasing expression of co-stimulatory molecules. LPS-stimulated dendritic cells exposed to saliva also had decreased IL-12 production but IL-10 secretion was unchanged (17). Their data suggest that saliva of some blood-feeding arthropods may regulate immunity by immunomodulating APC-TCR interactions, thereby suppressing cell mediated immunity.

Salivary Modulation of Host Infection by Parasites

Parasite/viral host infection by blood-feeding arthropods may be significantly enhanced by the presence of saliva. As discussed above, CMI involves the proliferation of T-cells, a process that is disrupted by SALP 15 and an IL-2 binding protein in saliva of *I. scapularis*, the predominant vector of the Lyme disease pathogen *B. burgdorferi* (36). To determine if IL-2 depletion affected parasite transmission, *I. scapularis* infected with *B. burgdorferi* fed on mice, and were simultaneously injected with IL-2, as a control mice were also not injected with IL-2. Supplementing IL-2 resulted in a 50% reduction in *B. burgdorferi* titers compared to controls without IL-2 supplementation (106). Similarly, *I. ricinus* SGE modulated a reduction in macrophage production of superoxide and nitric oxide by murine macrophages, resulting in a decrease in killing of *Borrelia afzelli* spirochetes (48).

Enhancement of parasite infection has been well studied in the sandfly/*Leishmania* system. For example, mice co-injected with *L. major* and *L. longipalpis* SGE had lesions that were five to ten times as large as mice injected with parasites without SGE. It is also important to note that saliva from other blood-feeding arthropods did not enhance the infection (93). Similar effects have been noted with SGE from *P. papatasi* (59). In both cases the effect is thought to be due to the effect of maxadilan (in the case of *L. longipalpis*) or adenosine (for *P. papatasi*) on production of NO by macrophages as described above.

Dipteran saliva also up-regulates a host antibody response, which may in some cases be deleterious to parasites or pathogens. Chickens pre-exposed to *Aedes fluviatilis* bites, and then co-injected with *Plasmodium gallinaceum* sporozoites and *A.*

fluviatilis SGE, had decreased parasitemia levels compared to chickens that were not pre-exposed to saliva (85). Additionally, anti-sporozoite antibodies were also isolated from the chickens (85).

Sandfly Saliva Up-regulates a DTH Response and Enhances Host Protection Against Parasitemia

The enhancement of *Leishmania* transmission by sandfly SGE, discussed above, was observed in immunologically naive mice. However, vertebrate hosts exposed to sandfly bites develop a strong delayed-type hypersensitivity (DTH) response. DTH responses are initiated by an accumulation of macrophages at the injury site; these macrophages secrete chemokines that recruit T-cells to the site after about 24 hrs in humans (12). The T-cells, macrophages, and other cell types (neutrophils, eosinophils) drive a strong inflammatory response that results in erythema and edema at the bite site. The DTH reaction is characterized by, and is dependent upon an increase in CD4+ and/or CD8+ T-cells, which can secrete either Th1 cytokines (in the case of a DTH1 response) or Th2 cytokines (in the case of a DTH2 response) (12).

Pre-exposure of mice to sandfly saliva, either through sandfly biting or by injection of SGE, has been found upregulate a DTH response characterized by a large increase in immune cells secreting IFN-γ (42). Overall these mice had a 5 fold increase in leukocytes recruited to the bite site upon parasite injection versus those not pre-exposed to saliva (7) (42). In contrast to the effect of saliva in naïve mice, this DTH response enhances host protection against *L. major* infection (8). Mice that were pre-exposed to sandfly saliva had reduced dermal lesions, and a significantly decreased parasite load compared with parasite burdens seen in naïve mice. These results were

the first to suggest that pre-exposure of the host to the vector's saliva may influence disease progression.

The DTH response seems to aid sandflies in feeding. *P. papatasi* fed twice as fast on human bite sites that had a DTH response due to a previous bite, compared to "virgin" skin sites. The previously bitten sites that had a DTH response sites had twice the normal blood flow compared to non-bitten sites (8). Feeding from DTH lesions is thought to aid the sandfly in expedited feeding time, thereby reducing the sandflies chance of being harmed by the host. Indeed the generation of a DTH response appears to be part of the feeding strategy of sandflies. The response is due to a specific salivary protein, which has been named SP15 (94). Immunization of naïve mice with this protein, or with a DNA vaccine that drives a response to this protein, results in a strong DTH response to sandfly saliva, and in greatly reduced *Leishmania major* transmission to both permissive (BALB/c) and refractory (B6) mice (94). The response was still seen in B-cell deficient mice, which suggests that protective immunity is from a cell mediated response and not antibody-mediated neutralization of the immunomodulators (95).

Effects of *Aedes aegypti* Saliva on Host immunity and Viral Transmission

A. aegypti saliva also has been shown to have immunomodulatory activity in murine models. In experiments by Cross (24), increasing concentrations of saliva causes dose-dependent inhibition of proliferation of mitogen- or antigen- stimulated T-lymphocytes. SGE causes decreases in the Th1 cytokines IL-2 and IFN-γ. However, the Th2 cytokines IL-4 and 5 seems unaffected by SGE. Another study showed inhibition of TNF-α secretion from rat mast cells (11). This inhibition was only observed

with female and not male SGE. The immunosuppressive factor was larger than 10 kDa and it was neutralized by boiling for 10 min. This suggests that the critical component in the immunosuppressive factor was a protein. As well, inhibition of the pro-inflammatory cytokine TNF-α is consistent with an up-regulation of a Th2 cytokine environment reported by Cross (24). *In vivo* experiments on *A. aegypti* modulation of mouse cytokine secretion have also been reported (108). Observation of the interaction between *A. aegypti* SGE and virus will further our understanding of SGE on host effector cell functions. Arbovirus susceptible mice fed on by *A. aegypti* had a decrease in the Th1 cytokine IFN-γ but an increase in the Th2 cytokines IL-4 and IL-10 (108).

A. aegypti saliva has been shown to potentiate transmission of Cache Valley virus (28), and the related mosquito *A. triseriatus* potentiates transmission of vesicular stomatitis and LaCrosse virus (64). The role of salivary immunomodulators has not been clearly defined in these models, but it is reasonable to speculate that such immunomodulators are likely to be involved in enhancing transmission. For example, *A. aegypti* SGE has been shown to enhance infection of vesicular stomatitis New Jersey (VSNJ) virus in mice, compared to virus infected mice that were not exposed to saliva (56). The effect of *A. aegypti* saliva on enhancing viraemia in IFN-α/β secretion in VSNJ infected cells was investigated (55). Inhibition of IFN-α/β secretion from cells infected with VSNJ and incubated with SGE had greatly enhances viraemia. Somewhat contradictory results were found in a study of Dengue virus infection of human dendritic cells, where pre-exposure of dendritic cells to *A. aegypti* saliva enhances protection against viral infection (1).

OBJECTIVES

The two studies described above (11, 24) provide only a preliminary indication of the effects of *A. aegypti* saliva on host immune function, and essentially no characterization of the specific molecules involved. We propose several studies that will clarify the effect of *A. aegypti* saliva on both mouse and human immune responses in the following objectives:

1). Determine the immunomodulatory effects of *A. aegypti* SGE on T and B-lymphocyte proliferation and cytokine secretion. To achieve this goal, murine splenocytes were incubated with various concentrations of SGE, followed by stimulation with Con A (BALB/c mice) or OVA (DO11 mice), and monitored for proliferation and secretion of pro-inflammatory, Th1, and Th2 cytokines. T-cell and dendritic cell viability were also investigated by staining with Annexin V and propidium iodide (PI) and then monitoring cell viability with flow cytometry.

2). Preliminary characterization of the immunomodulatory component of *A. aegypti* saliva. We quantified the immunomodulator in saliva and salivary gland extracts of female and male mosquitoes, and in SGE from fasted and freshly blood-fed females, by assaying splenocyte proliferation. We estimated the size of the salivary immunomodulator with gel filtration HPLC, followed by assaying the fractions for immunomodulatory effects on splenocyte proliferation and cytokine secretion. Finally, we determined the nature of the salivary immunomodulator by denaturation and proteolytic digestion.

The results of studies from Objectives 1 and 2 are presented in Chapter Two of this dissertation.

3. Examine in more detail the effects of *A. aegypti* SGE on murine macrophage effector functions. Stimulated macrophages incubated with *A. aegypti* SGE were monitored for cytokine secretion, phagocytosis, MHC Class I and II expression, and iNOS activity. Additionally, potential modes of action of A*. aegypti* SGE on murine macrophages were investigated by observing macrophage intracellular cAMP production and PGE_2-dependent secretion of TNF-α.

The results of studies from Objective 3 are presented in Chapter Three of this dissertation.

4. Quantify the effects of *A. aegypti* SGE on viability and effector functions, including proliferation and cytokine secretion, of human PBMCs, neutrophils, macrophages, and dendritic cells.

The results of studies from Objectives 4 are presented in Chapter Four of this dissertation.

	Arthropod Anti-Hemostatic Factor	Arthropod	Function of Anti-hemostatic
Anti-Platelet Aggregation	Apyrase	*A aegypti (82), Phlebotomies (96) , Lutzomyia (81), A. albopictus (58), R. prolixus (87)*	Inhibits ADP induced platelet aggregation by hydrolysis of ADP to AMP
	Nitrophorin	*R. prolixus (19)*, *Cimex lectularius (100)*	Dissociates nitric oxide (NO)
	Platelet Activating Factor (PAF) hydrolase	*Culex quinquefasciatus (74)*	Phosphorylcholine hydrolase
Vasodilators	*Simulium vittatum* erythema protein	*Simulium vittatum (26)*	Affects K+ channels
	Sialokinin	*A. aegypti (20)*	Tackykinin peptide
	Catachol/oxidase and peroxidase	*An. albimanus (79, 83), An. gambiae (80)*	Hydolysis of catecholamine and serotonin
Anti-Coagulants	Americanum	*An. americanum (109)*	Thrombin inhibitor
	Serpin	*A. aegypti (91)*	Factor Xa inhibitor
	Anophelin	*An. albimanus (98)*	Thrombin inhibitor

Table 1.1: Examples of Arthropod Salivary Anti-Hemostatic Factors and their Specific Inhibitory Activities.

Arthropod	**Salivary Immunomodulator**	**Host cells effected by immunomodulator**	**Host immune functions modulated by saliva**
Ticks			
I. ricinus (60)			Inhibits complement
I. scapularis (97)	Isac		Inhibits complement
I. scapularis (36)	IL-2 binding protein	T-lymphocytes	Suppresses proliferation
I. ricinus (51)	Iris	T-lymphocytes	Inhibit T-cell proliferation, induces a pro-Th2 environment, decrease in pro-inflammatory cytokines
ticks (4, 5, 47, 107)		PBMCs and mouse splenocytes	Decrease Th1 and increase Th2 cytokines
I. scapularis (3)	Salp 15	CD4+ T-cells	Inhibits IL-2 secretion
D. andersoni (57)		T and B-cells	Decrease in T-cell proliferation and development of a primary Ab response
ticks (29, 46, 57, 60, 94), mosquito (24, 103), sandfly (68)		T-cells	Suppresses proliferation
R. appendiculatus (38)		macrophage	Inhibits pro-inflammatory cytokines and IL-10
I. ricinus (44, 48)		macrophage	Inhibits IL-2 and TNF-α, NO and superoxide
R. sanguineus (17)		Dendritic cells	Inhibits dendritic cell maturation, Increases MHCII, Decreased co-stimulatory molecule expression, Decreased IL-12
Diptera			
An. stephensi (63)		neutrophils	Inhibits chemotactic

			activity
A. aegypti (24)		T- and B-cells; mast cells	Inhibits T- and B-cell proliferation, and inhibits Th1 and Th2 cytokine secretion; Inhibits TNF-α secretion
L. longipalpis (53, 54, 90)	Maxidilan	Macrophages	Inhibits nitric oxide, hydrogen peroxide, and TNF-α, Th1 response; Agonist of the PACAP receptor, increases PGE_2 expression
P. papatasi (102)		macrophage	Down regulates iNOS gene; inhibits IFN-γ
Simulium vittatum (25)		T-cells, eosinophils	Decreased IL-5 and-10, eosinophils, and splenocyte proliferation
Hemiptera			
R. prolixus (41)		T-cells	Decreased splenocyte proliferation

Table 1.2: The Effect of Arthropod Salivary Immunomodulators on Host Immune cells Effector Functions

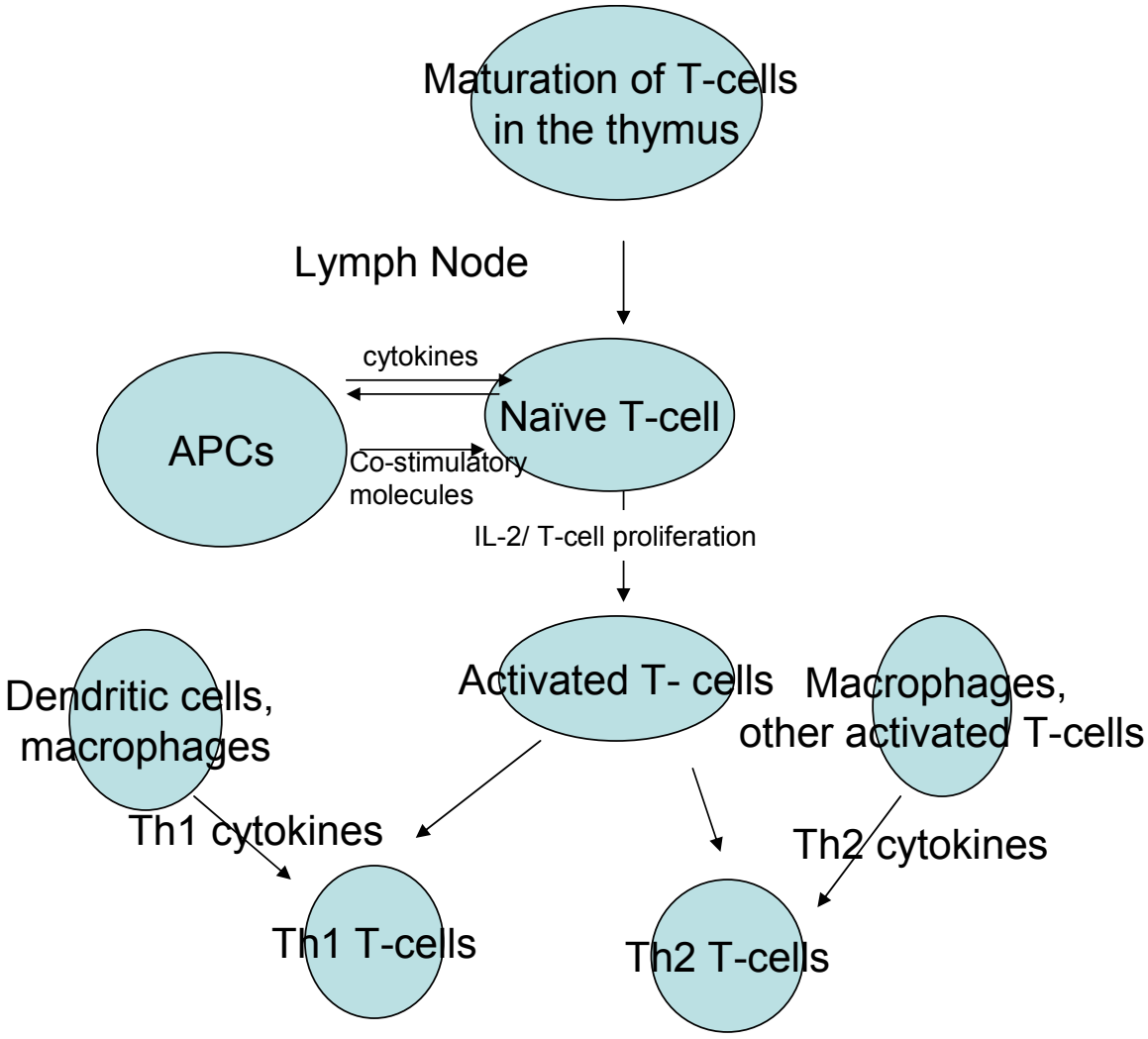

Figure 1.1: Interaction between T-cells and Antigen Presenting Cells (APCs). T-cells originate from the bone marrow and then mature in the thymus. From the thymus, they migrate to the lymph node were CD4+ T-cell receptors may eventually interact with MHC Class II on APCs to become effector T-cells. Depending upon the cytokine environment, these activated T-cells may differentiate into Th1 or Th2 cells.

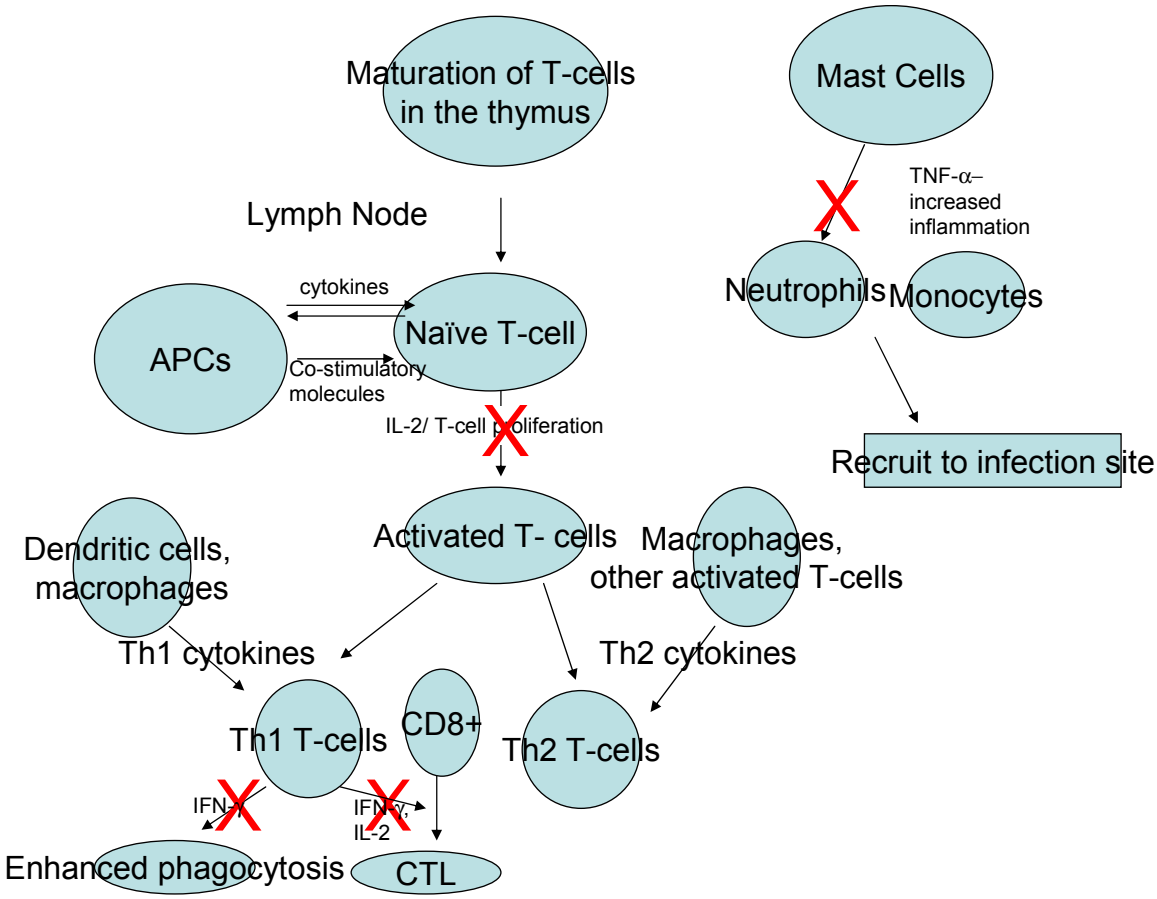

Figure 1.2: *Aedes aegypti* SGE modulates host immune cells effector functions. *A. aegypti* SGE *in vitro* inhibits antigen and mitogen stimulated mouse T-cell proliferation. Stimulated T-cells had a decrease in the Th1 cytokines, IFN-γ and IL-2, however there the Th2 cytokines IL-4 and 5 seemed unaffected. In a separate experiment, rat mast cells incubated *in vitro* with *A. aegypti* saliva had decreased secretion of the pro-inflammatory cytokine TNF-α, compared to a positive control.

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CHAPTER 2

SALIVA OF THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI*, MODULATES MURINE LYMPHOCYTE FUNCTION[1]

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ABSTRACT

Saliva of many vector arthropods contains factors that inhibit haemostatic responses in their vertebrate hosts. Less is known about the effect of vector saliva on host immune responses. We investigated the effect of *Aedes aegypti* salivary gland extracts on antigen-stimulated responses of transgenic OVA-TCR DO11 mouse splenocytes in vitro. T-cell proliferation was inhibited in a dose-dependent manner, with greater than 50% inhibition at 0.3 salivary gland pair (SGP) equivalents/mL. LPS-stimulated B-cell proliferation was also inhibited. Secretion of the Th1 cytokines IL-2 and IFN-γ was reduced by 50% or more with 0.45–0.6 SGP/mL, as was secretion of the pro-inflammatory cytokines GM-CSF and TNF-α, and the Th2 cytokine IL-5. The Th2 cytokines IL-4 and IL-10 were similarly reduced with 0.6–2.0 SGP/mL. Inhibition of lymphocyte function involved modulation of viable T-cells at low salivary gland extract (SGE) concentrations, and decreased viability at higher concentrations. Dendritic cells were not killed by salivary gland extracts at concentrations as high as 25.0 SGP/ mL, but secretion of IL-12 was inhibited by 87% following exposure to 0.6 SGP/mL. Activity is present in saliva and extracts of female but not male salivary glands, and it is depleted from salivary glands of blood-fed mosquitoes. The activity is denatured by boiling and by digestion with the protease papain, indicating a protein; gel filtration HPLC indicates a mass of about 387 kDa. These results suggest that *A. aegypti* saliva exerts a marked immunomodulatory influence on the environment at the bite site.

Keywords
Saliva, immunomodulation, immunosuppression, blood-feeding, ectoparasite

INTRODUCTION

The process of feeding on blood requires that arthropods be able to inhibit haemostatic defenses in their vertebrate hosts. Anti-haemostatic mechanisms involve factors that are present in the saliva and are injected into the host, including platelet anti-aggregating factors, vasodilators, and anticoagulants (1–3). For example, the mosquito *Aedes aegypti* secretes a variety of salivary factors, including the enzyme apyrase, which hydrolyses both ATP and ADP (4,5), the vasodilatory tachykinins sialokinin I and II (6,7), and an anticoagulant with anti-Factor Xa activity (8,9). Numerous other proteins with unknown pharmacological activity are also present, including members of the D7 protein family (10,11). Valenzuela and co-workers (12) recently described an inventory of 31 secreted proteins in the saliva of this mosquito, based on salivary gland specific cDNA sequences with signal peptides and on amino terminal sequences of proteins present in saliva. Other blood-feeding arthropods, including ticks, triatomine bugs, blackflies and sandflies, have similar anti-haemostatic activities in their saliva (1–3). Blood feeding evolved independently in each of these groups, and the specific molecules responsible for the anti-haemostatic activities differ in each taxon (1,3). In a few cases these activities may depend on a molecule other than a protein, such as the salivary prostaglandins produced by ixodid ticks (13) or adenosine in saliva of *Phlebotomus papatasi* sandflies (14,15). However, in most cases, the anti-haemostatic molecules are peptides or proteins, and because they are injected into the vertebrate host they are potential antigens.

Such a challenge may be expected to elicit an initial innate response, which then stimulates an acquired immune response (16–18). Because these responses constitute

another set of barriers to successfully obtaining a blood meal, anti-inflammatory and immunomodulatory components may be secreted into the host during blood feeding (3,16–19). Such immunomodulators have been especially well characterized in ixodid ticks that remain attached to their hosts for 10 days or more, but this activity has also been described for insects that feed in only minutes, including phlebotomine sandflies and blackflies (16,17). The host does develop a response to salivary antigens, but in most cases the response is strongly Th2, and in the case of *Lutzomyia sandflies* and ixodid ticks specific salivary components have been shown to direct this Th2 bias (20–24).

Aedes aegypti has a world-wide distribution in tropical to warm temperate regions, where it is of pre-eminent medical importance as the primary vector of Yellow Fever and Dengue Fever (25). Both of these diseases have long afflicted humankind, and both have recently increased in frequency such that they are considered emerging or re-emerging diseases (26,27). *A. aegypti* saliva has been shown to have immunomodulatory activity in murine models (28): increased concentrations of saliva caused dose-dependent inhibition of proliferation of mitogen-stimulated T-lymphocytes. In addition, SGE caused decreases in the Th1 cytokines IL-2 and IFN-γ, but little effect was seen on levels of the Th2 cytokines IL-4 and 5. A second study showed inhibition of TNF-α secretion from rat mast cells by salivary gland extract from female but not male mosquitoes (29). Feeding by *A. aegypti* has been shown to induce a typical Th2 response in both murine (30,31) and human (30) hosts. In this paper, we further describe the immunomodulatory effects of *A. aegypti* SGE on murine lymphocytes *in vitro*, and partially characterize the nature of the responsible component.

MATERIALS AND METHODS

Materials

Chemicals were from Sigma Chemical Corporation (St Louis,MO), unless otherwise stated. Antibodies and recombinant cytokines were from BD PharMingen (San Diego, CA). Adult *A. aegypti* mosquitoes, from a colony established in 1968 at the Department of Entomology, University of Georgia, were reared at 27°C under a 16-h light/8-h dark cycle. Larvae were fed crushed, dry cat food and Tetramin fish flakes. Adults were fed a 10% sucrose solution but not blood unless indicated otherwise for specific experiments. OVA-TCR DO11 transgenic mice (referred to as DO11 mice) were purchased from Jackson Laboratories (Bar Harbor, ME). DO11 mice have a genetically fixed T-cell receptor that responds to ovalbumin; these mice were derived from BALB/c mice and are otherwise comparable in their immune functions to humans. For certain experiments, splenocytes from BALB/c mice were used; these mice were also obtained from Jackson Laboratories. We chose to work with these mice because Chen et al. (30) have shown that feeding by *A. aegypti* elicits a cytokine and antibody response in BALB/c mice that is similar to the human response. Mice were maintained at the University of Georgia Animal Care Facility under pathogen-free conditions, and were used in accordance with protocols approved by the University of Georgia Institutional Animal Care and Use Committee.

Preparation of salivary gland extracts.

Aedes aegypti salivary gland pairs were dissected in 10 mM HEPES, pH 7.4/150 mM NaCl from 3- to 10-day-old female or, for one experiment, male mosquitoes. The glands were stored at -70°C in aliquots of up to 20 pairs in 50 µL of dissection buffer in

Eppendorf tubes. Salivary glands were homogenized by holding the Eppendorf tube, in a beaker filled with water, beneath the tip of a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT). The SGP were disrupted by 10 ultrasound bursts, with the power setting at five and a 50% duty cycle. Homogenized salivary glands were centrifuged at 11 750 g for 5 min and the supernatants were pooled and diluted in buffer to yield the concentrations given for the various experiments. These supernatants are subsequently referred to as salivary gland extract (SGE), and the concentration of SGE is given in salivary gland pair (SGP) equivalents per mL of culture medium (RPMI).

Preparation of splenocytes. Spleens were dissected from BALB/c or OVA-TCR D011 transgenic mice, and macerated with a sterile syringe plunger in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Paisley, PA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 1% penicillin–streptomycin (Gibco), 1% L-glutamine (Gibco), and 0·5% gentamicin (Gibco) (referred to subsequently as 'complete RPMI'). Cells were centrifuged (GS-6R Centrifuge, Beckman) at 365 g for 7 min at 22°C, then the supernatant was decanted and the pellet disrupted. Erythrocytes contained in the splenocyte suspension were lysed by resuspending the pellet in 4.5 mL sterile H2O for 10 s, followed with an aliquot of 0.5 mL 10X.sterile PBS to arrest cell lysis. Erythrocyte membranes were removed and the cells were again centrifuged for 7 min at 22°C. The supernatant was discarded and the pellet resuspended in 4 mL complete RPMI. Cells were then diluted in complete RPMI to a final concentration of $5X10^6$ cells/mL.

Splenocyte proliferation assay.

Various concentrations of SGE were added to wells containing $5X10^5$ splenocytes in 100 µL complete RPMI in a 96-well plate (Corning, Corning, NY) and incubated for 2 h

(5% CO2, 37°C). DO11 splenocytes were stimulated with 1μM ovalbumin (OVA) peptide (residues 323–339 of chicken ovalbumin) (synthesized by the Molecular Genetic Instrumentation Facilities, University of Georgia). For some experiments, cells were stimulated with 1 μg/well of either Concanavalin A (Con A), or *Escherichia coli* lipopolysaccharide (LPS). Positive controls were stimulated with mitogen or peptide without exposure to SGE, and negative controls were not exposed to SGE, mitogen, or peptide. The final volume per well was adjusted to 200 μL with complete RPMI. After 56 h of incubation, 1 μCi tritiated [3H]-thymidine (Amersham) in 20 μL complete RPMI was added to each well. After an additional 18 h of incubation, cellular proliferation was determined by harvesting the cells (Type 7000 cell harvester; Skatron, Inc., Sterling, VA), and radioactivity incorporation was determined by scintillation counting.

Measurement of cytokine secretion.

Briefly, $5X10^5$ DO11 splenocytes in complete RPMI were aliquoted into a 96-flat well plate and incubated with various concentrations of *A. aegypti* SGE. Following a 2 h incubation, (5% CO_2, 37°C), OVA peptide (1 μM final concentration) was added. The final volume in all wells was 200 μL. Following an additional 48 h incubation, the cell culture medium was collected and centrifuged at 11 780 *g* for 5 min. Supernatants were stored at. 70 °C until analysis. Cytokine levels were measured using a mouse Th1/Th2 BioPlex kit (Bio-Rad) following the manufacturer's protocols. Aliquots (50 μL) of the supernatants were assayed in duplicate. Cytokine concentrations were determined by reference to a standard curve generated from known quantities of recombinant cytokines. Experiments were independently replicated three times. In some experiments size-fractionated SGE was assayed for effects on IL-4 and IFN-γ secretion by Sandwich

ELISA. For these experiments, splenocytes from BALB/c mice were treated with HPLC fractions (see HPLC below) for 2 h, followed by stimulation with 1 μg/well Con A. After 48 h, the culture medium was harvested and assayed for IL-4 and IFN-γ. Assays were carried out in flat-bottomed ELISA plates following PharMingen protocols. Capture and detection monoclonal antibody pairs were: for IFN-γ, clones R4-6A2 and XMG1·2; and for IL-4, BVD4-1D11 and BVD6-24G2. Each sample was assayed in duplicate and absorbance values were expressed in pg/mL as determined by comparison with a standard curve obtained from known quantities of recombinant cytokine standards (PharMingen, San Diego, CA).

Analysis of cell viability by flow cytometry.

We determined the effect of SGE on the viability of CD4+ and CD8+ T-cells, and cultured dendritic cells. Splenocytes ($5X10^5$ cells) in 200 μL final volume complete RPMI in a 96-well plate were exposed to the indicated concentrations of SGE, and stimulated with 1 μM of OVA peptide, as described above. After 72 h the cells were transferred to V-bottomed plates and washed twice with 200 μL PAB, followed by treatment with Fc Block (rat anti-mouse CD16/CD32 Fcγ Receptor clone 2·4G2) (BD PharMingen) for 15 min at 4°C. Cells were again washed with 200 μL PAB, centrifuged (1500 r.p.m., 7 min at 4°C) in a Beckman GS-6R centrifuge (365 *g*), then stained for cell surface markers with the appropriate antibody in 100 μL PAB for 30 min at 4°C. Antibodies used were FITC-labeled rat anti-mouse CD4 (clone GK1·5) or PE-labeled rat anti-mouse CD8α (Ly-2, clone 53–6·7) (BD PharMingen). Subsequently cells were washed twice in 200 mL PAB, and resuspended in 300 μL PAB for analysis by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Cell viability as

assessed by treating the cells with propidium iodide (PI). The effect of SGE on a dendritic cell line was also assessed. Murine fetal skin dendritic (FSDC) cells (ATCC CRL-11904) were grown in ATCC complete growth medium, supplemented with 20% fetal bovine serum and 5 ng/mL murine GM-CSF, in the presence of the indicated concentration of SGE. After 72 h, cells were scraped from the culture flasks, labeled with PI, and a minimum of 10 000 cells were analyzed by flow cytometry. Each treatment was set up in duplicate within an experiment, and the experiment was replicated in triplicate.

Brief exposure of BALB/c splenocytes to SGE.

BALB/c splenocytes were incubated with various concentrations of SGE under the same conditions as for the proliferation assays described above. Following the 2-hour exposure to SGE, the plate was spun at 365 *g* for 7 min, the RPMI and SGE were removed from the cells and replaced with fresh complete RPMI before the addition of Con A (1 µg/well).Cultures were assayed for proliferation by [3H]-thymidine incorporation as described above.

Blood-fed and male mosquito assay.

Salivary glands were dissected from female mosquitoes immediately after they had fed on a mouse, from unfed female mosquitoes, and from male mosquitoes, placed in 50 µL 10 mM HEPES pH 7·4/150 mM NaCl, and frozen at -70°C until use. Salivary gland extracts were prepared as described above, and then diluted with complete RPMI to various concentrations. SGE from fed and unfed female mosquitoes were assayed for proliferation using Con A-stimulated BALB/c splenocytes as described above. Extracts

from male and female salivary glands were tested at 2·5 and 5·0 SGP/mL equivalents.

***Aedes aegypti* saliva collection.**

Fifteen female *A. aegypti* were anaesthetized on ice and restrained on a microscope slide with double-sided adhesive tape. The proboscis was inserted into a capillary tube filled with mineral oil, and salivation was induced by application of 1 µL of 10 mM pilocarpine. Salivation was allowed to occur for 15 min at room temperature and then the mineral oil plus saliva was pooled, 50 µL of dissection buffer was added, and the mixture was centrifuged at 11 750 *g* for 5 min to separate the aqueous phase (containing the saliva) from the mineral oil. Saliva was kept at -70°C until needed. Saliva was diluted to the indicated concentrations and assayed for effects on Con A-stimulated BALB/c splenocyte proliferation.

Heating and protease treatments.

SGE equivalent to 0·5 and 1·0 SGP were boiled for 3 min. Controls used were unboiled SGE equivalent to 0·5 and 1·0 SGP. SGE was aliquoted into wells containing splenocytes from BALB/c or DO11 mice, and proliferation was assayed with Con A- or OVA-stimulated splenocytes as described above. Protease digestion was carried out on an immobilized papain column (MoBiTec). SGE equivalent to 10 SGP was digested according to the manufacturer's protocol. The protease-digested SGE was diluted with complete RPMI to various concentrations, aliquoted into wells containing $5X10^5$ BALB/c splenocytes, and assayed for effects on proliferation of Con A-simulated BALB/c splenocytes.

Gel filtration HPLC.

Aedes aegypti SGP (100 pairs) were lysed by sonication and then centrifuged as described above. SGE (250 µL) was then diluted with 150 mM NaCl/25 mM HEPES pH 7.0 to a final volume of 500 µL and fractionated using a BioSil SEC 250 gel filtration column. The HPLC consisted of a Thermo Separations ConstaMetric 4100 biocompatible pump, a SpectroMonitor 4100 detector set to monitor the eluent at 280 nm, and an integrator. Proteins were eluted with 150 mM NaCl/25 mM HEPES pH 7.0 at a flow rate of 0·5 mL/min, and fractions were collected at 1 min intervals. Aliquots (20 µL) of the collected fractions were assayed for effects on BALB/c splenocyte proliferation, and on IL-4 and IFN-γ secretion, as described above.

RESULTS

***Aedes aegypti* SGE suppresses antigen-stimulated proliferation and cytokine production.**

Antigen-specific stimulation occurs when antigen is processed by antigen-presenting cells (APCs), complexed with MHC Class II, and presented on the cell surface to interact with the T-cell receptor (TCR) of CD4+ T-cells. To model this interaction, we used DO11 mice that have their TCRs genetically fixed to respond to stimulation with ovalbumin peptide (OVA). OVA-stimulated splenocytes, incubated with increasing concentrations of female SGE, showed dose-dependent decreased proliferation compared to a positive control. Addition of as little as 0.15 SGP/mL caused 40% suppression in T-lymphocyte proliferation; 1.2 SGP/mL caused 95% suppression, and proliferation was abolished by SGE concentrations equivalent to 4.0 SGP/mL (Figure 2.1). Comparable inhibition was observed for Con A-stimulated BALB/c T-lymphocytes

(data not shown). SGE also decreased proliferation of mitogen stimulated B-lymphocytes: addition of up to 0.45 SGP/mL had no observable effect, but proliferation was reduced by 40% with 0.6 SGP/mL and completely inhibited with 4.0 SGP/mL (Figure 2.1). Treatment of OVA-stimulated DO11 T-lymphocytes with SGE resulted in a dose-dependent decrease in the secretion of all cytokines measured, but secretion of Th1 and pro-inflammatory cytokines was suppressed by lower concentrations of SGE than were required to inhibit secretion of Th2-type cytokines (Figure 2.2). For example, 0.6 SGP/mL suppressed IL-2 and IFN-γ by 55% and 54%, respectively. IL-12 secretion was lowered by 87% at the same SGE concentration. The pro-inflammatory cytokines TNF-α and GM-CSF were reduced by over 50% when splenocytes were exposed to 0.45 and 0.6 SGP/mL, respectively. IL-4 secretion was reduced by 49%, and IL-10 by 42%, with 0.6 SGP/mL. Secretion of all cytokines was reduced by over 95% by SGE equivalent to 4.0 SGP/mL or higher. Concentrations of SGE sufficient to inhibit cytokine secretion did not significantly affect cell viability (Figure 2.3). SGE equivalent to 0.6 SGP/mL did not result in an increase in PI-positive CD4+ T-cells, although these cells did decrease slightly as a proportion of the total splenocytes, probably because of inhibition of proliferation (Figure 2.3a). Similarly, the proportion of PI-positive CD8+ T-cells was not significantly increased in the presence of SGE up to 0.6 SGP/mL (Figure 2.3b). However, higher concentrations of SGE (2.5 SGP/mL and above) did result in a significant decrease in viability of both CD4+ and CD8+ T-cells, and both of these cell types decreased as a component of the total splenocyte population. In contrast to the effects on T-cells, concentrations of SGE as high as 25.0 SGP/mL had no effect on viability of a murine skin dendritic cell line (Figure 2.3c).

Short-term exposure of T-cells to SGE is sufficient for suppression of splenocyte proliferation.

The inhibitory activity of *A. aegypti* salivary gland extract could result from a direct interaction with splenocytes, such that cells no longer respond normally to stimulation. Alternatively, SGE could sequester a signal released from activated cells, such as IL-2, necessary to sustain proliferation and activation, as has been described for saliva of the ixodid tick *Ixodes scapularis* (21). In the latter case, SGE would have to be present throughout the incubation period for inhibition to occur. To distinguish between these possibilities, cells were exposed to SGE for two hours, after which the cells were centrifuged, washed, resuspended in fresh complete RPMI without SGE, and stimulated with Con A. Controls were exposed to SGE for the full duration of the experiment. A two-hour exposure to SGE was sufficient to inhibit splenocytes from proliferating, and the degree of inhibition was similar to that of cells exposed to SGE throughout the incubation period (Figure 2.4). For example, exposing splenocytes to 0.3 SGP/mL, followed by washing, resulted in a 45% decrease in proliferation, comparable to the 36% decrease seen with continuous exposure to the same concentration of SGE.

The inhibitory activity of SGE is associated with female saliva, and it is secreted into the host during blood feeding. If the activity described here is specifically associated with blood feeding, it should be present only in females (which blood-feed) and not males (which feed on plant sugars), and the activity should be detectable in saliva as well as salivary gland homogenates. Further, we expect the activity to be depleted in salivary glands immediately following a blood meal, reflecting the loss of saliva caused by secretion into the host. A comparison of female and male SGE, equivalent to 0.5 and

1.0 SGP/well, indicated that the activity was associated only with female salivary glands (Table 1). Female SGE inhibited Con A-stimulated proliferation by over 98%, similar to the results described above. On the other hand, male SGE stimulated splenocytes, so that proliferation was 119% of the positive control when cells were treated with either 0.5 or 1.0 SGP. Female mosquitoes were induced to salivate into mineral oil, and this saliva was found to inhibit splenocyte proliferation (Table 2.1). Saliva equivalent to 1.0 and 2.0 individual mosquitoes inhibited T-cell proliferation by 28% and 85%, respectively, compared to 99% inhibition with comparable amounts of SGE. It is difficult to compare the activity of such saliva directly with the salivary gland homogenates, as not all of the saliva is secreted, and some saliva is lost during collection, centrifugation, and separation from the mineral oil. The presence of the activity in saliva is sufficient to show that the activity is injected into the host during a blood meal. Finally, the activity is depleted from female salivary glands following a blood meal (Figure 2.5). Proliferation was inhibited by 50% with SGE equivalent to 2.0 SGP/mL from blood-fed mosquitoes, compared to 0.6 pairs/mL from non-blood-fed mosquitoes, suggesting that about 2/3 of the activity was secreted into the host in a blood meal.

Chemical characterization of the salivary immunomodulatory component.

Immunomodulatory components of vector saliva have, for the most part, been identified as peptides or proteins, but other salivary components with potential immunomodulatory activity have been identified, including adenosine and AMP (3,10), prostaglandins (13), and even the gas nitric oxide (15). We attempted to determine if the immunomodulatory component(s) of *A. aegypti* SGE is likely to be a protein, by assessing its susceptibility to denaturation by boiling, digestion by protease, and its size. We also attempted to

determine if the various immunomodulatory activities (inhibition of proliferation and inhibition of cytokine secretion) are likely to be due to a single or multiple component(s) of the saliva. Boiling for 5 min abolished the inhibitory effects of *A. aegypti* SGE on splenocyte proliferation (Table 2.2). Splenocytes incubated with boiled SGE (0.5 and 1.0 pairs), actually increased proliferation by 34% and 19%, respectively (OVA-stimulated DO11 lymphocytes) or 30% and 5% (Con A stimulated BALB/c lymphocytes). In contrast, unboiled female SGE (0.5 and 1.0 pairs), incubated with splenocytes, suppressed proliferation by 98·4% and 99·5%, respectively, in DO11 splenocytes and 99·8% and 99·4% in BALB/c splenocytes.

Digestion with the protease papain significantly reduced the ability of SGE to inhibit splenocyte proliferation, providing further evidence that the immunomodulator is a polypeptide. Low concentrations of papain-digested SGE (e.g. 0.15–0.6 SGP/mL), actually increased T-lymphocyte proliferation compared to the positive control, as was also observed for boiled and for male SGE. Higher concentrations of SGE were only partially digested, but the inhibitory activity was decreased by at least 70%. Digestion with trypsin did not inhibit the activity of the immunomodulatory factor, suggesting that the protein lacks trypsin cleavage sites on its surface, or that tryptic fragments retain immunomodulatory activity (data not shown). To estimate the size of the immunomodulatory protein(s), *A. aegypti* SGE was fractionated by gel filtration HPLC, and fractions (0.5 mL) were collected at 1 min intervals for bioassay. Splenocyte proliferation was suppressed by fractions 11–15, with the peak activity in fraction 12 (Figure 2.6). Reference to a regression equation derived from retention times of proteins of known size (Figure 2.6 inset) indicates an estimated size of 387 kDa. If a single

component of the saliva is responsible for all the observed immunomodulatory effects (inhibition of proliferation, Th1 and Th2 cytokine secretion), then each activity should be highly correlated with the others. On the other hand, if multiple salivary components are involved, there should be some fractions that inhibit one immune function but not others, and the activities should be poorly correlated between fractions. Aliquots from each gel filtration fraction were therefore assayed for their ability to inhibit secretion of IFN-γ and IL-4, and the degree of inhibition was compared to the inhibition of proliferation by regression analysis. Inhibition of IFN-γ was significantly correlated with both inhibition of IL-4 ($r^2 = 0.88$, $P < 0.05$) and inhibition of proliferation ($r^2 = 0.97$, $P < 0.01$). Similarly inhibition of IL-4 was correlated with inhibition of proliferation ($r^2 = 0.82$, $P < 0.05$). Inhibition of LPS stimulated proliferation of B-cells was also significantly correlated with inhibition of T-cell proliferation ($r^2 = 0.91$, $P < 0.01$). This indicates that a single component of the saliva, either a single protein or a complex, is responsible for inhibition of both proliferation and cytokine secretion.

DISCUSSION

To model the normal route of CD4+ T-cell stimulation, where salivary antigens are processed and displayed in complex with MHC class II by antigen-presenting cells for interaction with the T-cell receptor (TCR), we used DO11 transgenic mice whose TCRs have been genetically fixed to respond only to interaction with OVA peptide. *A. aegypti* SGE strongly inhibited antigen-stimulated CD4+ T-cell proliferation, with over 50% inhibition at 0.3 SGP/mL. Antigen-stimulated cytokine secretion was also inhibited: the Th1 cytokines IL-2 and IFN-γ, and the pro-inflammatory cytokines TNF-α and GM-CSF were reduced by at least 50% by 0.45–0.6 SGP/mL, as was the Th2 cytokine IL-5.

Secretion of the Th1-inducing cytokine IL-12 was also strongly inhibited, with secretion reduced by 67% in the presence of 0.45 SGE/mL. However, IL-4 required 1.2 SGP/mL, and IL-10 required 2.05 SGP/mL for 50% inhibition. These results indicate that Th1 and inflammatory responses may be inhibited by low SGE concentrations, and that concentrations above 1.0 SGP/mL can inhibit Th2 responses as well. The observed inhibition of T-cell proliferation, and secretion of IL-2 and IFN-γ, occurred at SGE concentrations that do not decrease cell viability, as indicated by the proportion of cells unable to exclude propidium iodide. On the other hand, inhibition of secretion of the Th2 cytokines IL-4 and IL-10 occurred at higher SGE concentrations where T-cell viability was reduced.

The decreased production of Th1 type cytokines may follow from the dramatically reduced secretion of IL-12 in splenocyte cultures. Viability of dendritic cells, the major source of IL-12, is not affected by SGE concentrations as high as 25.0 SGP/mL, indicating an inhibition of the affecter functions of this cell type. The dose-dependent decrease in TNF-α and GM-CSF production from splenocyte cultures suggests that other cell types, including macrophages and neutrophils, may also be affected by *A. aegypti* SGE. Taken together, these results indicate immunomodulation of dendritic cell function at all SGE concentrations, modulation of T-cell function at very low SGE concentrations, and immunosuppression due to increased T-cell death at SGE concentrations above 1.0 SGP/mL. Similar concentrations of SGE inhibit B-cell proliferation; this effect is probably caused by a direct effect on the B-cells, because they were stimulated with LPS, which does not require participation of CD4+ T-cells.

Our results are consistent with the report by Bissonnette et al. (29) that *A. aegypti* salivary gland extracts inhibit release of TNF-α from rat mast cells. On the other hand, they contrast with those reported for the same mosquito by Cross et al. (28). These authors reported that Con A stimulated secretion of Th1 cytokines was inhibited by 4.0 SGP, and that Th2 cytokines were not significantly reduced (although a non-significant trend to lower IL-4 and IL-5 levels was seen). They further reported that antigen stimulated cytokine secretion was not inhibited. Cross et al. found 45% proliferation inhibition with 4 SGP/well, suggesting that their salivary gland extracts were attenuated at least 50-fold relative to ours. This attenuation would bring their SGE into the concentration range where we also see effects on cytokine secretion similar to what they reported, including more pronounced inhibition of Th1 than Th2 cytokines. It is possible that the difference in salivary gland extracts is due to differences in preparation and handling of the SGE (freeze/thaw lysis vs. sonication and use of fresh extract), or to differences between laboratory strains of *A. aegypti* mosquitoes. Cross et al. (28) measured proliferation using a vital dye, 3-[4,5-diamethylthiazol-2-yl]-2·5-diphenyltetrazolium bromide (MTT) rather than [3H]-thymidine incorporation; one technique measures numbers of cells and the other newly synthesized DNA, and it is difficult to compare the results directly.

Interpretation of the immunological significance of these results requires consideration of the actual concentration of saliva in the host at the site of mosquito feeding. If we assume for simplicity that saliva diffuses through a spherical region with a radius of 1 mm in the skin, a volume of 4.2 µL would be affected. Mosquitoes use about 0.5–0.6 SGP in a blood meal (see below for discussion of this point), and in addition

they re-ingest saliva equivalent to about 0.2 SGP (personal observation), leaving 0.3–0.4 SGP in the skin, which would result in an effective concentration of saliva as high as 30–70 SGP/mL at the bite site. As this concentration is at least 50-fold greater than the concentrations necessary to inhibit immune cell function, we conclude that *A. aegypti* saliva can suppress inflammatory responses, as well as aspects of both innate and acquired immune responses in the immediate vicinity of the bite site. The pronounced reduction in IL-2 is likely to result in inhibition of CD4+ T-cell activation and proliferation, as well as a reduction in NK cell activation. Activation of CD8+ T-cells is enhanced by co-stimulation with IL-2 and IL-12 (32), so we surmise that this aspect of the local immune response would also be inhibited. Reduced titres of IFN-γ could be expected to result in reduced activation of macrophages in the vicinity of the bite. The effect of saliva on Th1 and Th2 responses is likely to depend on the specific local concentration of saliva in the skin, because IL-4 and IL-10 require slightly higher saliva concentrations for inhibition to occur. In the immediate vicinity of the bite, saliva concentrations would be sufficient to inhibit both types of response. As saliva diffuses into the surrounding skin, the concentration could fall to a point where Th1 responses would be selectively inhibited and the local environment would favor a Th2 response.

The immunomodulatory activity described in this report is likely to have significance in blood feeding, because it is associated only with female mosquitoes, it is present in saliva as well as salivary gland extracts, and it is depleted from salivary glands following a blood meal. The depletion of activity in salivary glands from freshly blood-fed mosquitoes suggests that about half of the total activity is secreted into a host during a blood meal. This is consistent with earlier studies that showed that a similar

proportion of soluble protein or the anti-haemostatic enzyme apyrase were secreted during a blood meal (15,33).

Our preliminary characterization of the immunomodulator indicates that all of the observed activities are probably due to a single 387 kDa protein or complex of proteins. Bissonette and co-workers (29) reported that inhibition of TNF-α release from rat mast cells was due to a salivary component that was sensitive to boiling and larger than 10 kDa, results consistent with our findings. Because gel filtration is a non-denaturing chromatographic technique, we cannot say if the activity is due to a single polypeptide or to a multimeric complex. A protein of approximately the calculated size of the immunomodulator can be seen on the gels figured by Racciopi and Spielman (34), and this band became radiolabelled when mosquitoes were injected with ^{35}S `methionine, indicating that it is synthesized when saliva is replenished following a blood meal. Zeidner et al. (31) reported that mice injected with sialokinin I, a tachykinin peptide with vasodilatory activity isolated from *A. aegypti* salivary glands (7), had reduced levels of Th1 cytokines, including IL-2 and IFN-γ. It is unlikely that the immunomodulatory activity we report is due to this peptide, as sialokinin is much smaller (1·4 kDa) than the immunomodulator, and unlike the immunomodulator it is not denatured by boiling or by reversed-phase buffers such as acetonitrile. *A. aegypti* saliva also contains an apyrase, which inhibits platelet aggregation at the bite site by converting ADP and ATP to AMP. Although AMP has known immunomodulatory properties (35), it is unlikely that the activity we report is due to AMP or an AMP-generating system. Firstly, the immunomodulator elutes from the gel filtration column well before the 65 kDa apyrase, so that the most immunomodulatory fractions lack any ATPase or ADPase activity (data

not shown). Secondly, *A. aegypti* saliva contains adenosine deaminase and purine nucleosidase activity (36,37), so instead of accumulating AMP, saliva drives the conversion of ATP and ADP to hypoxanthine and ribose, which lack immunosuppressant activity.

The mode of action of the *A. aegypti* salivary immunomodulator appears to differ from immunomodulatory molecules and activities known from other blood-feeding arthropods. The deer tick, *I. scapularis*, secretes a T-cell inhibitor that binds to IL-2, sequestering this signal and interrupting IL-2-driven proliferation (21). Similarly, other ixodid ticks have an IL-8 binding protein that inhibits the ability of this chemokine to drive inflammatory and Th1 responses (22). These inhibitors must be continuously present to affect immune cell function. In contrast we found that a transient exposure to *A. aegypti* SGE was sufficient to change the subsequent response of splenocytes to stimulation, and the SGE did not have to be present at the time of stimulation or subsequently for inhibition to occur. These results indicate that *A. aegypti* SGE does not sequester a cytokine or other activation signal secreted from immune cells.

I. scapularis saliva also contains a protein, Salp15, that specifically interferes with T-cell receptor (TCR) signaling in CD4+ T-cells, ultimately inhibiting IL-2 secretion (20). This protein abolishes antigen-specific stimulation without affecting responses to mitogen or ionomycin. The related tick *I. ricinus* has a salivary protein, Iris, that modulates T-cell and macrophage function by inhibiting secretion of Th1 and pro-inflammatory cytokines (38); it is likely that this protein accounts for the ability of *I. ricinus* to produce a strong Th2 response (23). The New World sandfly, *Lutzomyia longipalpis*, secretes a vasodilatory protein, maxadilan (39), that also has potent

immunomodulatory activity, inhibiting macrophage function by interacting specifically with the pituitary adenylate cyclase-activating peptide (PACAP) type 1 receptor (33). Other blood-feeding arthropods have also been described as having immunomodulatory activity, without identification of specific molecules or mechanisms of action (16–19). Examples include the blackfly *Simulium vittatum* (40,41), and numerous ixodid ticks (17–19,42,43). In each case the range of cell types and cytokine signals affected differs from the spectrum of activity of *A. aegypti* SGE.

The *Aedes* immunosuppressant activity is also unusual in that it includes inhibition of B-cell proliferation in response to LPS stimulation. This anti-B-cell activity co-elutes with, and is highly correlated with the anti-T-cell activity of the saliva, suggesting that the same component is responsible.

The response of the vertebrate host to *A. aegypti* feeding is consistent with the immunosuppressive activities described here. Despite the suppression of T- and B-cells at the immediate bite site, the host does develop an antibody mediated response to salivary antigens that is strongly Th2 in character, indicating immunomodulation but not systemic immunosuppression. In both BALB/c and C3H mice, antigen restimulation assays and measurement of *in vivo* circulating cytokines following mosquito feeding indicate lower IL-2 and IFN-γ, and elevated IL-4 and IL-10 levels (30,31). Two effects of *A. aegypti* saliva contribute to an understanding of this phenomenon. As discussed above, diffusion of saliva into the skin is likely to result in a ‘pro-Th2’ zone surrounding an immunosuppressed zone at the centre of the bite site. It is also suggestive that *A. aegypti* saliva is not toxic to dendritic cells, but it inhibits secretion of IL-12 in our splenocyte cultures. IL-12 is primarily secreted by antigen presenting cells, and in

addition to its pro-inflammatory role it is a key promoter of the development of Th1 responses (44). Following exposure to saliva at the bite site, these IL-12 deficient dendritic cells could be expected to migrate to the T-cell rich regions of lymphoid organs and to direct the development of Th0 cells into Th2 CD4+ T-cells.

The nature of the IL-12 suppressing activity needs further investigation; it may be due to the same molecule that inhibits T- and B-cell function, or it could be due to a different component of the saliva or to the joint action of a number of salivary components. It is interesting in this regard that Zeidner et al. (31) found that they could induce a Th2 response in mice by injecting synthetic sialokinin (the tachykinin vasodilator present in saliva) as well as by mosquito feeding. The search for the *Aedes* immunomodulator(s) will also be helped by the recent publication of a thorough inventory of all the cDNAs expressed in salivary glands of this important vector species (12).

Salivary immunomodulators have been shown to influence the process of pathogen or parasite transmission in several instances. Perhaps best studied is the effect of sandfly saliva, enhancing establishment of *Leishmania* parasites in immunologically naive hosts (45–48). This effect is probably due to the Th2-promoting activity of maxadilan in *Lutzomyia* saliva (24) and adenosine or AMP in *Phlebotomus* saliva (14,15). Similarly, the Th2-enhancing activity of *I. scapularis* saliva enhances transmission of the Lyme disease spirochete *Borrelia burgdorferi*, and the effect can be reversed by providing exogenous IL-2 and IFN-γ (49). A similar effect of *I. ricinus* saliva on transmission of tick borne encephalitis has also been demonstrated (50). Saliva of, or feeding by *A. aegypti* has been shown to enhance transmission of Cache Valley

virus, because injection of virus into sites on mice where mosquitoes had fed, within 4 h of the feeding, resulted in earlier and higher peak viraemias (51). If virus was injected at 8 h post-feeding or later, no enhancement occurred. Similarly, saliva of the related mosquito *Aedes triseriatus* enhances infection of LaCrosse virus in white-tailed deer and chipmunks, and vesicular stomatitis virus in mouse models (52), producing higher peak and longer lasting viraemias. The latter effect has been correlated with a decrease in IFN-α/β in cell culture assays (53). We hypothesize that these effects are due to local suppression of CD8+ and CD4+ T-cell mediated immunity at the site of virus introduction. The transient nature of the enhancement can be ascribed to the replacement of the CD8+ and CD4+ T-cells with cells migrating to the injury site from lymph nodes and elsewhere in the skin. *A. aegypti* is most notorious as a vector of Yellow Fever and Dengue Fever, both of which are serious and potentially fatal infections currently undergoing an increase in geographical distribution and number of cases (26,27). In the case of Dengue Fever, a Th1 response is associated with relatively mild symptoms and rapid resolution of the infection, and severe disease is associated with decreased IL-12 and a more Th2-like response (54,55). The onset of a protective Th1 response may be delayed until the virus disseminates away from tissues that have been influenced by saliva from the vector. Although this is an attractive hypothesis, it remains to be determined if the immunomodulatory and immunosuppressive effect of *Aedes* saliva, described in this report, influences transmission of these human pathogens. However, it seems possible that further characterization of the molecule responsible for the effect of *Aedes* saliva could contribute to the future development of transmission blocking drugs or vaccines.

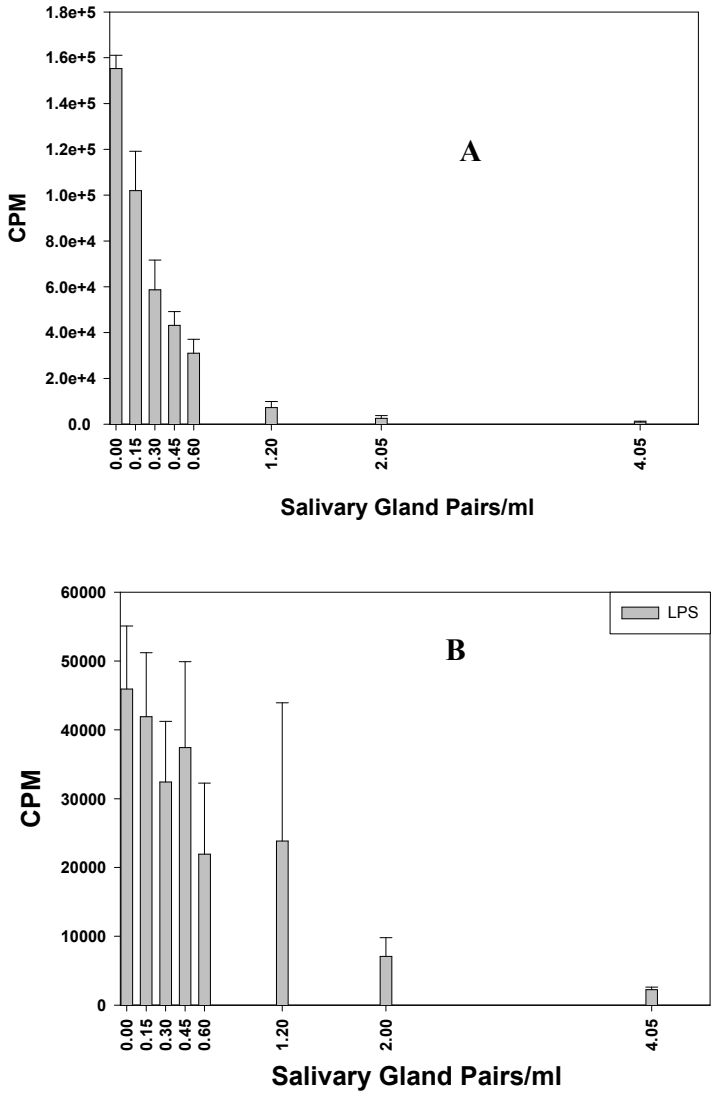


Figure 2.1: *Aedes aegypti* SGE suppresses proliferation of antigen-stimulated T-lymphocytes (**A**) and LPS-stimulated B-cells **(B**). DO11 splenocytes (5 x 105 cells) were cultured for 72 hr in the presence of the indicated concentrations of salivary gland extract and OVA peptide or LPS. Incorporation of [3H]–thymidine was used to determine splenocyte proliferation. Data points represent the mean counts per minute (CPM) ± standard error (n=3). Regression analysis indicates a significant treatment effect ($p < 0.01$).

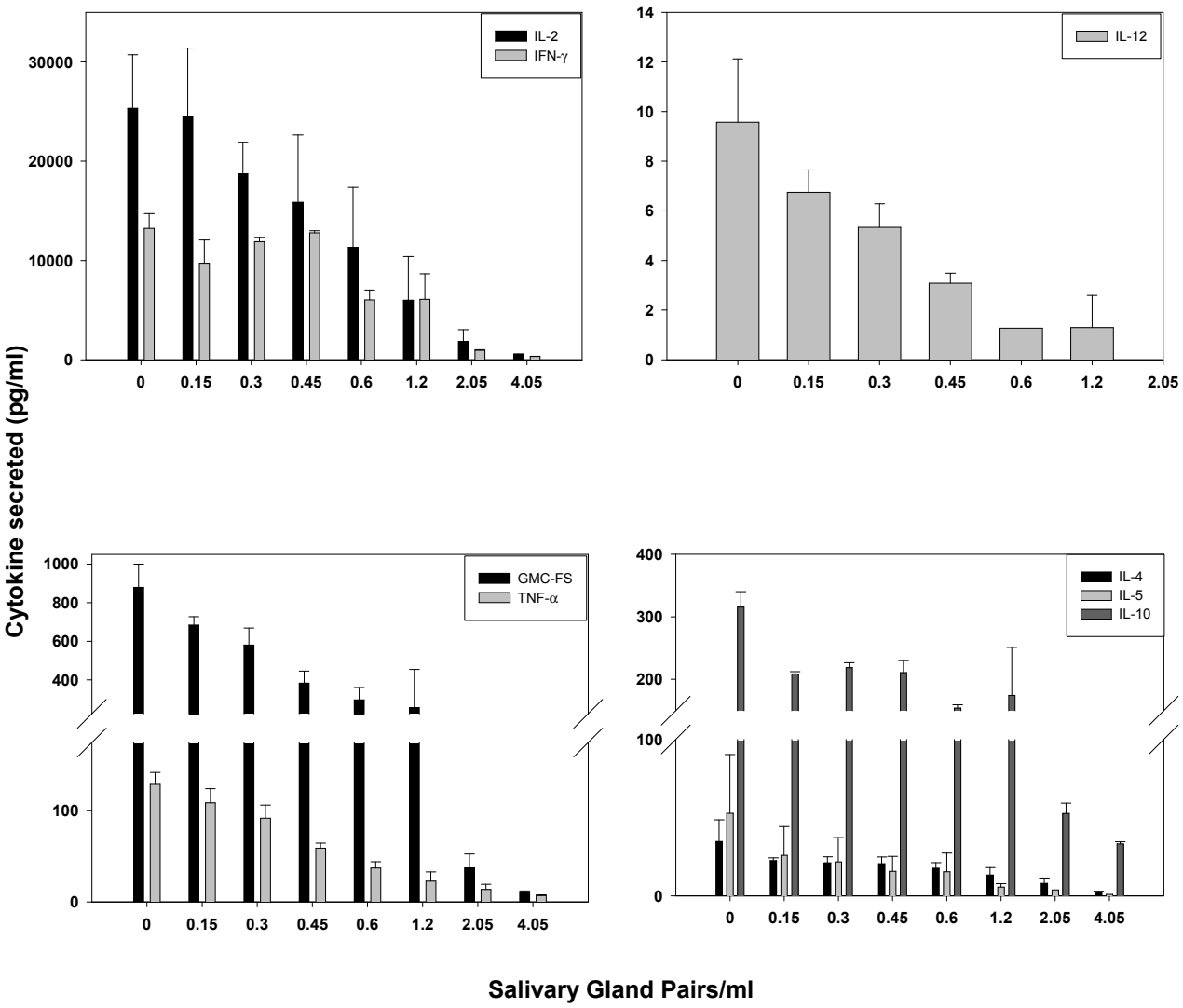
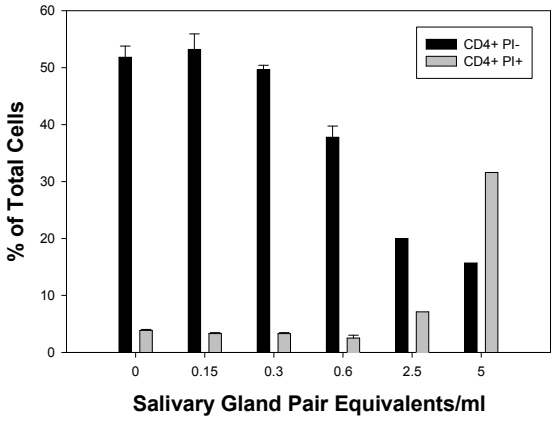
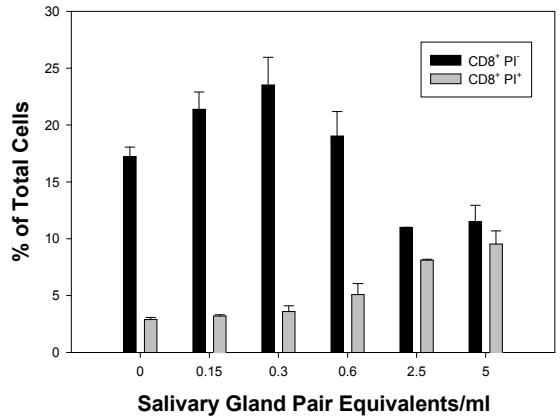


Figure 2.2: *Aedes aegypti* salivary gland extract suppresses pro-inflammatory, Th1, and Th2 cytokine secretion by OVA-specific T-lymphocytes. Supernatants from DO11 splenocytes (5 x 105 cells) cultured for 48 hr in the presence of indicated amounts of salivary gland pairs (SGP) and OVA peptide were assayed for: (A) the Th1 cytokines IL-2 and IFN-g; (B) IL-12; (C) the pro-inflammatory cytokines GM-CSF and TNF-a; and (D) the Th2 cytokines IL-4, IL-5, and IL-10. Data points represent the mean ± standard error (n=3). Regression analysis indicates a significant treatment effect ($p < 0.01$) for all cytokines.





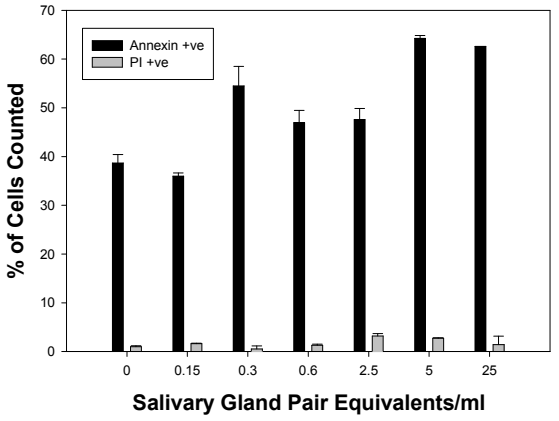


Figure 2.3: Salivary gland extract effects on viability of CD4+ and CD8+ T-cells and dendritic cells. Splenocytes were cultured for 72 hr with the indicated concentration of SGE (panels A and B). Subsequently cells were labeled with FITC-anti-CD4 (Panel A) or PE-anti-CD8 (Panel B) and counted by flow cytometry. Cell viability was determined by the ability to exclude propidium iodide (PI). Similarly, a murine dendritic cell line was cultured in the presence of SGE, and cell viability was assayed by binding of annexin V and by PI eclusion (Panel C).

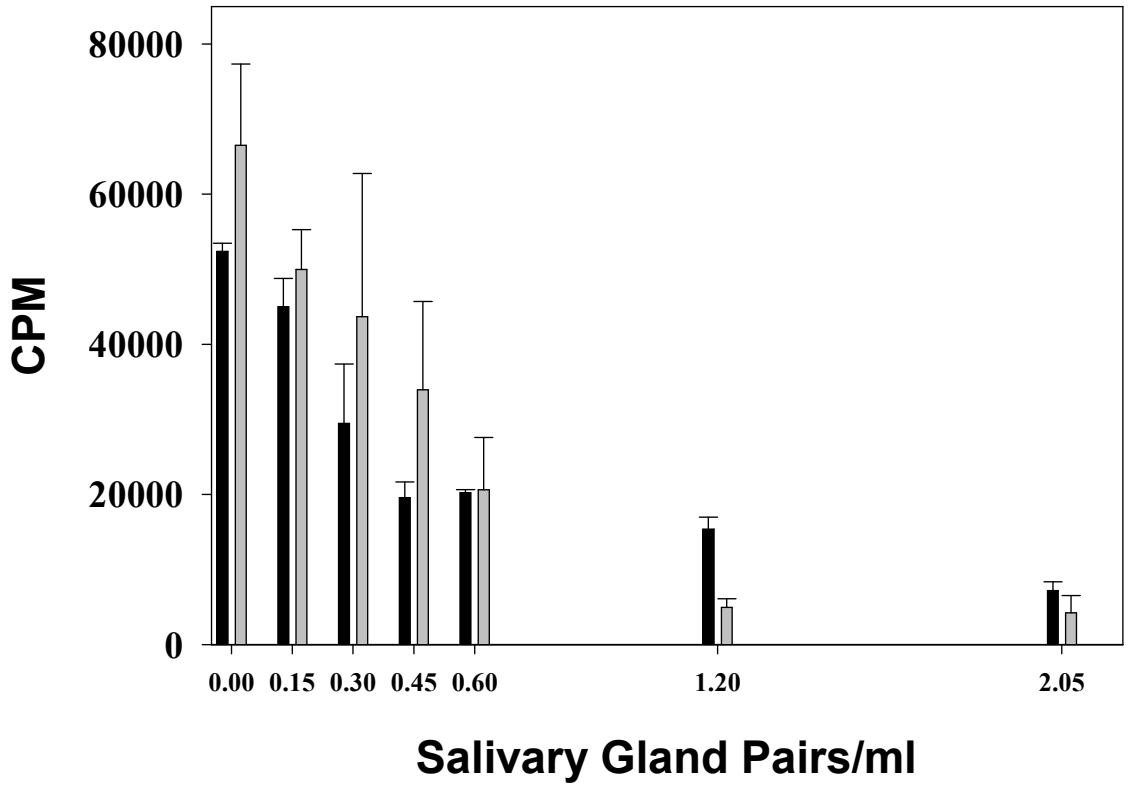


Figure 2.4: *Aedes aegypti* SGE affects immune cells directly. SGE in the indicated concentrations was added to splenocyte cultures for 2hr. The splenocytes were then washed and fresh RPMI was added to the cultures (dark bars). Control cultures (light bars) were exposed continuously to SGE. Splenocytes (5 x 105 cells) were cultured in the presence of Con A for 72 h. Incorporation of [3H]–thymidine was used to determine splenocyte proliferation. Data points represent the mean counts per minute (CPM) ± standard error (n=3). Regression analysis indicates no significant difference between the two treatment groups.

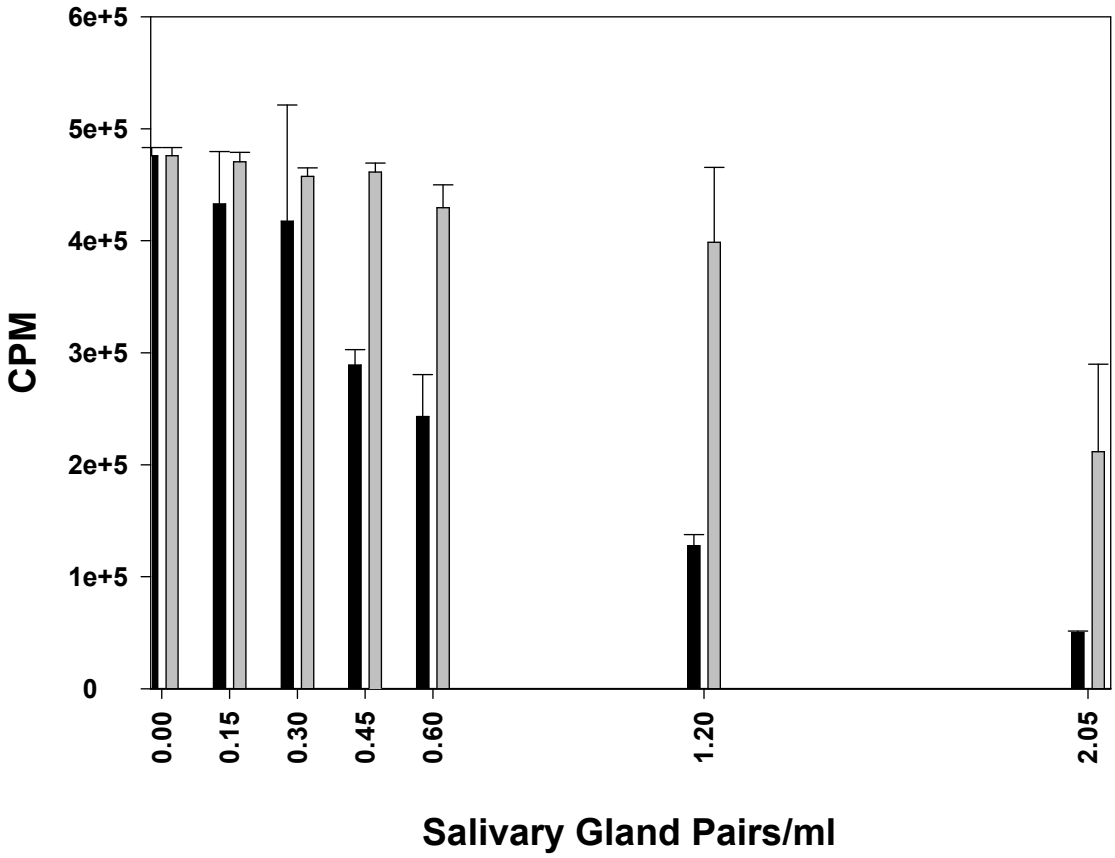


Figure 2.5: Blood feeding depletes the immunosuppressive activity from salivary glands. BALB/c splenocytes (5 x 105 cells) were cultured in the presence of *Aedes aegypti* SGE from blood-fed (light bars) and unfed mosquitoes (dark bars) and stimulated with Con A for 72 hr. Cellular incorporation of [3H]–thymidine was used to determine splenocyte proliferation. Data points represent the mean counts per minute (CPM) ± standard error (n=3). Regression analysis indicates a significant treatment effect ($P < 0.01$). Additionally, there is a significant difference between the two treatment groups ($p<0.01$).

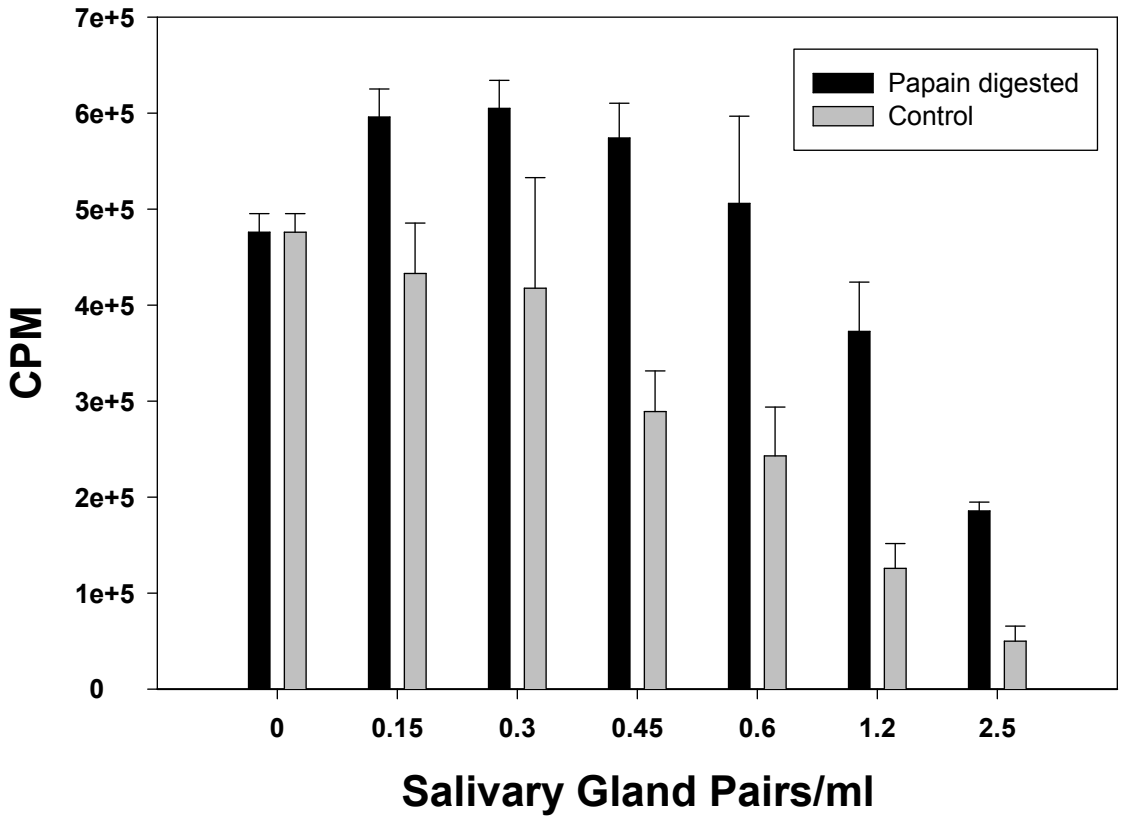


Figure 2.6: Protolytic digestion reduces the ability of salivary gland extract to inhibit splenocyte suppression. SGE was digested with papain and then incubated with BALB/c splenocytes (5 x 105 cells) and Con A for 72 h. Cellular incorporation of [3H]–thymidine was used to determine splenocyte proliferation. Data points represent mean counts per minute (CPM) ± standard error (n=3). Differences between the positive control and experimental treatments were significant ($p < 0.01$). Additionally, ANOVA indicates a significant difference between digested and control SGE ($p<0.01$).

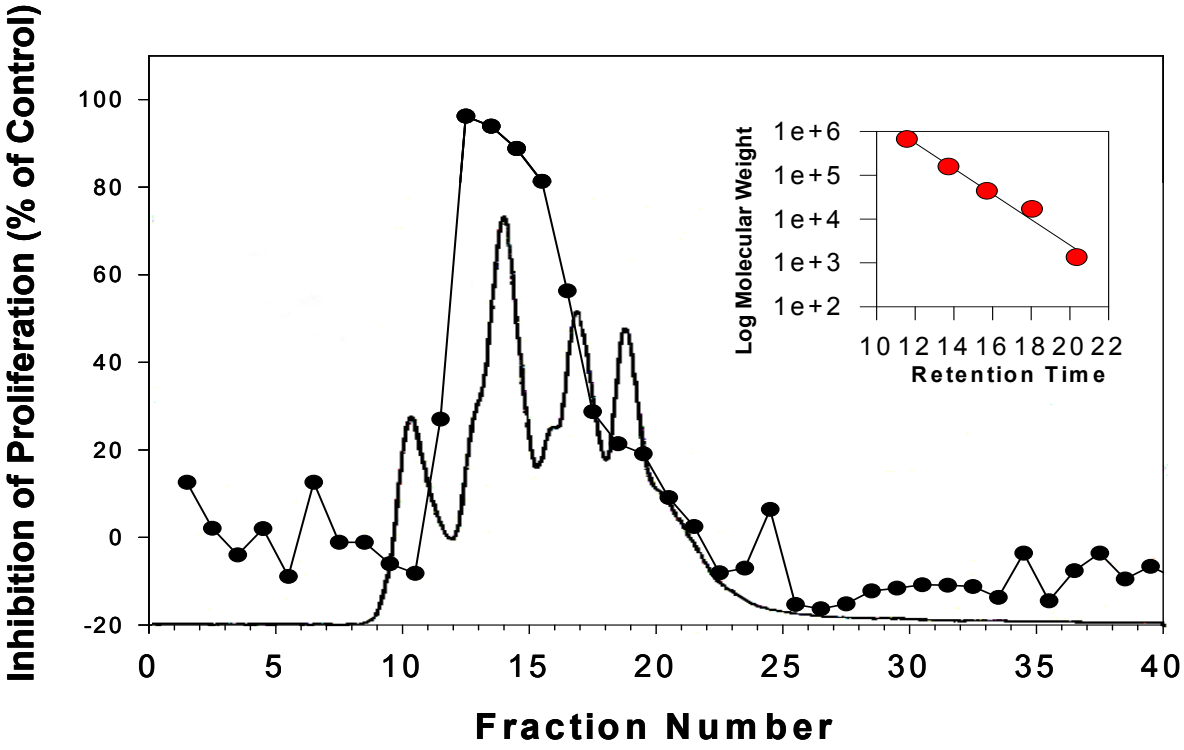


Figure 2.7: Gel filtration HPLC of *Aedes aegypti* SGE. Extracts from 100 SGP were chromatographed on a BioSil SEC 250 gel filtration column, run at 0.5 ml/min with PBS as the mobile phase. Fractions were collected at 1 min intervals, and 20 ml of each fraction was bioassayed using Con A-stimulated BALB/c splenocytes (5 x 105 cells) for 72 hr. Cellular incorporation of [3H]–thymidine was used to determine splenocyte proliferation. The chromatogram is shown with the thicker continuous line, and the assay results with the filled circles. A calibration curve of log molecular weight against retention time of protein standards is shown in the inset.

Salivary gland pairs	Saliva (% suppression)	SGE (% suppression)
Control	0 ± 1·73	0 ± 1·73
Male, 0·5	ND	−19·09 ± 9·77
Male, 1·0	ND	−19·75 ± 3·91
Female, 0·5	ND	99·78 ± 0·01
Female, 1·0	28·26 ± 3·06	99·59 ± 0·09
Female, 2·0	85·16 ± 7·31	99·78 ± 0·03

Table 2.1: Female and male *A. aegypti* salivary gland extract (SGE) (0·5 or 1·0 SGP), female saliva (1·0 or 2·0 SGP), or male SGE (0·5 or 1·0 SGP) was incubated with BALB/c splenocytes for 72 h. Incorporation of [^{3}H]-thymidine was used to determine splenocyte proliferation. Values represent mean suppression (as percentage of the positive control) ± standard error (n=3). Male SGE is significantly less inhibitory than female SGE (P< 0·001), and it is not different from the solvent control (P=0·8077). All concentrations of female saliva and SGE significantly reduced proliferation (P < 0·01) relative to the positive control. ND, not done.

Salivary gland pairs	DO11 (% suppression)	BALB/c (% suppression)
0·5, boiled	−34·64 ± 7·60	−29·67 ± 4·26
1·0, boiled	−19·33 ± 4·28	−5·26 ± 8·13
0·5	98·36 ± 0·70	99·78 ± 0·01
1·0	99·45 ± 0·13	99·36 ± 0·08

Table 2.2: Boiling the salivary gland extract causes a loss of immunomodulatory activity. Female salivary gland pairs were boiled and then added to BALB/c or DO11 splenocytes (5×10^5 cells/200 µL RPMI) for 72 h. Incorporation of [^{3}H]-thymidine was used to determine splenocyte proliferation. Values are mean inhibition of proliferation, relative to the positive control, ± standard error (*n* =3)

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CHAPTER 3

MODULATION OF MURINE J774 MACROPHAGES BY *AEDES AEGYPTI* SALIVA[1]

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ABSTRACT

The saliva of many vector arthropod species contains factors that inhibit hemostatic responses in their vertebrate hosts. However, less is known about the effect of vector saliva on the development of immune responses. We investigated the effect of *Aedes aegypti* salivary gland extracts (SGE) on several different macrophage effector functions including; phagocytosis, iNOS activity, MHC Class I and II representation, and cytokine secretion *in vitro*. The production of pro-inflammatory cytokines (Interleukin-1a [IL-1a], Interleukin-6 [IL-6], Interleukin-12 [IL-12], and Tumor Necrosis Factor-α [TNF-α]) was strongly reduced by salivary extract, with 50% or greater inhibition at only 1.0 SGP/ml. IL-12 secretion was suppressed by 50% with 0.5 SGP/ml, and almost completely with the addition of 2.0 SGP/ml. The addition of 2.0 SGP resulted in a 62% reduction of IL-6. In marked contrast, there was no significant modulation of IL-10 secretion, compared to the control, with the addition of up to 5.0 SGP/ml, although inhibition was seen at 10 SGP/ml and above. SGE significantly inhibited phagocytosis of *E. coli* by macrophages. MHC Class I and II expression was also decreased in a dose-dependent manner following incubation with SGE for 24 and 48 h. Nitric oxide (NO) production was not inhibited in macrophages that were incubated with SGE. Prostaglandin (PGE_2) and intracellular cAMP signal transduction pathways are not affected by exposure to *A. aegypti* SGE.

INTRODUCTION

Many insects, including *Aedes aegypti*, may inject parasites or other pathogens as well as saliva into the host upon blood-feeding. This mosquito is particularly known as a vector of Dengue and Yellow Fever virus (26) (18). *A. aegypti,* along with other bloodsucking arthropods, has evolved salivary anti-hemostatic components to circumvent host platelet aggregation, blood coagulation, and vasoconstriction defenses. Salivary anti-hemostatic components aid in increasing the size of the blood meal and decreasing feeding time, increasing the probability of feeding without the mosquito being detected and destroyed by the host (22).

Additionally, bloodsucking arthropod saliva contains immunomodulators that work to minimize the adverse fitness impact of the host's immune responses. Specifically, Lutzomyia longipalpis saliva contains maxadilan, which dilates blood vessels (Milleron 2004, Nef effects), thereby enhancing blood acquisition by the sandfly. In vitro and in vivo experiments showed that antibodies specific for maxadilan would decrease vasodilation thus inhibit blood acquisition. Saliva of *A. aegypti* has been reported to modulate cellular effector functions of the innate as well as the adaptive immune system. Activated rat mast cells had decreased secretion of TNF-α after exposure to *A. aegypti* saliva (2). *Aedes* saliva incubated with activated murine splenocytes modulated a decrease in T-cell proliferation and IL-2 secretion (7). In the previous chapter, we reported that exposure to saliva of this mosquito resulted in a dose-dependent decrease in pro-inflammatory and both Th1 and Th2 cytokines in both Con A stimulated BALB/c and OVA-stimulated DO11 mouse splenocytes (28). In

particular, secretion of IL-12 and TNF-α was markedly inhibited, suggesting an effect on antigen presenting cells (APCs), including macrophages and/or dendritic cells.

Macrophages are effector cells with roles in both innate and adaptive immunity. They are found in the epidermis where vector feeding and injection of pathogens takes place. Macrophages phagocytize pathogens, process and present pathogen derived proteins, and secrete various cytokines that influence the subsequent adaptive immune response. Specifically, IL-1, IL-12, and TNF-α are pro-inflammatory, and IL-12 regulates a Th1 response in CD4+ helper T-cells. On the other hand, the anti-inflammatory cytokine IL-10 promotes a Th2 response. Phagocytized pathogens are processed in the phagolysosome and the resultant peptide fragments are then bound to Major Histocompatability Complex (MHC) Class II and presented to CD4+ T- cells.

Our hypothesis is that the effector functions of APCs, in this case macrophages, are influenced by *A. aegypti* SGE. To test this, several different macrophage effector functions were examined including: phagocytosis, inducible nitric oxide synthase (iNOS) production, MHC Class I and Class II expression, and cytokine secretion. Sandfly saliva has been shown to modulate macrophage effector functions through cell signaling pathways that involve upregulation of cAMP or PGE_2 (25). We therefore tested the hypothesis that *A. aegypti* SGE's mode of action on macrophages was also dependent on cAMP or PGE_2 signaling pathways.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma Chemical Corporation (St Louis, MO), unless otherwise stated. Antibodies were purchased from BD PharMingen (San Diego, CA). Adult *A. aegypti* mosquitoes, from a colony established in 1968 at the

Department of Entomology, University of Georgia, were reared at 27°C under a 16-h light/ 8-h dark cycle and fed crushed, dry cat food and Tetramin fish flakes. Adults were fed a 10% sucrose solution.

Preparation of Salivary Gland Extracts. *A. aegypti* salivary gland pairs (SGP) were dissected in 10 mM HEPES (pH 7.4), 150 mM NaCl from 3- to 10- day old female mosquitoes. The glands were stored at –70°C in aliquots of up to 20 pairs per 50 μl of dissection buffer in Eppendorf tubes. Salivary glands were homogenized by a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT). The SGP were disrupted with 10 ultrasound bursts, with the power setting at five and a 50% duty cycle. Homogenized salivary glands were centrifuged at 11 750 *g* for 5 min and the supernatants were pooled and diluted to yield the concentrations used in these studies. The supernatants will be referred to as salivary gland extract (SGE) and the concentration of SGE is given in salivary gland pair (SGP) equivalents per ml.

Macrophage cultures. A BALB/c mouse macrophage cell line was used in these experiments. The J774.1 macrophage cell line was a gift from R. Tarleton (University of Georgia). The cell line was maintained at 37°C at 5% CO_2 in 250 ml tissue culture flasks. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Paisley, PA), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), 1% penicillin-streptomycin (Gibco), 1% L-glutamine (Gibco), and 0.5% gentamicin (Gibco) (referred to subsequently as "complete RPMI").

Phagocytosis.

Escherichia coli preparation.

Macrophage phagocytosis of SYTOX (Molecular Probes) labeled *E. coli* was performed as described previously (10). Briefly, *E. coli* were grown in LB broth overnight at 37°C at 250 rpm. Cells were pelleted by centrifugation at 8,800 *g* for 3 min and spectrophotometrically adjusted to a final absorbance of 0.37 (wavelength= 480nm) in phosphate buffered saline (PBS). Aliquots (1ml) of cells were then permeabilized with 70% ethanol for 1 h at room temperature, pelleted by centrifugation, washed twice in PBS, and stored until use. Cells were stained with 5 μM SYTOX green (Molecular Probes) for 10 min at room temperature in the dark. The cells were pelleted by centrifugation, washed twice with PBS, and then opsonized with 1 ml of fetal calf serum (heat inactivated) for 20 min at room temperature. The cells were then washed twice with PBS.

Phagocytosis assay.

Macrophages were removed from the tissue culture flasks by incubation with cold 0.02% EDTA in PBS for 10 min at 4°C. The cells were then gently pipetted out of the flask into a Falcon tube and the flask was washed again with 10 ml RPMI complete and added to the Falcon tube. The cells were centrifuged for 7 min at 365 *g* (GS-6R Beckman Centrifuge, Beckman) at 4°C. The supernatant was removed and the cells were diluted in RPMI complete to a final concentration of $5X10^5$ cells/ml. In a 96-well round-bottom plate, 100 μl containing $5X10^4$ cells was incubated with various concentrations of *A. aegypti* SGE. The cells were then stimulated with lipopolysaccharide (LPS) (2 μg) to make a final volume 200 μl. The cells were

incubated for 2 h at 37°C at 5% CO_2 and then 50 μl of SYTOX labeled *E. coli* was added to each well. The plate was centrifuged at 365 *g* for 15 min at 37°C then placed in a temperature controlled shaker for an additional 20 min at 37°C at 50 rpm. The cells were then pelleted by centrifugation, the supernatant was removed, and the cells were washed twice with a solution of PBS (pH=7.5) containing 1% BSA and 0.009% sodium azide (PAB). Cells were suspended in 200 μl of PAB, and 200 μl of trypan blue was added to the sample to quench florescence from unphagocytized *E. coli* immediately before cell analysis. The samples were analyzed on a DakoCytomation flow cytometer. Phagocytosis was characterized by macrophages that were SYTOX positive. Experiments were replicated in triplicate.

MHC Class I and II expression.

Macrophages were gently scraped from a tissue culture flask and centrifuged at 365 *g* for 7 min at 4°C. The supernatant was removed and the cells were resuspended in RPMI complete to a final concentration of $5X10^5$ cells/ml. In a 96-well round-bottom plate, 100 μl of $5X10^4$ cells were incubated with various concentrations of *A. aegypti* SGE. The cells were stimulated with 2 μg LPS and 4 U IFN-γ to make a final well volume of 200 μl. The cells were incubated for 24 h and 48 h at 37°C and 5% CO_2. The plate was then centrifuged at 365 *g* at 4°C for 7 min. Cells were washed twice with PAB, and Fc block (1 μl/100 μl PAB) (Molecular Probes) was added to each sample and incubated for 20 min on ice. The cells were washed twice with PAB and stained for MHC Class I (2 μl/ 200 μl PAB) (FITC conjugated mouse anti-mouse $H\text{-}2D^d$ Mab) or MHC Class II (2 μl/ 200 μl) (FITC conjugated mouse anti-mouse $I\text{-}A^d$). Cells were incubated on ice for 30 min in the dark, washed twice with PAB, and resuspended in

100 μl 2% paraformaldehyde in PAB for 10 min on ice in the dark. The cells were washed twice, resuspended in 200 μl PAB and analyzed on a DakoCytomation flow cytometer. Unstained stimulated macrophages were used to set the negative gate and stimulated macrophages, that were single Ab-stained, were used to set the positive gate. All experiments were replicated at least twice.

Inducible Nitric Oxide Synthase Activity.

Briefly, 5X 10^5 cells/ml (100 μl) in complete RPMI were aliquoted into a 96-well round-bottom plate with 2 μg LPS and 4 U IFN-γ and incubated for 24 h with various concentrations of *A. aegypti* SGE at 5% CO_2 and 37°C in a final volume of 200 μl. The positive control consisted of recombinant murine iNOS (1 Unit/ 200 μl) (Cayman Chemicals). ^{3}H-Citruline, produced in equimolar ratio with nitric oxide, was measured with a NOS assay kit (Cayman Chemicals) following the manufacturer's protocol. ^{3}H-Arginine (Amersham) was repurified prior to use in the assay. Experiments were replicated in triplicate.

Measurement of intracellular cAMP.

Macrophages were plated out at a concentration of 2 X 10^6 cells/ml in RPMI complete (100 μl) into a 96- well flat-bottom plate and incubated overnight at 37°C at 5% CO_2.. Cells were then treated with isobutylmethylxanthine and SGE, and cAMP was measured as described previously (25). A positive control consisted of 1 μM forskolin incubated with the macrophages (25). Briefly, isobutylmethylxanthine (1mM) was added to the cells and incubated for an additional 30 min. SGE was then added to a final volume of 200 μl and incubated for 15 min. The medium was removed and the cells were washed with PBS. The amount of cAMP was determined using the

Catchpoint cAMP Fluorescent Assay kit (Molecular Devices). Experiments were replicated in triplicate.

PGE_2 Dependent TNF-α Secretion

Macrophages were plated out at a concentration of 2 X 10^6 cells/ml in RPMI complete (100 μl) into a 96- well flat-bottomed plate. Macrophages were incubated with SGE and 2 μg LPS at 37°C at 5% CO_2 in a final volume of 200 μl for either 6 h or 24 h. A negative control consisted of indomethocin (1 μg/μl), 2 μg LPS, and SGE added to the macrophages. The positive control consisted of LPS-stimulated macrophages. Supernatants were collected and TNF-α concentrations were determined with a BioPlex kit (BioRad). Experiments were replicated in duplicate.

Measurement of Cytokine Secretion. Briefly, 5X 10^5 cells/ml (100 μl) in complete RPMI were aliquoted into a 96- well flat-bottomed plate with 2 μg LPS and 4 U IFN-γ and incubated for 24 h with various concentrations of *A. aegypti* SGE at a final volume of 200 μl. Following incubation at 37°C at 5% CO_2, the supernatant was collected and centrifuged at 11780 *g* for 7 min. The cell free supernatants were stored at –70°C until analysis.

Secreted IL-1, IL-6, TNF-α and GM-CSF were measured using a mouse Inflammation Panel BioPlex kit (BioRad) following the manufacturer's protocols. Aliquots (50 μl) of supernatants were assayed in duplicate. Experiments were individually replicated three times. Cytokine concentrations were determined by reference to a standard curve generated from known quantities of recombinant cytokines.

IL-12 and IL-10 secretion was assayed using a Sandwich ELISA. Assays were carried out in flat-bottomed ELISA plates following PharMingen protocols. IL-12 capture and detection monoclonal pairs were clones C15.6 and C17.8, and IL-10 antibody pairs were JESS-2A5 and MP5-32C11. Aliquots of supernatants were assayed in duplicate. Experiments were individually replicated three times. Absorbance values were converted to pg/ml as determined by comparison with a standard curve obtained from known quantities of recombinant standards (PharMingen, San Diego, CA).

Statistical Analysis

Data were analyzed for significance using nonlinear or linear regression. Additionally, some data were analyzed for significance using pairwise comparisons with Student's t-test. Comparisons or regressions with $P < 0.05$ were considered to indicate significant treatment effects.

RESULTS

***Aedes aegypti* saliva modulates cytokine production by LPS/ IFN-γ stimulated macrophages.** Previously, we found that *A. aegypti* saliva suppresses IL-12 and TNF-α secretion from stimulated murine BALB/c and DO11 splenocytes (28). These results suggested that saliva may be modulating cytokine secretion in

macrophages as well as T-cells. Given these results, we decided to examine if *A. aegypti* SGE would effect cytokine secretion in stimulated macrophages. Treatment of LPS-stimulated macrophages with SGE resulted in a significant dose-dependent decrease in the secretion of all cytokines measured: IL-1a (R=0.45) (P< 0.04); IL-6 (R=0.52) (P< 0.03); IL-10 (R=0.97) (P< 0.0001); IL-12 (R=0.92) (P< 0.0001); GM-CSF (R=0.80) (P< 0.0001); and TNF-α (R=0.86) (P< 0.0001)(Figure 3.1). Addition of 1.25 SGP/ml suppressed TNF-α and IL-6 secretion by 33% and 17%, respectively. Additionally, the addition of 10 SGP/ml resulted in a significant decrease in IL-1a by 54% (P< 0.03), IL-10 by 62% (P< 0.03), IL-12 by 95% (P<0.0001), GM-CSF by 43% (P< 0.03), and TNF-α by 75% (P< 0.0003) compared to the positive control. Interestingly, it took the addition of 10 SGP/ml to suppress IL-10 secretion; the addition of 1.25, 2.5, and 5 SGP/ml had no significant effect on secretion of this cytokine (P> 0.1). This is in marked contrast to the other cytokines that had a significant decrease in secretion after the addition of only 2.5-5.0SGP/ml.

***Aedes aegypti* saliva suppresses phagocytosis by macrophages.**

Macrophages are the dominant effector cells of a later response to microbes in innate immunity, typically persisting at the site of an infection for one to two days. Macrophages bind to and phagocytose microbes such as *E. coli*. Efficiency of phagocytosis is enhanced by opsonization of *E. coli* by IgG antibodies that are bound by the Fcγ macrophage receptor. To determine if *A. aegypti* SGE inhibited phagocytosis, macrophages were incubated with various concentrations of SGE and then presented with opsonized *E. coli*. Phagocytosis was suppressed in a dose-dependent manner upon addition of increased concentrations of SGE (Figure 3.2). The control, which

consisted of macrophages incubated without SGE prior to the addition of *E. coli*, was 50% *E. coli* positive. The addition of as little as 2.5 SGP/ml resulted in a decrease of phagocytosis by macrophages, (43% *E. coli*-positive cells) compared to the control, and incubation with 25 SGP/ml resulted in a two fold decrease of phagocytosis to 25% *E. coli*-positive.

Expression of MHC Class I and II by macrophages is suppressed by *A. aegypti* saliva.

The MHC-peptide complex interaction with TCRs is a critical step in the development of the acquired cellular immune response. If *A. aegypti* saliva modulates MHC expression, thereby preventing MHC-TCR interaction, subsequent immune responses could be delayed or inhibited. Macrophages incubated for either 24 or 48 h in the presence of *A. aegypti* SGE showed a dose-dependent decrease in MHC Class I expression (Figure 3.3). Macrophages stimulated with LPS and IFN-γ were 88% and 83% MHC Class I positive at 24 and 48 h, respectively. Macrophages incubated for 24 h with 5 SGP/ml had a 31% decrease in MHC Class I expression compared to the positive control, and with 48 h of incubation there was a 15% decrease. The addition of 25 SGP/ml to stimulated macrophages for 24 h and 48 h resulted in a 53% decrease and a 46% decrease in MHC Class I expression, respectively. Macrophages incubated for 48 h with saliva had an increase in MHC Class II expression with the addition of 2.5 SGP or less. The addition of 1.25 SGP increased MHC II by 5%, and 15% with the addition of 2.5 SGP/ml. Conversely, the addition of 10 SGP/ml resulted in a 31% decrease in MHC Class II expression after 24 h and a 33% decrease after 48 h. Stimulated macrophages

that were incubated for 24 h or 48 h were 73% and 60% MHC Class II positive, respectively.

***Aedes* saliva does not inhibit iNOS activity.**

Nitric oxide synthase (NOS) activity is upregulated in activated macrophages, and is an important element of the parasite killing activity of macrophages. We tested the hypothesis that *Aedes* saliva also inhibited upregulation of iNOS in activated macrophages. We determined this by counting the amount of ^{3}H-citruline which is equal to the amount of nitric oxide produced in the reaction. There was no significant difference in iNOS activity between activated macrophages incubated with or without SGE (R= 0.207) (P= 0.4) (Figure 3.4).

***A. aegypti* saliva does not modulate macrophage function through cAMP or PGE_2-dependent signaling pathways.**

To test the hypothesis that *A. aegypti* saliva modulates macrophage function by upregulating intracellular PGE_2 synthesis, we incubated LPS stimulated macrophages with SGE, in the presence or absence of indomethocin, and measured TNF-α secretion. As indomethocin blocks PGE_2 synthesis by inhibiting cyclooxygenase, any PGE_2-dependent effects of SGE should be abolished in the presence of this drug. We found that *A. aegypti* SGE inhibited TNF-α secretion in a dose-dependent manner, and this effect was not altered by indomethocin (Figure 3.5). LPS-stimulated control macrophages secreted TNF-α (6 h [TNF-α]= 496 pg/ml; 24 h [TNF-α]= 1402 ±166 pg/ml; mean + SEM), and this secretion was inhibited in LPS-stimulated macrophages treated with SGE (e.g. with 10 SGP/ml, at 6 h [TNF-α]= 288 pg/ml and at 24 h [TNF-α]= 348 pg/ml). There was no difference between the indomethocin and SGE treated LPS-

stimulated macrophages (indomethocin treated; 2.5 SGP/ml [TNF-α]=360 pg/ml at 6 h and 730 pg/ml ± 166 SEM at 24 h), and LPS-stimulated macrophages treated with only SGE (e.g. 2.5 SGP/ml [TNF-α]= 340 pg/ml at 6 h and 733 pg/ml ± 82 SEM at 24 h).

Maxidilan, a peptide from sandfly saliva, induces an increase in macrophage cAMP levels by activating the pituitary adenylate cyclase activating polypeptide (PACAP) type I receptor (25). To determine if *A. aegypti* saliva has a similar affect, we examined the effect of SGE on intracellular cAMP levels. Macrophages that were incubated for 15 min with 25 SGP/ml had 0.037 pmol ± 0.004 SEM intracellular cAMP ($P > 0.3$), the control (0 SGP/ml) had 0.040 pmol ± 0.006 SEM intracellular cAMP, and the positive control, containing forskolin (which strongly stimulates cAMP synthesis), had 0.159 pmol ± 0 SEM intracellular cAMP ($P < 0.07$).

DISCUSSION

Previously we reported on the immunomodulatory effects of *A. aegypti* saliva in a murine model (28). To reproduce a more natural route of CD4+ T-cell stimulation, DO11 transgenic mice were used in those experiments. DO11 mice contain CD4+ T-cell receptors that will only recognize and interact with MHC Class II bound with the peptide ovalbumin. We found that *A. aegypti* saliva inhibited antigen stimulated T-cell proliferation and secretion of a range of both Th1 and Th2 cytokines in a dose-dependent manner. Additionally, antigen stimulated cytokine secretion of the Th1 inducing cytokine IL-12 was strongly inhibited in a dose-dependent manner. Further, there was also a dose-dependent decrease in TNF-α and GM-CSF production from the splenocyte cultures; TNF-α and GMC-SF secretion were decreased by at least 50% by 0.45-0.6 SGP/ml. These results agree with those reported by Bissonnette, who found

that *A. aegypti* saliva also modulates a reduction in TNF-α secretion by rat mast cells (2). The reduction in the T-cell secreted Th1 cytokines therefore may be due to the decrease in the Th1 stimulating cytokine IL-12, at least in low SGE concentrations. As these cytokines and growth factors are secreted by APCs including macrophages, but not by T-cells, we hypothesized that *A. aegypti* SGE modulates macrophage function independently of its direct effects on T-cells.

Macrophages are important effector cells in both innate and adaptive immunity. Both macrophages and neutrophils are involved in phagocytosis; however, macrophages are effector cells that respond as rapidly as neutrophils but persist longer at the inflammation site (4). Activation of an innate immune response involves macrophage recruitment to the site, followed by phagocytosis and destruction of pathogens. One method of pathogen destruction by LPS and IFN-γ activated macrophages is achieved by nitric oxide in the phagolysosome. Proteins processed in the macrophage phagolysosome are mainly displayed on the cell surface in a complex with MHC Class II, but some protein is transported to the macrophage cytosol, ubiquinated, and displayed associated with MHC Class I (17). The MHC-peptide complex and co-stimulatory molecules interact with the T-cell receptor of a naive T-cell. This interaction activates the naive T-cell to mature into an effector T-cell and subsequently activates T-cell mediated acquired immunity (17).

In this report we demonstrate that *Aedes* saliva immunosuppresses a variety of functions in murine macrophages, relevant to both innate and adaptive immunity. To determine the effect of *A. aegypti* saliva on phagocytosis, macrophages were pre-incubated with saliva and then incubated with opsonized *E. coli*. Phagocytosis was

suppressed in a dose-dependent manner upon addition of increasing concentrations of SGE. The control, which consisted of macrophages pre-incubated without SGE for 2 h prior to the addition of *E. coli*, were 50% *E. coli* positive. The addition of as little as 2.5 SGP/ml reduced *E. coli* phagocytosis by macrophages to 43% *E. coli* positive, and incubation with 25 SGP/ml resulted in a two fold decrease of *E. coli* phagocytosis to 25% *E. coli* positive. As stated in Chapter 1, mosquitoes secrete approximately 60-90 SGP/ml into the host, therefore the amount of SGE used in these experiments is well within a reasonable physiological limit. This result suggests that macrophages at the bite site may be compromised in their ability to phagocytose pathogens transmitted by the feeding mosquito.

MHC Class I and II presentation by LPS/IFN-γ-stimulated macrophages was also inhibited by *A. aegypti* saliva. Macrophages incubated for 48 h with 2.5 SGP or less saliva had a modest increase in MHC Class II presentation. However the addition of 10 SGP/ml modulated a marked decrease in MHC Class II presentation after 24 h and 48 h by 31% and 79%, respectively, compared to the control without SGE, and 25 SGP/ml decreased MHC Class II presentation by 41% and 86%, respectively. MHC Class I presentation was also inhibited. The addition of 5 SGP/ml resulted in a 31% decrease in MHC Class I presentation after 24 h of incubation and a decrease of 15% after 48 h compared to the positive control. The addition of 25 SGP/ml to stimulated macrophages modulated a 53% decrease (after 24 h) and a 46% decrease (after 48h) in MHC Class I presentation, respectively, compared to the positive control. Inhibition of MHC Class II presentation could interfere with the ability of macrophages to present

antigen to CD4+ T-cells, and inhibition of MHC Class I would inhibit the interaction between macrophages and CD8+ T-cells.

Following phagocytosis, parasites/pathogens are killed by NO produced by an inducible iNOS, as well as other bioactive molecules. *A. aegypti* saliva had no significant effect on macrophage iNOS activity. The positive control, consisting of macrophages stimulated with IFN-γ/LPS, had an average DPM= 88,000; stimulated macrophages incubated with 10 SGP/ml had an average DPM= 85,000 ($P > 0.9$); and the recombinant iNOS positive control had an average DPM= 390,373 ($P < 0.02$).

We observed a significant decrease in secretion of the pro-inflammatory cytokines TNF-α and IL-6, the Th1 including cytokine IL-12, and the growth factor GM-CSF following exposure to *A. aegypti* saliva. For example, 1.25 SGP/ml suppressed TNF-α and IL-6 secretion by 33% and 17%, respectively, compared to the positive control. The addition of 10 SGP/ml resulted in a significant decrease in IL-1a by 54%, IL-10 by 62%, IL-12 by 95%, GM-CSF by 43%, and TNF-α by 75%. In contrast, it took the addition of 10 SGP/ml to suppress IL-10 secretion; the addition of 1.25, 2.5, and 5 SGP/ml were without effect. These results suggest that *A. aegypti* SGE has an anti-inflammatory effect on macrophage responses to stimulation. Macrophages exposed to SGE at concentrations below 10 SGP/ml responded to stimulation with normal (i.e. positive control) levels of IL-10, but IL-12 secretion was markedly impaired, suggesting that a strongly pro-Th2 environment may be generated at the bite site.

This is the first report that mosquito saliva modulates a decrease in phagocytosis by macrophages. Indeed, only one previous study has examined the effect of vector saliva on this aspect of immune function. Saliva of the tick *Ixodes scapularis* (reported

as *I. dammini*) was found to suppress several functions of rat neutrophils including phagocytosis of *Borrelia burgdorferi* spirochetes, anaphylatoxin-induced aggregation, FMLP-induced granule enzyme secretion, and zymosan-induced superoxide secretion (23).

Following phagocytosis, pathogen/parasite proteins are processed and presented by MHC Class II, and to some extent MHC Class I. This aspect of macrophage function was also inhibited by *A. aegypti* SGE. In this study, we observed a dose-dependent decrease in both MHC Class I and II with increased doses of SGE. Similarly, blackfly saliva from *Simulium vittatum* was found to suppress MHC Class II presentation on spleen cells following *in vivo* inoculation of SGE into mice (8). Contrary results were found when the effect of sandfly saliva on macrophage MHC Class II presentation was determined *in vitro*. IFN-γ stimulated macrophage cultures from C57BL/6 mice were incubated with *Leishmania major* promastigotes and *P. papatasi* saliva, and there was no observable effect on MHC Class II presentation (13). This variation may reflect real differences between mosquitoes and blackflies on the one hand and sandflies on the other. The comparison is also complicated by methodological differences in the experiments, such as the use of a BALB/c mouse macrophage cell line in our study vs macrophages harvested from C57BL/6 mice in the sandfly experiments, as well as concentration differences, as only 0.5 SGP/ml *P. papatasi* saliva was used by Hall and Titus (13) and our lowest dose was 1.5 SGP/ml.

Our study found a dramatic suppressive effect of *Aedes* saliva on cytokine secretion by macrophages. *Aedes* saliva modulates a dose-dependent suppression of the pro-inflammatory cytokines, IL-1a, IL-12, GM-CSF, and TNF-α, which in turn could

be expected to lead to a decrease in recruitment of neutrophils and monocytes to the area affected by saliva. These findings are similar to those seen previously with a variety of tick species. *Rhipicephalus appendiculatus* saliva was incubated *in vitro* with a mouse macrophage JA-4 cell line and LPS for 24 h, resulting in decreased levels of secreted as well as transcribed IL-1 and TNF-α (12). Saliva from another tick, *Dermacentor andersoni*, inhibited IL-1 and TNF-α secretion, and *I. ricinus in vivo* experiments also have shown a decrease in the pro-inflammatory cytokines IL-6 and GM-CSF (21). Overall, most arthropod saliva that has been experimentally investigated modulates a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines (8, 12, 29, 32). On the other hand maxidilan, a vasodilatory salivary gland peptide cloned from the sandfly, *Lutzomyia longipalpis*, produces a more complex response, inhibiting the release of TNF-α (an anti-inflammatory effect), but increasing IL-6 secretion from macrophages (25).

IL-10 is an anti-inflammatory cytokine produced by activated macrophages and some CD4+ T-cells. This cytokine antagonizes the effects of IFN-γ, leading to the development of a Th2 type environment (14). As well, IL-10 can down-regulate the expression of MHC Class II and the co-stimulatory molecules B7 and B7.2 (3). IL-10 can inhibit production of PGE_2 and NO as well as the pro-inflammatory cytokines TNF-α, IL-1, and IL-6 in macrophages (19). We found that macrophage secretion of IL-10 is immunosuppressed by *Aedes* saliva only with the addition of 10 or more SGP/ml. These findings are in contrast to the inhibition of the pro-inflammatory cytokines including the Th1 cytokine IL-12, which were suppressed with as little as 1.25 SGP/ml. These results are consistent with our observation that *A. aegypti* SGE inhibited IL-10

secretion from antigen-stimulated mouse splenocytes (Chapter 2). *L. longipalpis* saliva has been reported to immunomodulate an increase in IL-10 secretion in mouse bone marrow derived- macrophages (20). However, *R. appendiculatus* saliva immunosuppressed macrophage secretion of IL-10 *in vitro* (12). The differences in salivary effects on IL-10 expression, including upregulation by sandflies, no modulation of macrophage IL-10 secretion by *A. aegypti* SGE and a decrease from *A. aegypti* SGE incubated with mouse splenocytes, and a decrease by tick saliva may be attributed to species differences as well as the inherent differences in the different macrophage lineages.

Maxidilan, isolated from *L. longipalpis* saliva, increases intracellular PGE_2 production and decreases macrophage TNF-α secretion. Macrophages treated with both indomethocin and maxidilan had an increase in TNF-α secretion and a decrease in PGE_2. As indomethocin blocks PGE_2 synthesis by inhibiting cyclooxygenase, this result suggests that maxidilan inhibits TNF-α secretion by upregulating PGE_2 synthesis (25). To determine if *Aedes* saliva also inhibits TNF-α macrophage secretion through a PGE_2 signaling pathway, we treated macrophages with indomethocin in the presence of *A. aegypti* saliva. We did not see an increase in TNF-α production in these indomethocin-treated macrophages compared to a control consisting of LPS-stimulated macrophages, implying that *A. aegypti* SGE does not inhibit TNF-α secretion by a PGE_2-dependent pathway.

Additionally, *Aedes* saliva may immunomodulate macrophage cytokine secretion via the regulation of intracellular cAMP levels. It has been reported that elevated levels of cAMP may inhibit the secretion of TNF-α from macrophages (1, 24). Additionally,

maxadilan binds to the PACAP type I receptor and stimulates a dose-dependent increase in intracellular cAMP (25). Taken together it is a logical step to determine if *Aedes* saliva also modulates an intracellular increase in macrophage cAMP. However, we did not observe a difference in cAMP levels between the control and SGE treated macrophages. Our results preclude a cAMP-dependent mechanism, including binding to the PACAP receptor, for *A. aegypti* saliva.

Our results indicate that *A. aegypti* saliva (or SGE) profoundly modifies almost every aspect of macrophage function relevant to their role in both innate and adaptive immunity. Phagocytosis of pathogens and signaling to both CD4+ and CD8+ T-cells are inhibited. Cytokine signals that influence the type of acquired immunity are modified in a manner that biases towards a Th2 response. It is likely that these effects will greatly inhibit the ability of the vertebrate host to respond to pathogens or parasites transmitted with the mosquito bite. In this regard it is interesting that *A. aegypti* saliva has been shown to potentiate transmission of Cache Valley virus (9), and the related mosquito *Oclerotatus* (formerly *Aedes*) *triseriatus*, which potentiates infection of LaCrosse virus in reservoir hosts (chipmunks and white-tailed deer) (16). We hypothesize that these effects are, at least in part, due to the immunomodulatory effects of *A. aegypti* saliva on macrophage function described here. Limesand (15) also showed that *A. aegypti* SGE enhances titers of vesicular stomatitis virus in Vero cell cultures, and that this enhancement correlates with inhibition of IFN-α secretion by Vero cells. Mononuclear phagocytes are the primary source of this cytokine, which has potent antiviral activity through activation of the JAK/TAT pathway. It seems reasonable to hypothesize that *A.*

aegypti SGE also inhibits IFN-α secretion in macrophages, and we plan to test this hypothesis.

A. aegypti is a geographically widespread vector for the potentially fatal Dengue and Yellow Fever viruses (11). Dengue virus has been found to infect primarily tissue macrophages and blood monocytes (6). The role of *A. aegypti* SGE in the transmission of this disease is not well understood. On the one hand, the effects of SGE reported here would seem to indicate a role in enhancing transmission. The elimination of viruses and other intracellular pathogens from the mammalian host requires a Th1 response. In Dengue infections, a Th1 response is associated with a relatively mild disease, and severe disease is associated with a more Th2-like response (5). *Aedes* saliva promotes a Th2 type response from macrophages, which would seem to favor a more severe disease. However, *A. aegypti* SGE has also been shown to inhibit Dengue virus infection of human dendritic cells *in vitro* (1). The net effect of saliva in transmission of this disease may depend on the complex interaction between different cell types with differential responses to SGE.

The nature of the SGE component or components affecting macrophage function is yet to be determined. In the previous chapter we showed that the effects of *A. aegypti* SGE on splenocyte cultures could be associated with a protein of about 387 kDa. It is possible that the same molecule affects macrophages, but this activity could equally well be due to other components of the saliva. Valenzuela and coworkers have described an inventory of 31 secreted proteins in the saliva of this mosquito, and the function of all but five of these are unknown (27). We plan to pursue the characterization of the responsible salivary component in future studies.

In conclusion, the results reported here point to a clear modulation of macrophage effector functions by *Aedes* saliva. This immunomodulation leads to a decrease in pro-inflammatory cytokines as well as inhibiting the macrophage from phagocytosis of *E. coli* and upregulation of MHC presentation. Experiments are underway to characterize the *Aedes* salivary immunomodulator of T-lymphocytes and macrophages. Further study is needed to determine the mode of action of *Aedes* saliva on modulation of macrophage effector functions. Nevertheless, it is clear that saliva of this mosquito vector modulates macrophage function in a manner likely to have major consequences for host responses to challenge with vector-borne pathogens or parasites. Ultimately, understanding these effects of saliva could help in the development of a transmission-blocking vaccine against dengue and yellow fever.

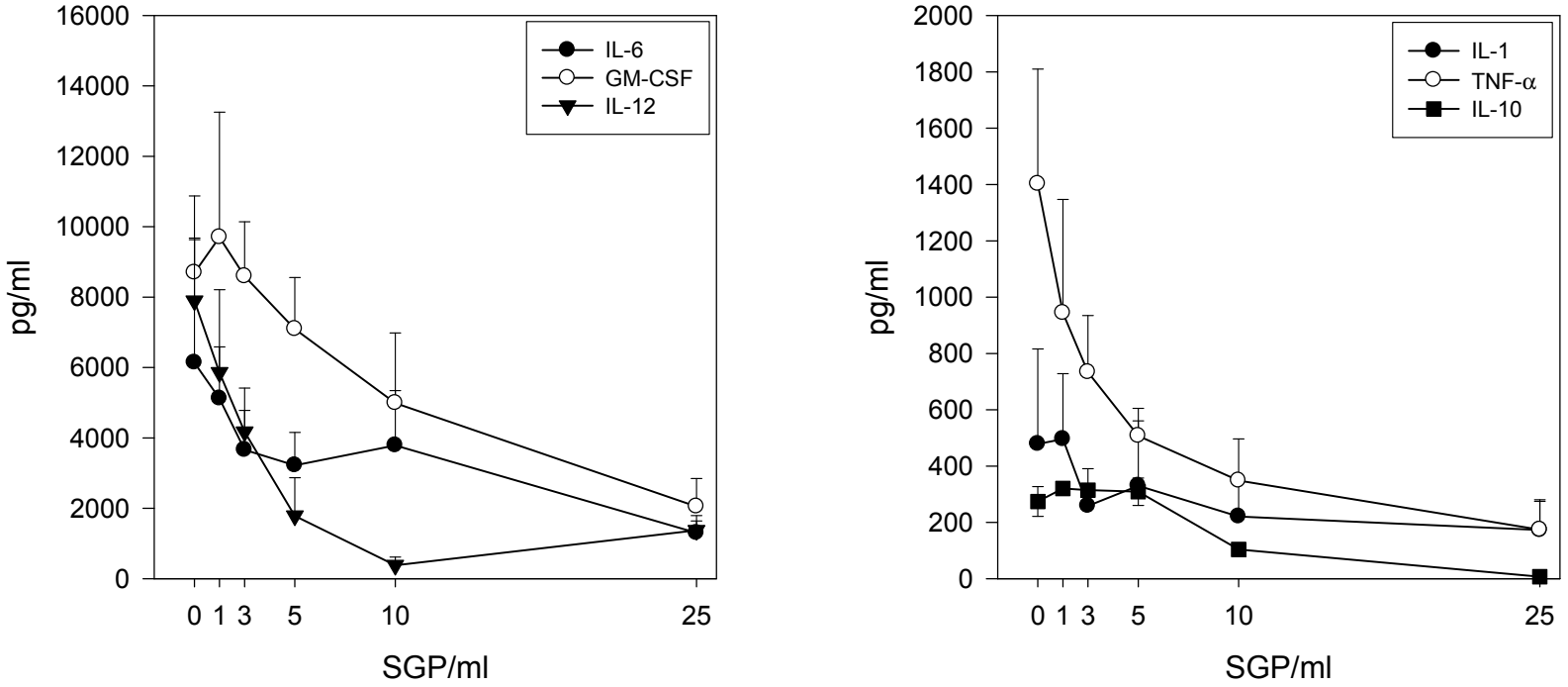


Figure 3.1: *A. aegypti* saliva modulates macrophage secretion of IL-1, IL-6, IL-10, IL-12, TNF-α, and GM-CSF. 2 μg LPS and 4 U IFN-γ was added to macrophage cultures and incubated for 24 h with various concentrations of *A. aegypti* SGE, and then cell-free supernatants were collected. IL-1, IL-6, TNF-α and GM-CSF concentrations were determined by BioPlex analysis, and IL-10 and IL-12 were determined by ELISA. Values shown are pg/ml from two independent experiments, with three replicates per experiment.

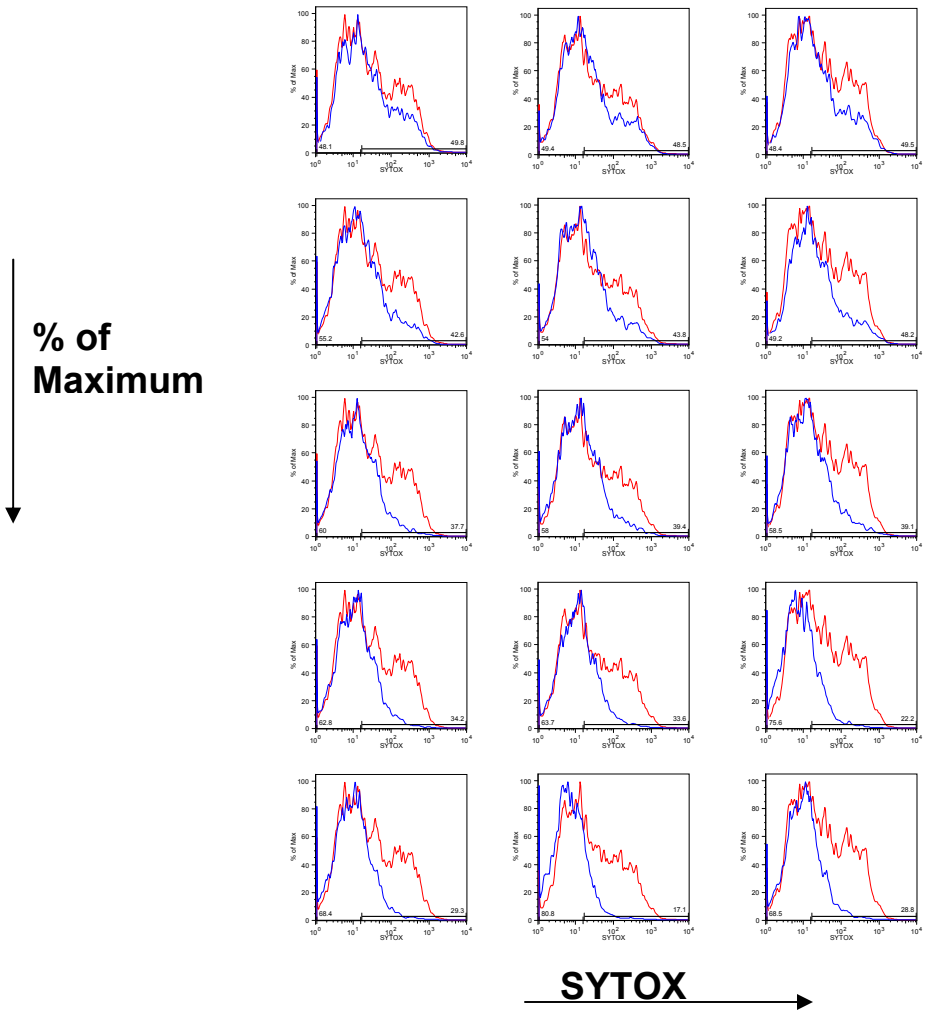
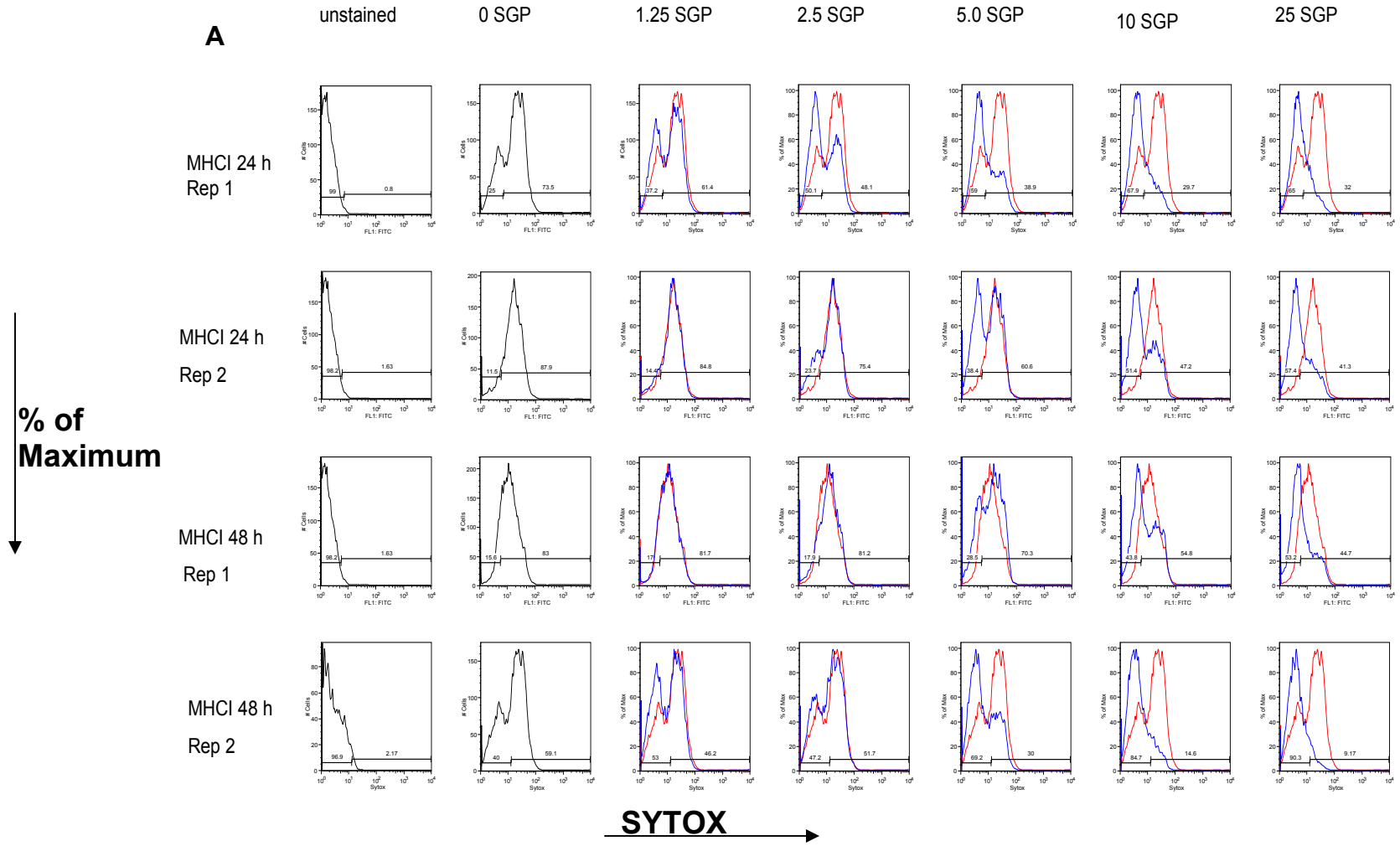


Figure 3.2: *Aedes* saliva immunosuppresses macrophage phagocytosis. LPS (2 μg) activated macrophages were incubated with various concentrations of *A. aegypti* SGE. The cells were incubated for 2 h and then SYTOX labeled *E. coli* was added to each well and incubated for 35 min. The samples were analyzed for fluorescence indicating phagocytized *E. coli* on a DakoCytomation flow cytometer. Results shown are from one representative experiment of three independent experiments performed (R1=Rep 1; R2=Rep 2; R3=Rep 3). The red histograms represent the controls and the blue histograms represent the SGE treated macrophages.


A
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1.25 SGP
2.5 SGP
5.0 SGP
10 SGP
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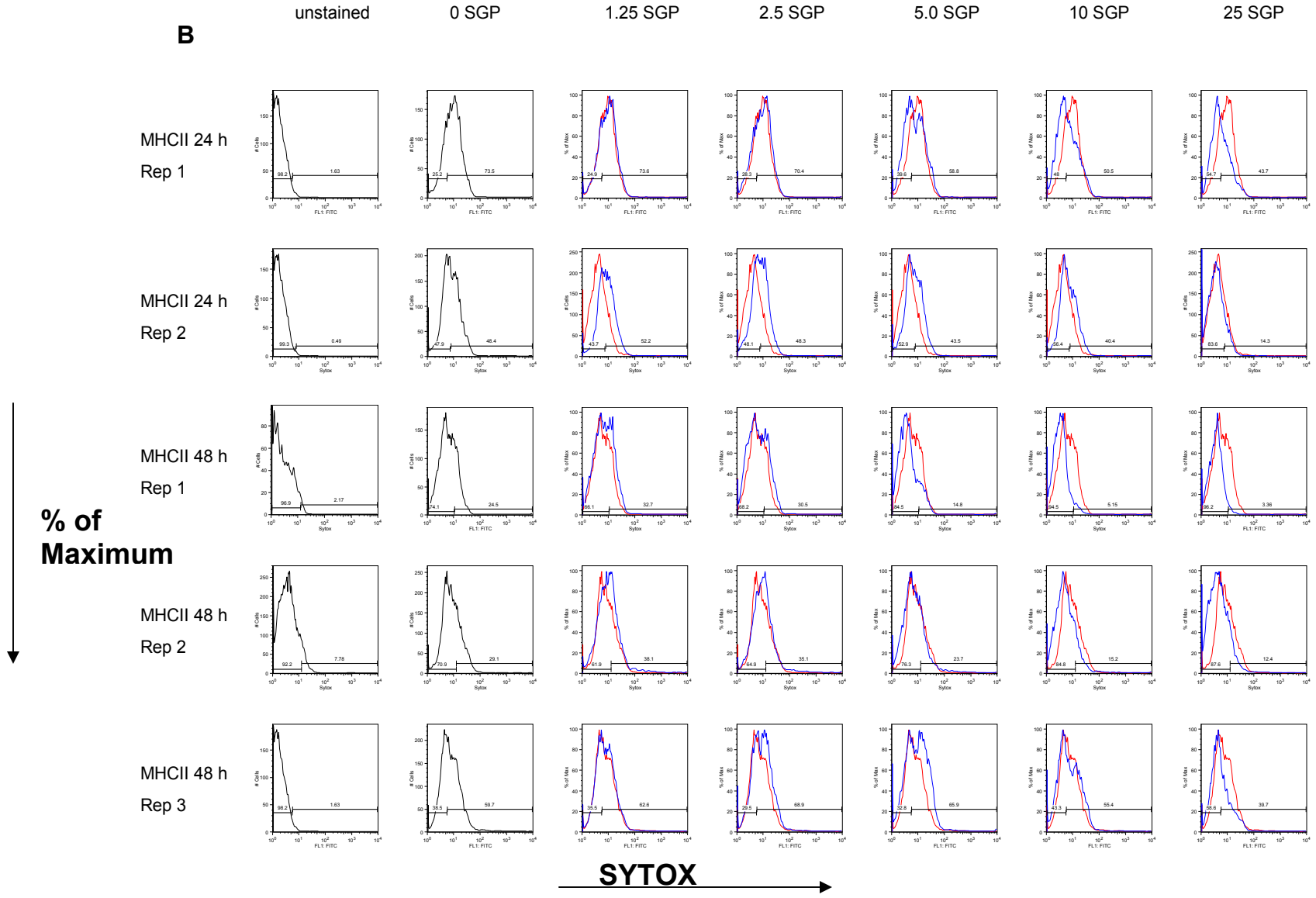
MHCI 48 h
Rep 1
MHCI 48 h
Rep 2
% of
Maximum
SYTOX

B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Rep 2
MHCII 48 h
Rep 1
MHCII 48 h
Rep 2
MHCII 48 h
Rep 3
% of Maximum
SYTOX

Figure 3.3: *A. aegypti* saliva decreases LPS/IFN-γ activated macrophage presentation of MHC Class I and II. 2 μg LPS and 4 U IFN-γ was added to macrophages incubated with the indicated concentrations of *A. aegypti* SGE. The cells were incubated for 24 h and 48 h. The cells stained for MHC Class I (2 μl/ 200 μl PAB) (FITC conjugated mouse anti-mouse H-2D^{d}) (Panel A) and MHC Class II (2 μl/ 200 μl) (FITC conjugated mouse anti-mouse I-A^{d}) (Panel B). Cells were analyzed on a DakoCytomation flow cytometer. Phagocytosis was characterized by macrophages that were SYTOX positive. Results shown are from one representative of two independent experiments performed. The red histograms represent the controls and the blue histograms represent the SGE treated macrophages.

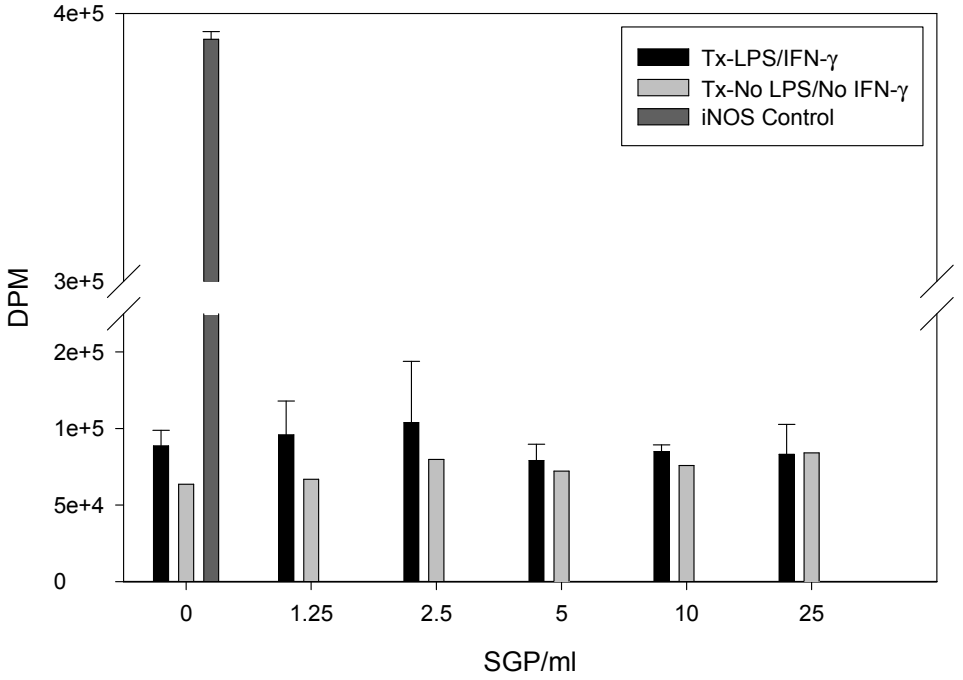

Figure 3.4. *Aedes* saliva does not inhibit iNOS production in activated macrophages. LPS and IFN-γ were added to macrophages with the indicated concentrations of *A. aegypti* SGE and incubated for 24 h and then bioassayed for iNOS activity. Data represent the mean of three separate experiments. Tx= treatment.

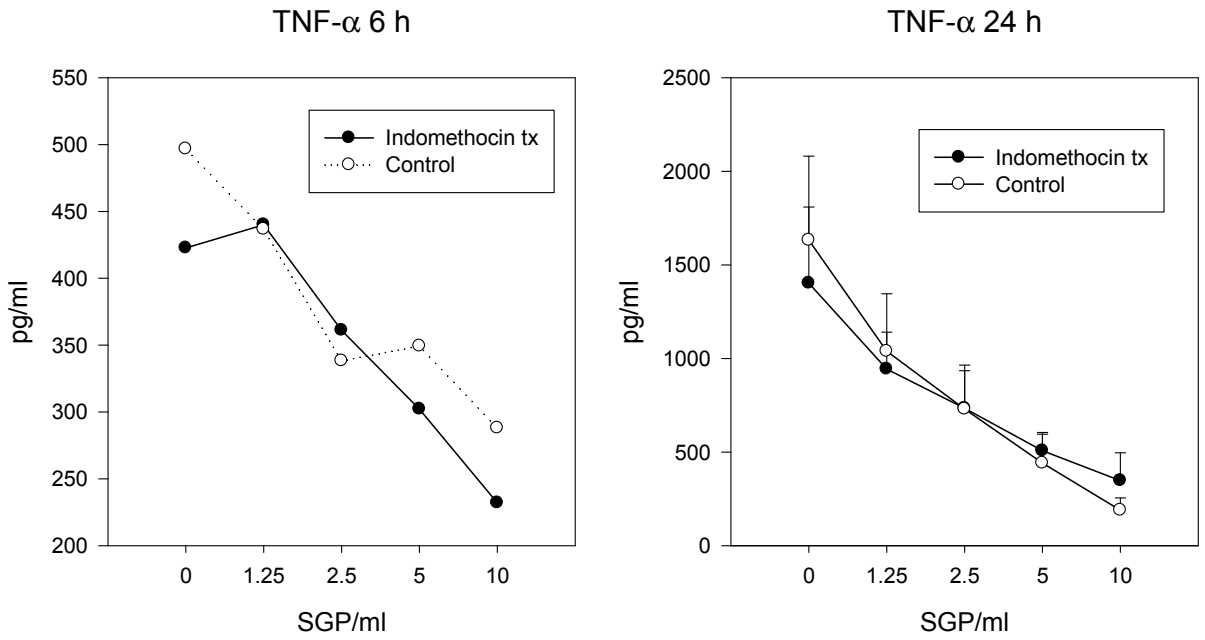

Figure 3.5: ***A. aegypti*** **saliva does not modulate macrophage PGE_2.**

Macrophages were incubated with indomethocin (1 μg/μl), 2 μg LPS, and SGE for either 6 h or 24 h. A negative control consisted of macrophages incubated with SGE and 2 μg LPS. Supernatants were collected and tested for levels of TNF-α with a BioPlex kit (BioRad).

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CHAPTER 4

MODULATION OF HUMAN PBMC FUNCTION BY *AEDES AEGYPTI* SALIVA[1]

[1] Wasserman, H.A. and Champagne, D.E. 2005. To be submitted to *The American Journal of Tropical Medicine and Hygiene*.

ABSTRACT

A. aegypti modulation of mouse immune cell effector functions has been previously studied, but the effect of saliva on human immune cells and their effector functions has not. In this study, we observed the modulatory effect of *A. aegypti* salivary gland extract (SGE) on human peripheral blood mononuclear cells (PBMC's) cytokine secretion, cell proliferation, and cell viability. First, we found that exposure of PBMCs to the equivalent of 1.25 SGP/ml resulted in a significant reduction of proliferation of compared to the control. However, lower doses of SGE did not effect cell proliferation. Carboxyfluorescien diacetate succinimidyl ester (CFSE) labeled CD4+ and CD8+ PBMCs incubated with SGE had an increase in cell division with low doses of SGE compared to the control, however 10 SGP/ml or more decreased cell division. Addition of SGE to stimulated PBMCs modulated some of the Th1 cytokines resulting in an increase in IL-12 secretion, a decrease in IL-2 cell secretion, and no significant difference in IFN-γ production. Among the Th2 cytokines addition of SGE resulted in a dose-dependent decrease in IL-5 and IL-13 and no significant difference in IL-4. Secretion of the inflammatory cytokine IL-10 was slightly stimulated by low SGE concentrations, but concentrations of 5 SGP/ml and above were inhibitory. Cytokine secretion from isolated human neutrophils, dendritic cells, and monocytes was also modulated by SGE. Addition of low concentrations of SGE to stimulated monocytes induced an increase in IL-10 secretion while a significant decrease in IL-10 secretion was observed in monocytes treated with 5 SGP/ml or more. There was an overall dose-dependent decrease in IL-10 production from dendritic cells and neutrophils. In contrast, low doses of SGE modulated an initial increase TNF-α secretion by neutrophils, and

then decreased secretion with 20 SGP/ml or more. Dendritic cells had a SGE dose-dependent decrease in TNF-α production. Low doses of SGE modulated an increase in neutrophil IL-6 secretion, but the addition 10 or more SGP/ml decreased secretion. Finally, stimulated dendritic cells did not produce a sufficient quantity of detectable IL-6. Addition of saliva to CD4+ cells stimulated a dose-dependent increase in Annexin+/PI- cells but the percent of Annexin-/PI+ cells did not markedly increase or decrease with the addition of SGE. Addition of saliva to CD8+ cells modulated a dose-dependent increase in Annexin+/PI- and Annexin+/PI+ cells.

INTRODUCTION

The mosquito, *Aedes aegypti*, is one of many blood sucking arthropods that are vectors of pathogens and is best known as a vector of Dengue and Yellow Fever virus (21, 27). During blood-feeding, pathogens are transmitted to the host along with saliva injected by the mosquito to facilitate the blood-feeding process. The saliva of *A. aegypti* contains several well-characterized anti-hemostatic proteins that aid in circumventing host hemostatic defenses (8, 9).

Additionally, many hemotophagous arthropods produce saliva that contains immunomodulatory factors (23). For example, saliva of the sandfly *Leishmania longipalpis* contains the immunomodulator maxadilan (17, 18), which inhibits secretion of Th1 cytokines and enhances the production of the Th2 cytokine IL-6 from stimulated human peripheral blood mononuclear cells (PBMCs) (25). The presence of an immunomodulatory activity in *A. aegypti* saliva is well established from studies utilizing rodent models (6, 11, 19, 20). Saliva of this mosquito inhibits the release of TNF-α from rat mast cells (6) and modulates a dose-dependent decrease in murine splenocyte proliferation (11, 30). We have recently reported the effect of *A. aegypti* SGE on mouse antigen specific T-cells as well as mitogen stimulated splenocytes (30). Saliva modulated a dose-dependent decrease in secretion of Th1, Th2, and inflammatory cytokines by stimulated murine splenocytes (30). Interestingly, low concentrations of *Aedes* saliva did not affect CD4+ and CD8+ T-cell viability, but higher concentrations decreased cell viability through an increase in apoptosis. Decreases in cytokine secretion as a consequence of exposure to low concentrations of saliva are therefore not attributable to loss of viable CD4+ and CD8+ T-cells (31). Dendritic cells are antigen

presenting cells whose function is to present MHC-peptide complexes to naive T-cells and induce a cell mediated response to foreign antigens. Inhibition of dendritic cell functions would greatly inhibit this cell mediated response. We found that high concentrations (e.g. 25 salivary gland pairs (SGP)/ml) of saliva had no effect on dendritic cell viability, but concentrations as low as 0.6 SGP/ml significantly decreased IL-12 secretion (30). IL-12 is a key cytokine in the elaboration of a Th1 response. Suppression of IL-12 secretion would likely shift the immune response to a Th2 response.

Recently, Ader et al. (1) reported that *A. aegypti* saliva inhibited dengue virus (DV) infection of human dendritic cells, and enhanced production of IL-12 and TNF-α. This study, the only one to address the effect of *A. aegypti* saliva on human cells, used a single concentration of saliva and a single cell type. Indeed, very few studies have examined the effect of saliva of any vector species on human immune cell functions. In the present study, we examine the effect of *A. aegypti* saliva on PBMC, dendritic cell, monocytes, and neutrophil cytokine secretion, and CD4+ and CD8+ T-cell proliferation and viability.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma Chemical Corporation (St Louis, MO), unless otherwise stated. Antibodies were purchased from BD PharMingen (San Diego, CA). *A. aegypti* mosquitoes, from a colony established in 1968 at the Department of Entomology, University of Georgia, were reared at 27°C under a 16-h light/ 8-h dark cycle. Larvae were fed crushed, dry cat food and Tetramin fish flakes. Adults were fed a 10% sucrose solution.

Preparation of Salivary Gland Extracts. *A. aegypti* salivary gland pairs (SGP) were dissected in 10 mM HEPES (pH 7.4), 150 mM NaCl from 3- to 10- day old female mosquitoes. The glands were stored at –70°C in aliquots of up to 20 pairs per 50 μl of buffer in Eppendorf tubes. Salivary glands were homogenized by a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT) with 10 ultrasound bursts, with the power setting at five and a 50% duty cycle. Homogenized SGP were centrifuged at 11 750 *g* for 5 min and the supernatants were pooled and diluted to yield the concentrations used in these studies. The supernatants will be referred to as salivary gland extract (SGE) and the concentration of SGE is given in salivary gland pair (SGP) equivalents per mL of Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Paisley, PA), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), 1% penicillin-streptomycin (Gibco), 1% L-glutamine (Gibco), and 0.5% gentamicin (Gibco) (referred to subsequently as "complete RPMI").

Dendritic Cells. A BDCM human dendritic cell line (American Type Culture Collection # CRL-2740) was used (14). Cells were maintained at 5% CO_2 at 37°C in 250 ml tissue culture flasks in complete RPMI. Cells were grown to confluence and passaged every 3 days.

Human PBMC preparation and proliferation. Blood was obtained from three healthy human volunteers in heprinized vacutainers. PBMCs were isolated using a standard Ficoll-Verografin gradient method. PBMCs ($5X10^6$ cells/ml) were diluted in complete RPMI.

Human neutrophil and monocyte isolation and culture. Blood was obtained from two healthy individuals, which are replicate 1 and 2, in heprinized vacutainers. Red

blood cells were lysed by briefly suspending the cells in 4.5 ml water, and the process was stopped with 0.5 ml 10X phosphate buffered saline (PBS). Neutrophils and monocytes were isolated using Polymorphprep (Greiner Bio-One, Longwood, FL) density gradient centrifugation. Monocytes were in the first layer containing PBMCs and neutrophils were contained in the second layer between the plasma/density gradient interface. Cells were collected and washed twice in Hank's Balanced salt solution.

Monocyte isolation

Polymorphoprep separated PBMCs were incubated with 20% human serum (Gibco) in PBS for 20 min at room temperature. Cells were centrifuged at 300 *g* for 5 min and washed twice in a solution of PBS (pH 7.5) containing 1% BSA and 0.009% sodium azide (PAB). Cells were then stained with PE-Cy7-labeled mouse anti-human CD14 (clone M5E2), and FITC-labeled mouse anti-human CD3 (clone UCHT1). CD14+ cells were sorted and collected using a MOFLO cell sorter (DakoCytomation) in the Flow Cytometry Facility at the University of Georgia, Athens, GA.

Neutrophils

Neutrophils were stained with PE-labeled mouse anti-human CD16b (clone CLB-gran 11.5). Expression of CD16b was assessed by immunofluorescence staining of fluorochromes using a DakoCytomation flow cytometer. Neutrophils were counted and diluted in RPMI complete to a final concentration of $5X10^6$ cells/ml.

PBMC Proliferation Assay. Varying concentrations of *A. aegypti* SGE were added to wells containing $5\ X10^5$ PBMCs (100 μl) in a 96-well plate (Corning, Corning, NY) and incubated for 2 h (5% CO_2, 37°C). PBMCs were stimulated with 1 μg/well of Concanavalin A (Con A) or lipopolysaccaride (LPS). Positive controls were stimulated

with mitogen without exposure to SGE, and negative controls were not exposed to SGE or mitogen. The final volume per well was 200 μl in complete RPMI. After 56 h of incubation, 1 μCi H^3thymidine (ICN Pharmaceutical) in 20 μl complete RMPI was added to each well. After an additional 18 h of incubation, cellular proliferation was determined by harvesting the cells (Type 7000; Skatron, Inc., Sterling, VA) cell harvester and radioactivity incorporation was determined by scintillation counting.

Measurement of Cytokine Secretion. Briefly, 5X 10^5 PBMCs, dendritic cells, neutrophils, or monocytes in complete RPMI were aliquoted into a 96-well flat bottomed plate and incubated with various concentrations of *A. aegypti* SGE. Following a 2 h incubation, (5% CO_2, 37°C), a cell stimulant (Con A (1 μg/well) for PBMCs; 1 μg/well LPS for dendritic cells, neutrophils, and monocytes) was added. The final volume in all wells was 200 μl. Following an additional 48 h incubation, the cell culture medium was collected and centrifuged at 11 780 *g* for 5 min. The cell free supernatants were stored at –70°C until analysis.

Cytokine levels were measured using a human BioPlex kit (BioRad) following the manufacturer's protocols. Aliquots (50 μl) of supernatants were assayed in duplicate. Cytokine concentrations were determined by reference to a standard curve generated from known quantities of recombinant cytokines. Experiments were individually replicated at least twice.

Flow cytometric CFSE assay

PBMCs (2X10^7 cells/ml) were labeled with 1 μM carboxyfluorescien diacetate succinimidyl ester (CFSE)(Molecular Probes) in PBS for 8 min at 37°C. An equal volume of human serum was added, incubated for an additional 1 min, and then

centrifuged for 7 min at 22°C. Cells were incubated 5 min in 20% human serum. PBMCs were centrifuged, washed twice in PBS, and resuspended in complete RPMI to a final concentration of $5X10^6$ cells/ml.

Varying concentrations of SGE were added to a plate containing $5 X10^5$ CFSE labeled PBMCs in 100 μl in a 96-well plate and incubated for 2 h (5% CO_2, 37°C) followed by stimulation with 1 μg/well of Con A. Positive controls were stimulated with Con A without exposure to SGE, and negative controls were not exposed to SGE or mitogen. The final volume per well was 200 μl complete RPMI. Cells were incubated for 72 or 96 h and then transferred to a 96-well V-bottom plate and washed twice with 200 μl PAB. Cells were incubated with 20% human serum in PAB for 20 min, centrifuged and washed twice with PAB. Cells were stained for cell surface markers with the appropriate antibody in 100 μl PAB for 30 min at 4°C. Antibodies used were PE-Cy5-labeled mouse anti-human CD4 (clone RPA-T4) or APC-labeled mouse anti-human CD8 (clone RPA-T8). Cells were washed twice in 200 μl PAB, and resuspended in 300 μl PAB for analysis using a DakoCytomation flow cytometer. Cell compensation in all experiments was performed using the data analysis program FlowJo (Macintosh version 6.2).

CD4+ and CD8+ T-cell Annexin/PI Staining.

Cell apoptosis was determined by the presence of external phosphatidylserine as detected by annexin labeling, and the presence of dead cells was determined by propidium iodide (PI) labeling. Human PBMCS were stained for CD4+ and CD8+ T-cells as described above. Cells were incubated with SGE for 2 h as previously described. This time course was used because prior experiments involving mouse splenocyte

proliferation showed elevated annexin binding in mouse splenocytes exposed to SGE for 2 h (30). Cells were then gently pipetted onto a 96 well V-bottomed plate and centrifuged for 7 min at room temperature. Cells were resuspended in annexin binding buffer (Gibco) and washed twice. Cells were incubated with annexinV-FITC at 3.5 μl/100 μl annexin binding buffer for 15 min in the dark at room temperature. Cells were centrifuged and then washed with annexin binding buffer, 5 μl of 50 μg/ml PI was added, and cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson) within 1 h of staining.

Statistics

Statistical differences between controls and SGE treatments were determined using Student's t-test. Dose/response relationships were analyzed by nonlinear regression using SigmaStat software or software built into the SigmaPlot graphics package.

RESULTS

***A. aegypti* SGE suppresses PBMC proliferation.** Con-A-stimulated human PBMCs were incubated with various concentrations of *A. aegypti* saliva for 72 h, and cell proliferation was measured by incorporation of ^{3}H-thymidine. Exposure to *A. aegypti* SGE resulted in a significant dose-dependent decrease in PBMC proliferation ($R=0.6162$) ($P< 0.003$) (Figure 4.1). Exposure of PBMCs to the equivalent of 1.25 SGP/ml resulted in a significant reduction of proliferation of 16% compared to the control ($P< 0.05$). Additional increases in SGE dose amounts further decreased PBMC proliferation. The addition of LPS to PBMCs did not stimulate measurable levels of cell proliferation (data not shown), probably because B-cells comprise only about 10% of total blood lymphocytes (5).

Con A-stimulated proliferation may be expected to involve mainly T-cells. Our PBMC preparations included approximately 29% CD4+ and 9% CD8+ T-cells, which is within the normal range (5). To determine saliva effects on these cell types, CFSE-labeled PBMCs were stimulated with Con A and incubated with saliva for 72 h or 96 h, labeled to identify CD4+ and CD8+ T-cells, and counted by flow cytometry. CFSE binds irreversibly to intracellular and cell surface proteins via lysine and other amine groups of the parent cell and upon cell division the approximate amount of CFSE in the daughter cell is half that found in the parent cell. CFSE labeling of the cells allowed us to determine if the saliva interacting with CD4+ or CD8+ cells for an extended time period affects cell division. CD4+ cellular division in the 72 h stimulated control had up to three cell divisions, and after 96 h there were up to five divisions (Figure 4.2a). The proportion of G0 CD4+ cells in the control was 35% after 72 h of cell division and 18% after 96 h of division. The addition of 5 SGP/ml decreased the G0 cells in both of the 72 h and 96 h treatments to 26% and 11%, respectively, compared to the untreated control. Finally, there was a dramatic increase in the percent of G0 CD4+ cells with the addition of 40 SGP/ml; there was an increase to 45% and 34% with the addition of 40 SGP/ml after 72 h and 96 h, respectively.

A. aegypti SGE effects on CD8+ T-cell proliferation was also investigated. CD8+ T-cells are known to divide at a higher relative rate than CD4+ T-cells (26) and our results corroborate this, with a higher number of overall generations observed in CD8+ cells. After 72 h of stimulation, the CD8+ cells had four cell divisions and after 96 h stimulation CD8+ T-cells divided five times (Figure 4.2b). The proportion of G0 CD8+ T-cells in the control was 47% after 72 h of cell division; this decreased to 37% after 96 h

of division. However, the addition of 5 SGP/ml decreased the G0 cells in both of the 72 h and 96 h treatments to 46% and 21%, respectively. Finally, there was a dramatic increase in the percent of G0 CD4+ T-cells with the addition of 40 SGP/ml; there was an increase to 67% and 51% with the addition of 40 SGP/ml after 72 h and 96 h, respectively.

***A. aegypti* saliva promotes secretion of DTH cytokines from stimulated human PBMCs.** We wanted to determine if *A. aegypti* saliva produces a DTH cytokine response from stimulated PBMCs. Human PBMCs were stimulated with Con A in the presence of varying doses of SGE, and incubated for 48 h (Figure 4.3). The cell free supernatants were then bioassayed for various cytokines.

***Th1 Cytokines*:** Addition of SGE to stimulated PBMCs led to an increase in IL-12 secretion but a significant decrease in IL-2 cell secretion ($R=0.5774$) ($P< 0.05$). PBMC IL-2 secretion was suppressed by 56% with the addition of 20 SGP/ml ($P< 0.05$). However, there was no decrease in IL-2 secretion until 5 SGP/ml or more was added. Conversely, IL-12 secretion increased by two-fold with the addition of 5 SGP/ml ($P< 0.05$), and by 11-fold with the addition of 40 SGP/ml. There was no significant difference ($R=0.2966$) ($P>0.6$) observed in IFN-γ production between cells treated with only Con A and cells treated with Con A and varying amounts of saliva.

***Th2 Cytokines*:** PBMCs incubated with varying doses of SGE secreted significantly less IL-5 ($R=0.7424$) ($P< 0.001$) and IL-13 ($R=0.7871$) ($P< 0.001$). The addition of 5 SGP/ml suppressed IL-5 secretion by 68% ($P< 0.03$) and IL-13 secretion by 50% ($P< 0.08$) compared to the control. In contrast, there was no significant difference

(R=0.3824) (P>0.3) in IL-4 levels in control versus saliva treated PBMCs at concentrations up to 40 SGP/ml.

***Pro-Inflammatory and Anti-inflammatory cytokines*:** Secretion of the anti-inflammatory cytokine IL-10 appeared to be slightly stimulated by low SGE concentrations, but concentrations of 5 SGP/ml and above were inhibitory. IL-10 production was slightly increased with the addition of 0.625 and 1.25 SGP/ml (P< 0.7), but IL-10 production was suppressed 56% compared to the control with the addition of 10 SGP/ml (P< 0.03). PBMC production of pro-inflammatory cytokines, TNF-α and GM-CSF was also observed. Saliva had no significant effect on TNF-α secretion (R=0.3376) (P>0.1), however there was a modest dose-dependent decrease in GM-CSF levels (R=0.4647) (P=0.07) with the addition of increasing amounts of saliva.

Cytokine Secretion by Dendritic cells, Neutrophils, and Monocytes

PBMCs contain a variety of cell types including T-cells, monocytes, neutrophils, and some dendritic cells. These cells contribute to inflammatory responses and innate immunity through secretion of cytokines. We determined the effect of SGE on secretion of IL-6, IL-10, or TNF-α on dendritic cells, neutrophils, and monocytes by Bio-Plex analysis.

IL-10; IL-10 was mainly secreted by monocytes, which produced as much as 1400 pg/ml with the addition of 1.25 SGP/ml (Figure 4.4a). Addition of SGE to stimulated monocytes induced an increase in IL-10 secretion with addition of 1.25 and 2.5 SGP/ml. There was a significant decrease in IL-10 secretion between by monocytes treated with 5 SGP/ml (P< 0.04), 10 SGP/ml (P< 0.05), and 20 SGP/ml (P< 0.01). In replicate 2

there was a 14% and 78% suppression of IL-10 compared to the control with the addition of 5 and 20 SGP/ml, respectively.

Additionally, IL-10 secretion was negligible in dendritic cells and neutrophils stimulated for 48 h (Figure 4.4b and c). There was an overall dose-dependent decrease in IL-10 production from dendritic cells and neutrophils; In replicate 2, addition of as little as 1.25 SGP/ml decreased IL-10 secretion by 13% from dendritic cells and 22% from neutrophils. The addition of 2.5 SGP/ml or more to dendritic cells and neutrophils led to a significant reduction ($P < 0.04$) in IL-10 secretion.

TNF-α; Of the three individual cell types observed, monocytes produced the largest quantity of TNF-α (Figure 4.4a). Monocytes incubated with 2.5 SGP/ml secreted 760 pg/ml of TNF-α, however, there was no significant difference between the positive control and the saliva treated cells ($P > 0.05$). In contrast, low doses of SGE modulated an initial increase TNF-α secretion by neutrophils, and then decreased secretion with 20 SGP/ml or more (Figure 4.4b; Rep 1). In replicate 1, secretion of TNF-α by neutrophils was significantly increased by 66% with the addition of 1.25 SGP/ml ($P < 0.04$) and significantly decreased by 35% with the addition of 20 SGP/ml ($P < 0.02$) compared to the positive control. Finally, dendritic cells had a SGE dose-dependent decrease in TNF-α production (Figure 4.4c). In replicate 2 the addition of 2.5 SGP/ml or more lead to a significant decrease by 68% ($P < 0.05$) in secretion of TNF-α by dendritic cells compared to the positive control and 20 SGP/ml almost complete suppressed all TNF-α secretion by dendritic cells.

IL-6; We also determined the production of IL-6 from these individual cell types. The greatest production of IL-6 was from the stimulated monocytes, which produced as

much as 80,000 pg/ml with the addition of 10 SGP/ml (Figure 4.4a). There was no significant difference in IL-6 production between the control and the SGE treated monocytes ($P > 0.5$), but there is an observable dose-dependent increase in monocyte IL-6 secretion in both replicate 1 and 2. The addition of 1.25 SGP/ml to stimulated neutrophils led to a significant 20 fold initial increase ($P < 0.05$) in IL-6 secretion compared to the control in replicate 2 (Figure 4.4b). However, there was a significant dose-dependent decrease in neutrophil IL-6 production with the addition of 10 or more SGP/ml ($P < 0.05$) compared to neutrophils incubated with low doses of SGE. Only in replicate 2, neutrophils incubated with 1.25 SGP/ml secreted an average of 200 pg/ml IL-6, in contrast neutrophils incubated with 20 SGP/ml secreted an average of 81.5 pg/ml IL-6. Finally, stimulated dendritic cells did not produce a sufficient quantity of IL-6 to be detected by the Bio-Plex kit.

Higher dosages of *A. aegypti* saliva modulates cell death in stimulated CD4+ and CD8+ human PBMCs, however lower doses of saliva promote a DTH response.

We wanted to determine if the reduction in cytokine secretion, and CD4+ and CD8+ cell proliferation was a product of cell apoptosis. PBMCs were incubated for 2 h with varying doses of SGE and then stained with Annexin/PI to determine cell viability and apoptosis (Figure 4.5). Addition of saliva to CD4+ cells stimulated a dose-dependent increase in Annexin+/PI- cells with the addition of 2.5 SGP/ml or more. However, the percent of Annexin-/PI+ cells did not markedly increase or decrease with the addition of SGP; For example the positive control had 0.58% Annexin-/PI+ CD4+ cells and cells treated with 20 SGP/ml had 0.47% CD4+ cells. There was an observed change to the percent of cells that were Annexin+/PI+ compared to the non-SGE treated control. The percent of

viable Annexin-/PI- CD4+ cells markedly decreased with the addition of 2.5 SGP/ml or more.

Addition of *A. aegypti* saliva to human PBMCs also stimulated CD8+ apoptosis. Addition of saliva to CD8+ cells modulated a dose-dependent increase in Annexin+/PI- and Annexin+/PI+ cells with the addition of as few as 0.625 SGP/ml. However, the percent of Annexin-/PI+ cells did not markedly increase or decrease until the addition of 20 SGP/ml; the positive control had 2.19% Annexin-/PI+ CD8+ cells and cells treated with 20 SGP/ml had 4.38% CD8+. The percent of viable Annexin-/PI- CD8+ cells markedly decreased with the addition of 2.5 SGP/ml.

DISCUSSION

In previous chapters of this dissertation, we reported that *A. aegypti* saliva strongly immunomodulates a variety of important immune functions, including antigen specific and mitogen-stimulated T-cell proliferation and secretion of both Th1 and Th2-type cytokines, in murine splenocytes. In those experiments, a mouse model was used to determine the interaction between *A. aegypti* saliva, antigen specific T-cells, and antigen presenting cells. In the present study I address the effects of *A. aegypti* saliva on cells involved in human immunity.

PBMC Proliferation and Viability

Antigenic stimulation of immune responses is characterized by the proliferation of lymphocytes, a process that may be mimicked with polyclonal activators such as the mitogen Con A. *A. aegypti* SGE at concentrations of 1.25 SGP/ml or more decreased PBMC proliferation in a dose-dependent manner. However some proliferation (40% of the positive control) was still observed in PBMCs exposed to 20 SGP/ml. A variety of

cell types are found in human PBMCs; we found that there were between 29-40% CD4+ and 8-18% CD8+ T-cells. As it is largely this cell population that proliferates in response to Con A stimulation, and may influence the effector functions of the other PBMCs, we investigated the effect of SGE on Con A-stimulated proliferation of CD4+ and CD8+ T-cells. CD4+ and CD8+ PBMCs were labeled with CFSE and incubated with *Aedes* saliva for 72 or 96 h. *A. aegypti* saliva at concentrations up to 10 SGP/ml weakly stimulated or did not affect CD4+ division at 72 or 96 hours; proliferation was markedly inhibited by 20 or 40 SGP/ml at both time points. Similar results were seen with CD8+ T-cells. Annexin binding by saliva-treated CD4+ T-cells increased modestly, from 5.4% annexin-positive cells in the control to 15.8% of the cells exposed to 40 SGP/ml (Replicate 1; similar results were obtained for Replicate 2). CD8+ PBMCs showed a similar trend, from 8.22% to 24.2% annexin-positive cells (Replicate 1). Viability of these cells was not affected by exposure to saliva, as there was almost no change in the proportion of PI positive cells at any saliva concentration. Although the increase in Annexin+ cells at high SGE concentrations may indicate apoptosis, the lack of dying PI positive cells argues against this interpretation. In addition, inspection of the results from the flow cytometry analysis does not indicate the changes in cell size or granularity characteristic of apoptotic cells. Further, CD4+ and CD8+ T-cells do not change their proportional representation in the population of PBMCs, as would be expected if these cells were being depleted by apoptosis. However, to definitively include or exclude apoptosis as a result of exposure to SGE, it will be necessary to further investigate effects of SGE on up-regulation of pro-apoptotic proteins such as the caspases, and on PARP cleavage, or on suppression of anti-apoptotic proteins such as

Bcl-2. The lack of an increase in PI-positive cells also suggests that the inhibition of proliferation, as well as effects on cytokine secretion discussed below, are due to immunomodulation and not just an increase in cell death.

Effects on Cytokine Secretion by PBMCs

Interaction between components of the immune system is mediated by cytokines. These proteins are critical in both innate and adaptive immunity. Cytokines up-regulate the effector functions of immune cells, stimulate cell proliferation and differentiation, and enhance effector cell microbial killing. Addition of SGE to stimulated PBMCs modulated a marked dose-dependent increase in the pro-inflammatory and Th1-stimulating cytokine IL-12. This does not represent a classical Th1 response however, as SGE also inhibits IL-2 secretion at concentrations of 10 SGP/ml and above. This inhibition may account for the inhibition of T-cell proliferation, a process that is dependent on this cytokine (2). Further, secretion of both IFN-γ and TNF-α from Con A-stimulated PBMCs is not influenced by SGE at concentrations up to 40 SGP/ml. On the other hand, SGE decreased production of the pro-inflammatory cytokine GM-CSF. Similar mixed effects were seen with the Th2 cytokines. Both IL-5 and the anti-inflammatory cytokine IL-13 were markedly reduced in a dose-dependent manner. A trend towards higher IL-10 concentrations was seen with PBMCs treated with SGE at concentrations up to 1.25 SGP/ml, but concentrations of 5 SGP/ml and above were strongly inhibitory. The Th2-inducing cytokine IL-4 was not affected by SGE at any of the tested concentrations. The net effect of saliva in this system appears to be induction of a pro-inflammatory response.

Dendritic Cells/Monocytes/Neutrophils

Dendritic cells, monocytes, and neutrophils are phagocytic cells that play key roles in innate immunity, and, in the case of dendritic cells, subsequently induce acquired immune responses by stimulating T-cells. Neutrophils, macrophages, and dendritic cells are also important cells in DTH reactions, which will be discussed later. Each of these cell types was affected by exposure to SGE, but the response differed between different cell types.

Neutrophils act in an initial innate immune inflammatory response to phagocytize microbes. Secretion of IL-10 by neutrophils was inhibited in a dose-dependent manner, with a significant reduction in cells treated with only 1.25 SGP/ml SGE, and 90% inhibition after treatment with 20 SGP/ml. On the other hand, secretion of IL-6 was stimulated almost four-fold by 1.25 SGP/ml. This effect was reduced in a dose-dependent manner with higher SGE concentrations, but levels were above control values (LPS-stimulated without SGE) at all concentrations. A similar effect was seen with TNF-α, but the stimulation was not as strong and TNF- α levels declined below control values in the presence of 20 and 40 SGP/ml. The effect of SGE varied between individuals, as neutrophils from Replicate 2 produced no or barely detectible amounts of all three cytokines, despite the fact that monocytes and dendritic cells from this individual functioned normally.

Monocytes are attracted to an inflammatory site and then differentiate into macrophages. Macrophages, important in both innate and adaptive immunity, phagocytize microbes and secrete inflammatory cytokines. Secretion of IL-10 was stimulated above control values with 1.25 and 2.5 SGP/ml, but concentrations of 10 SGP/ml and above were highly inhibitory. TNF-α secretion was unaffected by SGE,

and secretion of IL-6 trended towards higher levels with increased SGE. These patterns were seen with both individuals tested, although there were differences in the absolute amount of cytokine produced.

Dendritic cells phagocytize or pinocytize antigens and then migrate to the lymph node where they display the MHC-Class II-peptide complex for interaction with an antigen-specific TCR of CD4+Th0 T-cells. If IL-12 secretion is upregulated, Th0 T-cells will be induced to differentiate into IFN-γ secreting Th1 CD4+ cells. Although we did not measure IL-12 secretion directly from monocytes or dendritic cells, these cell types are the predominant source of this cytokine, and it is likely that they account for the elevated secretion of IL-12 that we observed in SGE-treated PBMCs. The CD4+ T-cells function in cell mediated immunity to further increase inflammation and phagocytosis. In contrast to the monocytes and the neutrophils, secretion of TNF-α was sharply inhibited in a dose-dependent manner. Effects on IL-10 secretion were similar to the pattern seen with monocytes. However, dendritic cells produced less that 1% the amount of IL-10 that was secreted by equivalent numbers of monocytes, and only 1/20 of the amount secreted by neutrophils.

Overall, there seems to be a pro-inflammatory response in the presence of low doses of SGE in PBMCs and in the individual cell types observed. Neutrophils, which account for the majority of white blood cells, as well as monocytes and dendritic cells, secrete large quantities of the pro-inflammatory cytokines IL-6 and TNF-α. For example, the addition of 10 SGP/ml to monocytes induced the secretion of 80,000 pg/ml of IL-6. Secretion of the anti-inflammatory IL-10 cytokine was also observed with low doses of SGE. Although monocytes were the most abundant producers of this cytokine

(eg 1.25 SGP/ml modulated the secretion of 1400 pg/ml IL-10), they are less plentiful compared to other white blood cells, and will therefore have less influence the overall cytokine environment than the combined cytokine effects of the neutrophils, dendritic cells, and other PBMCs.

The combined results of the PBMC and individual cell cytokine data indicate that saliva is immunomodulating an overall pro-inflammatory reaction. The PBMC data indicate an increased secretion of the Th1 cytokine IL-12, which is crucial in upregulating an inflammatory response. There is also no inhibition of the Th1 cytokine IFN-γ, which is associated with inflammation. IL-2 secretion is inhibited only in the presence of higher SGE concentrations. Although we do observe a small stimulation of IL-10 secretion at low SGE concentrations by both PBMCs and monocytes, the effects of the other pro-inflammatory and Th1 cytokines are likely to overwhelm this cytokine's ability to inhibit a pro-inflammatory response. In addition, the anti-inflammatory effects of IL-10 and IL-13 would be lost at slightly higher SGE concentrations, where secretion of both cytokines is strongly inhibited.

Is this consistent with a DTH response?

It is well documented that mosquito saliva induces an inflammatory, delayed type hypersensitivity response (DTH) *in vivo* (22). A typical immune response to intermittent mosquito bites has 4 phases, which include: no reaction, observed in an individual that is naive for that specific antigen → delayed type hypersensitivity (DTH), seen in those individuals that have had some antigen exposure→ immediate type hypersensitivity, seen in those individuals that have had regular antigen exposure → desensitization, seen only in those individuals that have chronic, consistent, antigen exposure (23).

Mosquito saliva also elicits an increase in IgE and IgG antibodies, lymphocyte proliferation, an immediate wheal and flare skin response, and delayed induration (22).

A DTH response is activated by circulating, antigen-specific memory CD4+ and CD8+ T-cells, inducing erythema and induration at the site of antigen injection. Classically, a DTH response is initiated by an influx of macrophages and neutrophils to the antigen affected area, followed by the accumulation of memory CD4+ and CD8+ T-cells and blood monocytes to the area within 12 hours (7). In a skin sensitization reaction, such as those seen with mosquito bites, dendritic cells and macrophages present antigen to memory CD4+ and CD8+ T-cells in the bite area as well as to Th0 CD4+ T-cells in the lymph node. The affected area begins to swell with accumulated fibrin as well as T-cells and monocytes causing induration, which lasts for approximately 48-72 hours in humans. Depending upon the initial antigen stimulating the DTH reaction, both Th1 and Th2 cytokines can be upregulated and influence the immune response (7).

In contrast to our *in vitro* experiments, the DTH response can be studied only *in vivo*. Therefore, the limitations of our *in vitro* studies need to be defined when comparing data. One restriction is the constant presence of SGE antigen to the PBMCs in our *in vitro* assays, vs the eventual dissipation of antigen in an *in vivo* system. Constant antigen stimulation of T-cells could lead to anergy and loss of a cell-mediated response. Secondly, there is a lack of APC migration between the lymph node and the inflamed bite site with a cell culture experiment. The interaction of the APC/MHC-peptide complexes with TCR's will lead to T-cell clonal expansion and up-regulation of Th0 cells to either Th1 or Th2 CD4+ cells as observed in a DTH response. However, as

both naive and memory CD4+ and CD8+ T-cells are found in the peripheral blood lymphocytes, it is possible to obtain a suitable number of T-cells for both an antigen-stimulated memory response as well as naive T-cell clonal expansion. Finally, *in vitro* assays do not permit us to observe erythema and induration, the hallmarks of a DTH reaction, at the site of antigen entry. Despite these limitations, our results are consistent with an antigen stimulated *in vivo* DTH response. Low concentrations of SGE stimulated proliferation of both CD4+ and CD8+ T-cells, as would be expected for a cell-mediated response *in vivo*. We saw an overall increase in the Th1/pro-inflammatory cytokine IL-12, and secretion of both IFN-γ and TNF-α is not inhibited by as much as 40 SGP/ml, all consistent with an inflammatory response at the site of antigen (i.e. saliva) deposition in the skin. Despite the inhibition of IL-13 and, at higher SGE concentrations, IL-10, IL-4 is not inhibited and IL-6 is somewhat stimulated in PBMCs exposed to SGE. These results suggest that the human response could retain some Th2 character. In this regard it is noteworthy that the human systemic response to *A. aegypti* bites is a Th2 response, based on high titers of saliva-specific IgE antibodies (10). It should also be noted that all the PBMC donors for these experiments have a history of mosquito bites, and one individual in particular has had extensive exposure to this immune challenge.

Comparison with Murine Immune Responses

Proliferation of murine splenocytes (Chapter 2) and human PBMCs was inhibited by *A. aegypti* SGE; in both species CD4+ and CD8+ T-lymphocytes were affected. However the murine cells were much more sensitive, as splenocyte proliferation was inhibited by 50% in the presence of only 0.6 SGP/ml, compared to almost 20 SGP/ml for

the human PBMCs. Murine CD4+ and CD8+ T-cells exposed to 2.5 SGP/ml or higher had an increase in cell death, indicated by an increase in cells unable to exclude PI (Chapter 2), and this increase in mortality was due to an increase in apoptosis (unpublished data). On the other hand there was only a small (<2%) increase in PI positive human CD4+ T-cells, and only about a 10% increase in annexin binding, following exposure to SGE equivalent to 40 SGP/ml. A similar result was seen for human CD8+ T-cells, although the increase in PI positive (4-12%) and annexin positive (16-17%) cells was slightly higher than the increases seen with CD4+ T-cells. This result suggests that human T-cells are highly resistant to the apoptotic effect of *A. aegypti* SGE, compared to murine cells.

The effect of *A. aegypti* SGE on cytokine secretion also indicated substantial differences between mice and humans. Most striking was a dose-dependent increase in IL-12 secretion in human PBMCs, compared to a strong inhibition of IL-12 secretion in mouse splenocytes. Secretion of IL-2, IFN-γ, and TNF-α by mouse splenocytes was inhibited in a dose-dependent manner, but only IL-2 was inhibited in human PBMCs, and that required substantially higher concentrations of SGE. Mouse and human cells (including macrophages) responded similarly with regard to secretion of IL-10 and GM-CSF, although again mouse cells were sensitive to lower concentrations of SGE. Another significant difference was in IL-4 secretion, which was decreased in a dose-dependent manner in mouse splenocytes, but unaffected in the case of human PBMCs. Altogether these results suggest that human cells produce a mixed inflammatory/Th2 response to challenge with *A. aegypti* SGE, whereas mice inhibit Th1 responses and produce a net Th2 response.

These differences are likely to be intrinsic to the two species, but it is important to note that the cellular composition of PBMCs differs from that of splenocytes, and perhaps more importantly, the human donors for the PBMCs all have a history of exposure to mosquito bites, in contrast to the immunologically naive mice. Nevertheless, one should be cautious about using mice or other species as models for human immune responses. However, the use of mice and other animals is often a necessity, especially in determining *in vivo* interactions between hosts, vectors, and pathogens. In addition, variation in genetic background and immunological history contributes to variability in the human response, which can confound attempts to quantify the effects of exposure to saliva. This problem is less of an issue with the genetically uniform mice.

Comparison with other vectors

Relatively few studies have addressed the effects of vector saliva on human immune responses. Kover et al. (15) found that high doses (5 SGP/ml or more) of *I. ricinus* SGE immunomodulated a dose-dependent decrease of proliferation in Con A-stimulated human PBMCs, but low doses (e.g. 0.5 SGP/ml) had no significant effect. This is similar to the effect of *A. aegypti* SGE that we observed. *I. ricinus* SGE induced a strong Th2 polarization in cytokines secreted by PBMCs, with increases in the Th2 cytokines IL-4, -6, and -10 and decreased secretion of the Th1 cytokines IL-2 and INF-γ. These human PBMC results were similar to what they had previously described in a murine model (16). These results contrast with the more complex effects we observed with *A. aegypti* SGE, which did not clearly favor either a Th1 or a Th2 pattern but rather suggested an inflammatory response complicated by uninhibited IL-4 secretion. These

differences likely reflect a different mechanism underlying the immunomodulatory effect, which may be correlated with inherently different feeding strategies between *A. aegypti*, an insect and a "fast-feeder" vs *I. ricinus*, a tick and a "slow feeder".

Our results may be compared to the immunomodulatory effects of feeding by sandflies (3). In these experiments, BALB/c mice, fed on by *P. papatasi* sandflies and subsequently injected with the equivalent of 0.2 *P. papatasi* SGP, had a significant DTH response that included induration of the injection site and an 82-fold increase in CD4+ T-cells. Humans fed on by *P. papatasi* had a significant increase in blood flow, itching, and redness at the bite site, also indicating a DTH response (4). However, in cell culture experiments, and in naive mice, *P. papatasi* saliva is immunosuppressive, inhibiting murine macrophage nitric oxide and IFN-γ production (13, 29). The discrepancy was resolved by the finding of a specific protein, which has been named SP15, present in the saliva of this insect (28). SP15 specifically drives a strong DTH response, which results in increased numbers of IFN-γ secreting CD4+ T-cells at the bite site (28). Sandflies use the DTH response in their feeding strategy; because of the enhanced blood flow at these sites, the flies can feed more quickly, and they are more likely to ingest a large meal (4). In this case there appears to be a selective advantage in initiating a DTH response in the host. The DTH response also results in a less favorable environment for transmission of *Leishmania* parasites, a consequence which has lead to the development of SP15 as a transmission-blocking vaccine candidate (28). It is not known if the DTH response generated following the bite is also advantageous to the feeding strategy of mosquitoes.

Effects on pathogen transmission

Arthropod saliva has been repeatedly shown to have a significant influence on pathogen transmission (23, 24). *A. aegypti* bites have been reported to enhance transmission of Cache Valley virus (12). Mice that were bitten by *A. aegypti* and then injected with virus at the bite site developed an earlier and higher peak viremia. SGE enhanced infection with vesticular stomatitis New Jersey (VSNJ) virus in mouse L929 fibroblast cells, which express IFN α/β receptors, but not in Vero cells, which lack IFN α/β receptors (19). The effect correlated with an inhibition of IFN- α/β secretion by the fibroblast cells. The immunomodulatory effects reported here could also be consistent with this increase in virus infectivity. *A. aegypti* is known principally as a vector of Dengue and Yellow Fever (21). The role of saliva in transmission of these viruses is presently unclear. These viruses have a trophism for monocytes and dendritic cells, so recruitment of these cells to a DTH response at the bite site would be likely to increase the number of susceptible cells for viral invasion. Curiously, *A. aegypti* SGE has been reported to reduce the infectivity of Dengue virus in dendritic cell cultures (1). However, the *in vivo* effects of *A. aegypti* SGE may result in an environment that is, on balance, more favorable for virus transmission. This possibility remains to be explored.

Future studies

We recently reported that *A. aegypti* saliva contains an immunosuppressive protein component with an estimated size of 387 kDa that suppresses mitogen stimulated BALB/c T-cell proliferation (30). Further study is needed to determine if the same molecule affects function of human PBMCs or antigen-presenting cells. Ultimately, identification, cloning, and expression of the immunomodulatory components of SGE

will permit more detailed studies of SGE effects on specific aspects of the human immune response. It is conceivable that such information could lead to vaccines that perturb the natural host response to *A. aegypti* feeding, in a manner designed to inhibit the transmission of mosquito-borne pathogens.

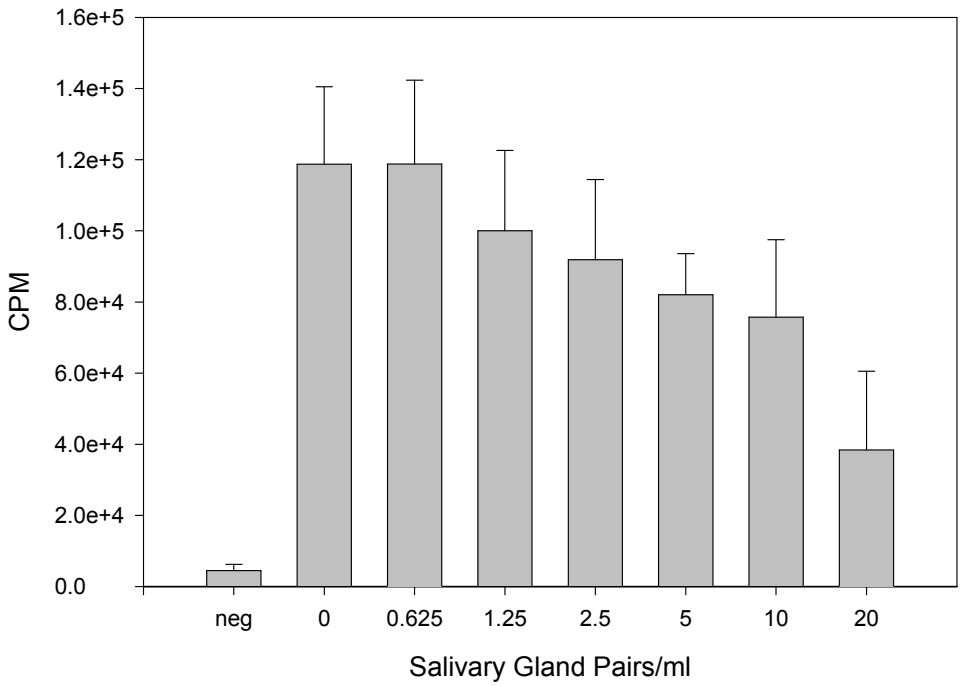
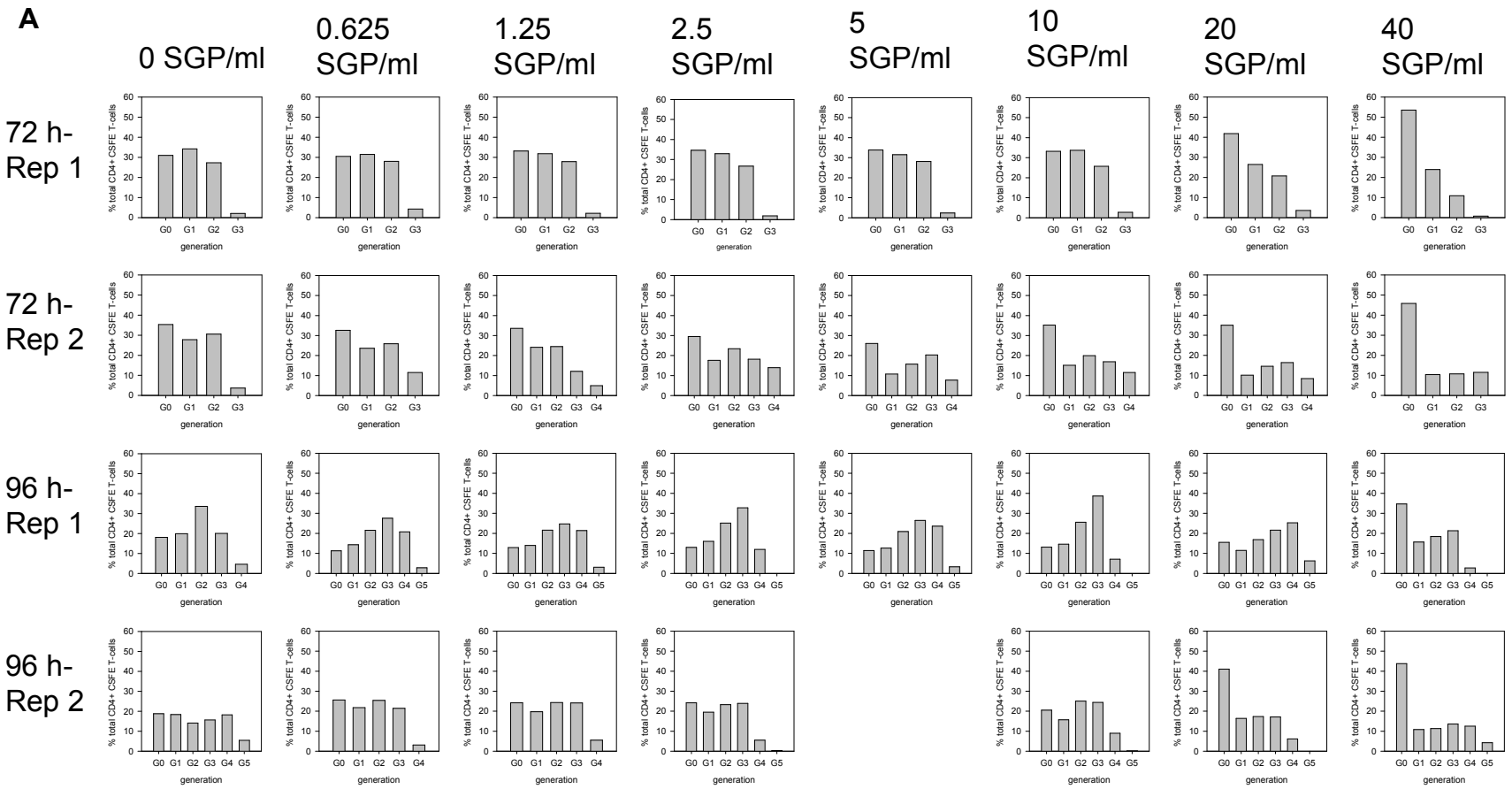


Figure 4.1: *Aedes aegypti* SGE modulates a dose-dependent decrease in Con A stimulated PBMC proliferation. PBMCs (5 x 105 cells) were incubated with varying concentrations of SGE for 2 h, followed by stimulation with 10 μg/ml Con A. The negative control received no Con A. Incorporation of [3H]–thymidine was used to determine PBMC proliferation. Data points represent the mean counts per minute (CPM) ± standard error (n=3). Regression analysis indicates a significant treatment effect ($p < 0.01$).


A
0 SGP/ml
0.625 SGP/ml
1.25 SGP/ml
2.5 SGP/ml
5 SGP/ml
10 SGP/ml
20 SGP/ml
40 SGP/ml
72 h-Rep 1
72 h-Rep 2
96 h-Rep 1
96 h-Rep 2
% total CD4+ CSFE T-cells
generation

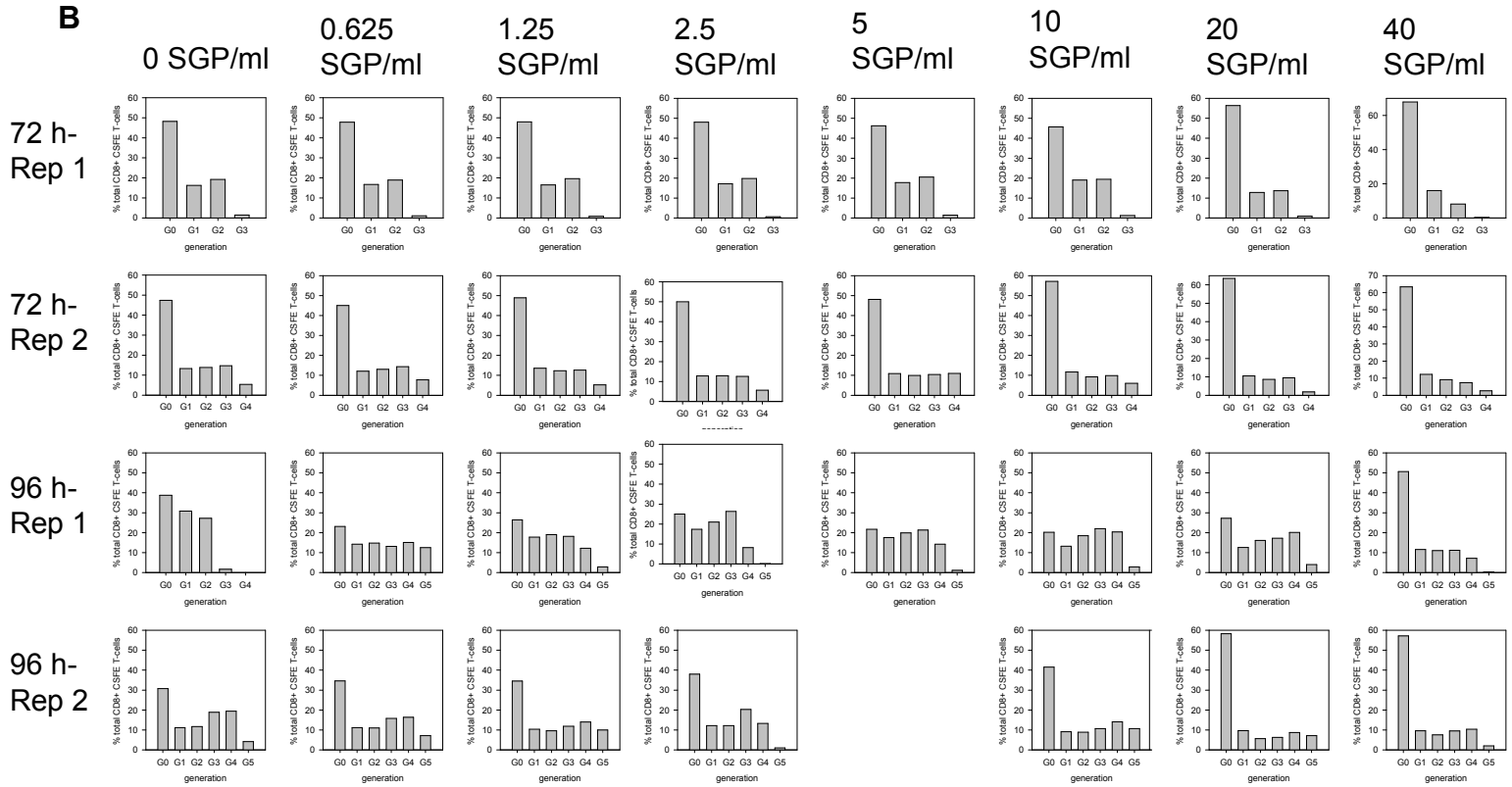


Figure 4.2: CFSE labeled CD4+ and CD8+ cell division decreases with higher dosages of *A. aegypti*. CFSE labeled PBMCs were incubated with varying concentrations of SGE and 10 μg/ml Con A for 72 and 96 h. Cells were stained with PE-Cy5-labeled mouse anti-human CD4 (RPA-T4) (Figure 4.2a) or APC-labeled mouse anti-human CD8 (clone RPA-T8) (Figure 4.2b). Cells were washed twice in 200 μl PAB, and resuspended in 300 μl for analysis with a DakoCytomation flow cytometer.

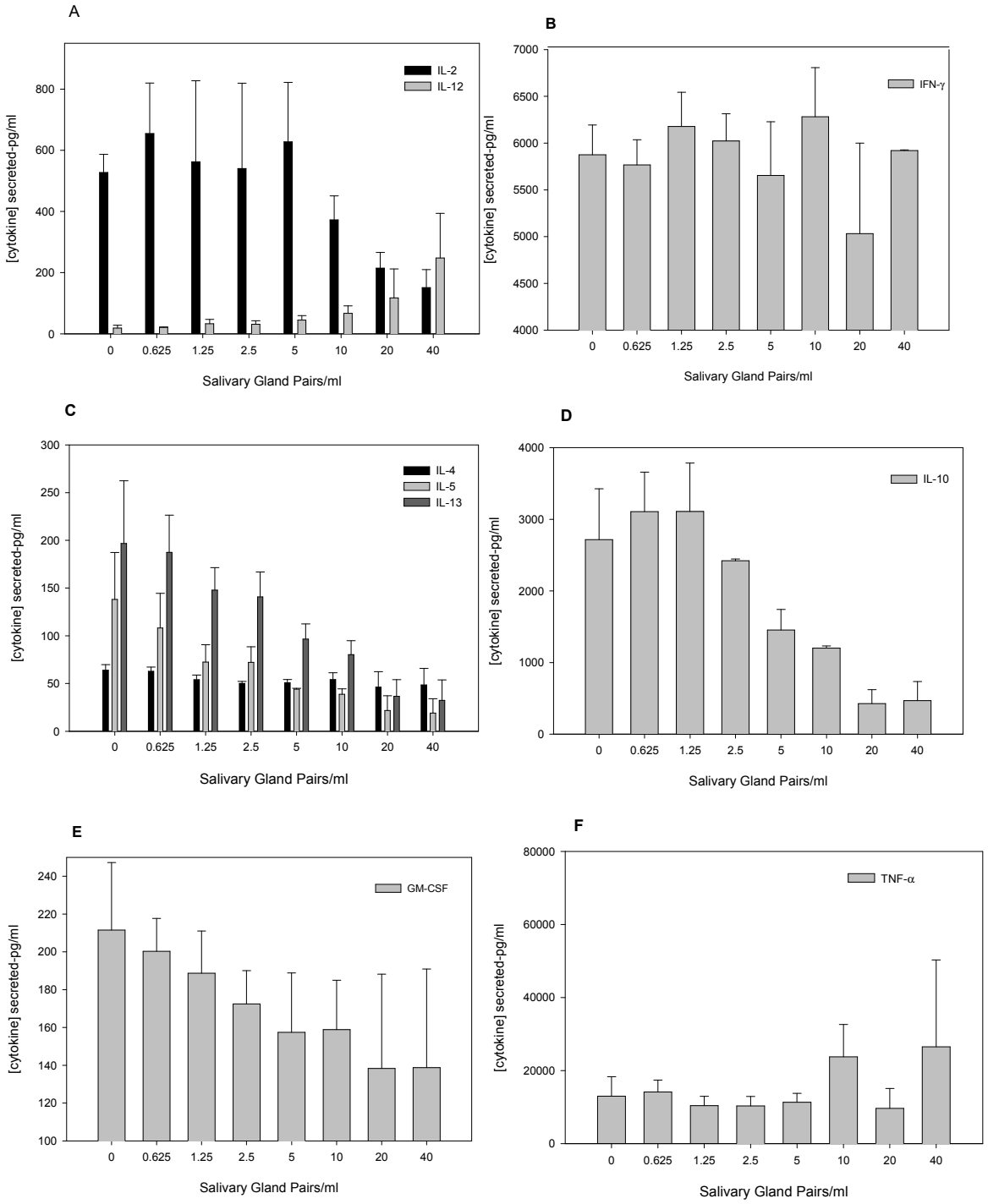


Figure 4.3: *A. aegypti* saliva modulates human PBMCs to secrete cytokines that up-regulate a DTH-like response. PBMCs (5 x 10^5 cells in 200 µl RPMI) were incubated with varying concentrations of SGE for 2 h followed by stimulation with 5 µg/ml Con A for 48 h. The cell culture supernatant was bioassayed for cytokine concentrations using a BioRad BioPlex Th1/Th2 kit.

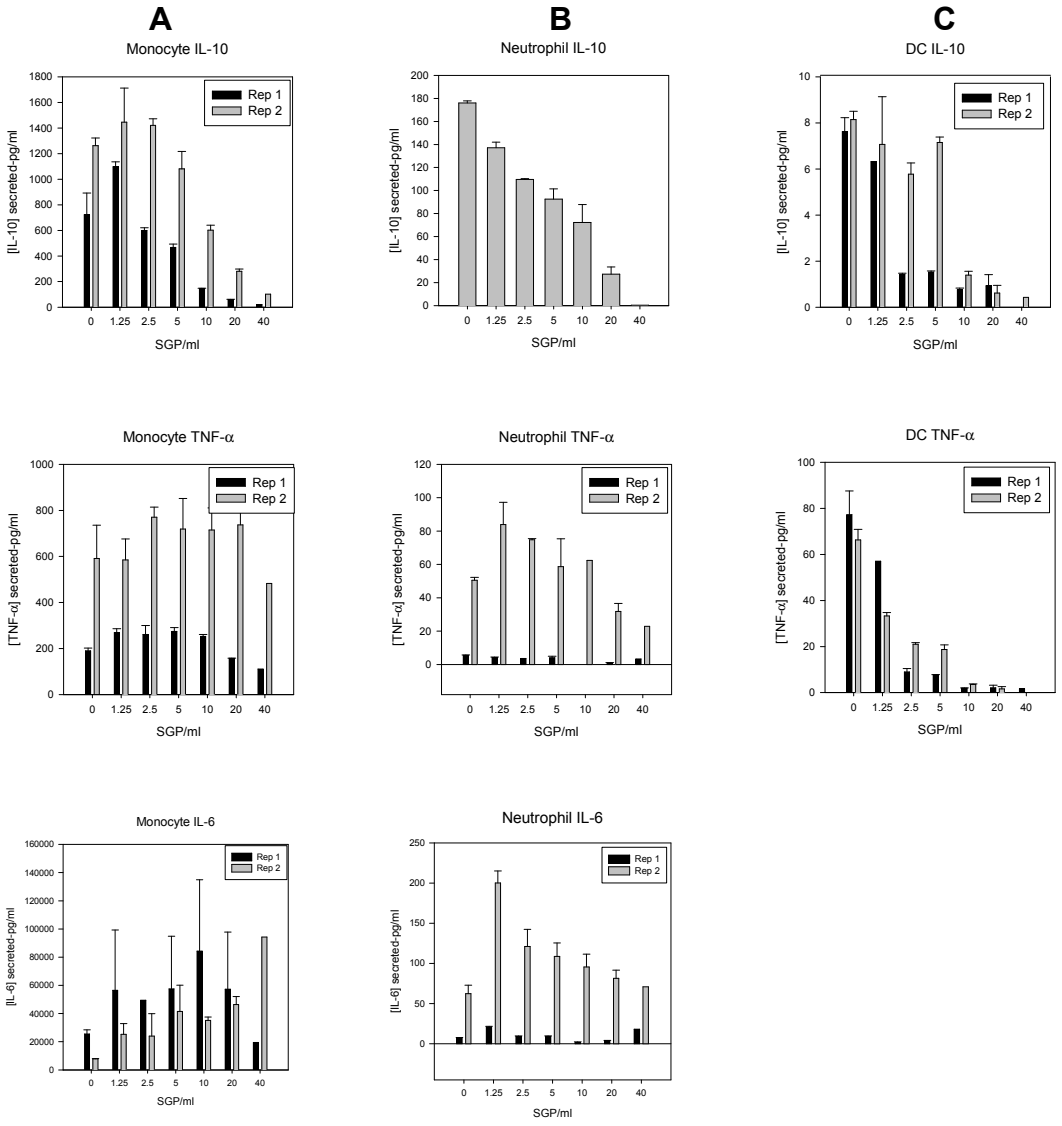
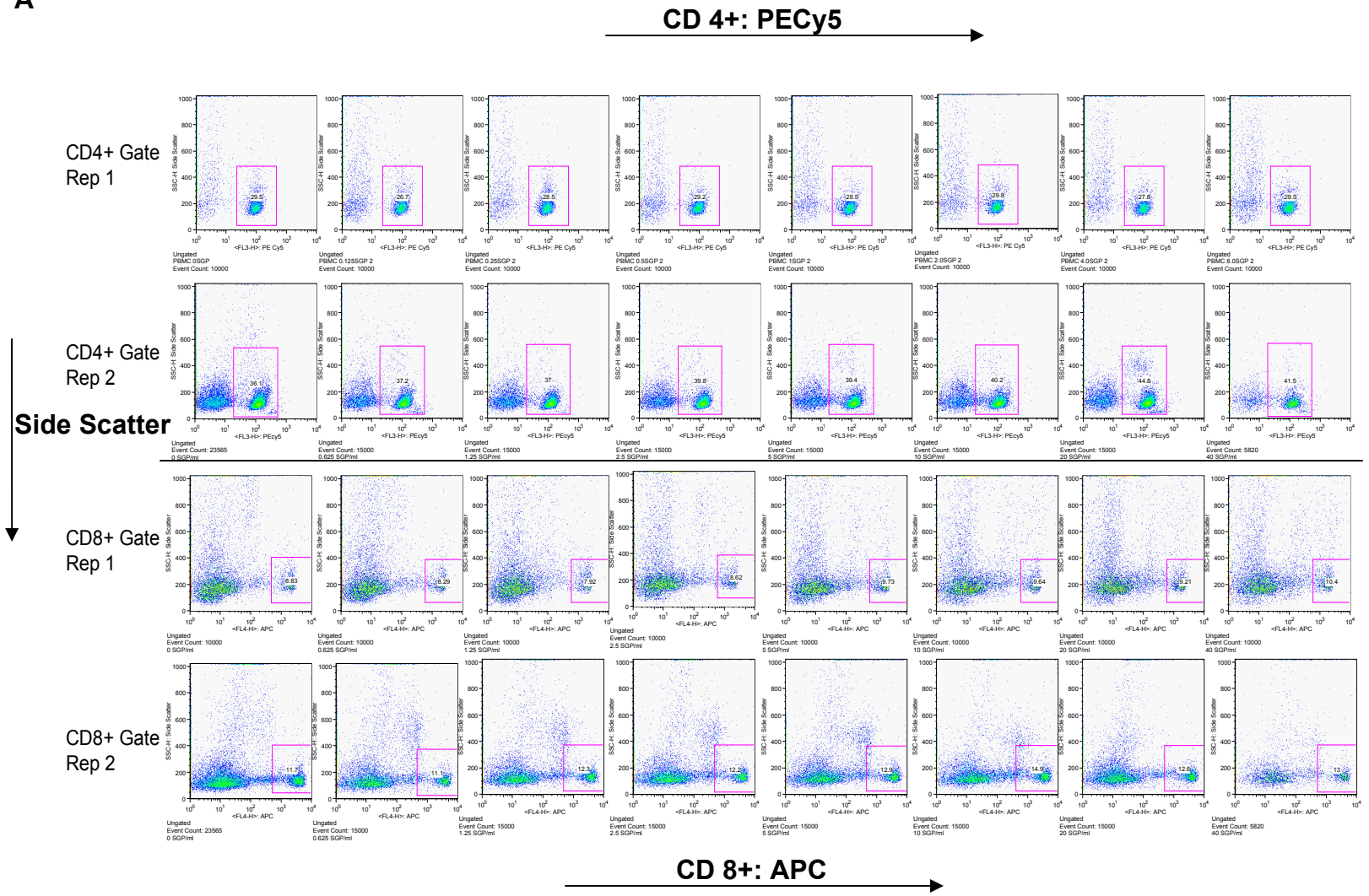


Figure 4.4: *Aedes aegypti* saliva both increases and decreases cytokine secretion by isolated neutrophils, monocytes, or dendritic cells. Human monocytes (Column A), neutrophils (Column B), and dendritic cells (Column C) were incubated with varying concentrations of SGE for 2 h followed by stimulation with 5 μg/ml LPS for 48 h. The cell culture supernatant was bioassayed for cytokine concentrations using a BioRad BioPlex kit. Replicate 1 and 2 represent two separate individuals.

A


CD 4+: PECy5
CD4+ Gate Rep 1
CD4+ Gate Rep 2
Side Scatter
CD8+ Gate Rep 1
CD8+ Gate Rep 2
CD 8+: APC

B

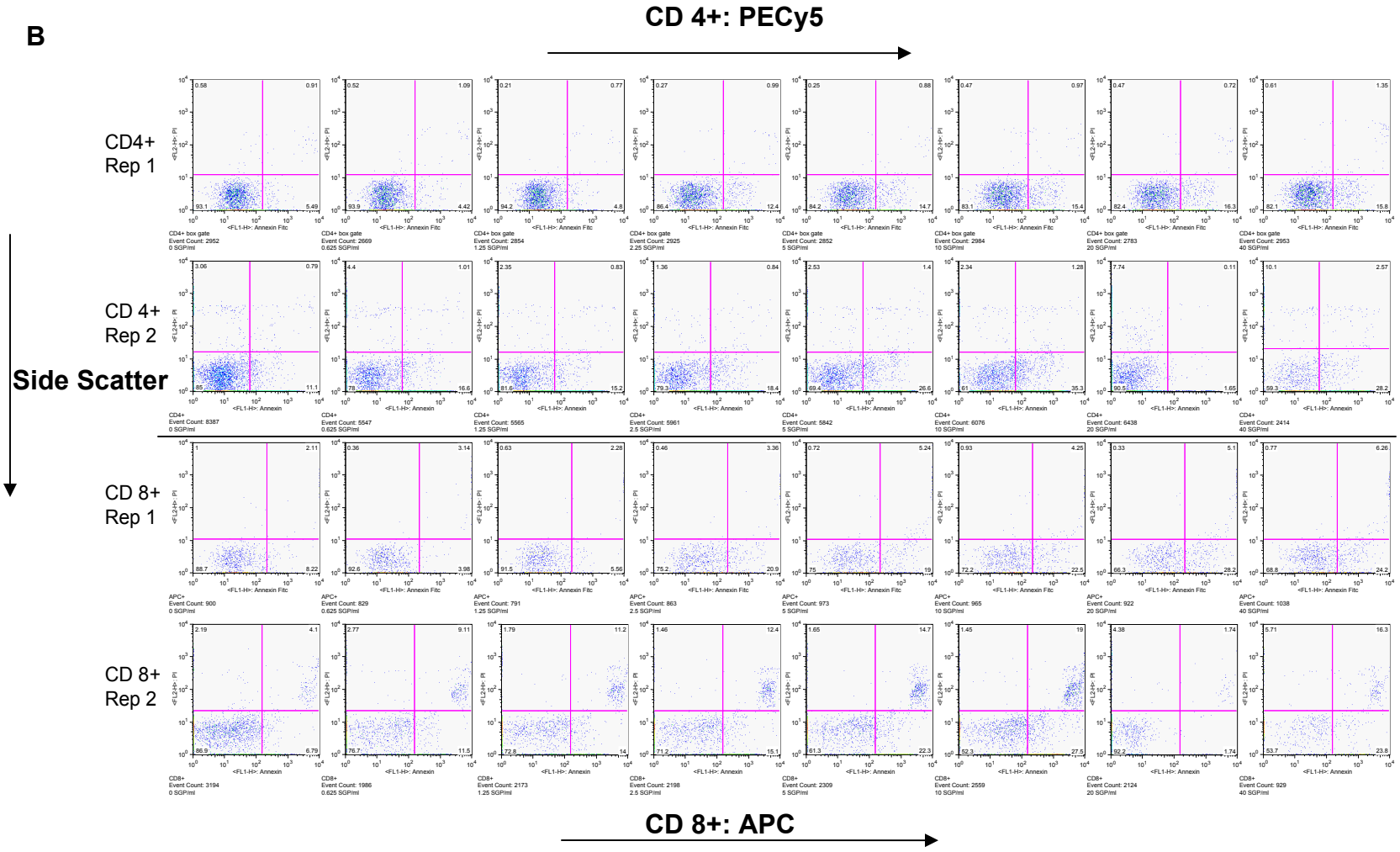

CD 4+: PECy5
CD4+ Rep 1
CD 4+ Rep 2
Side Scatter
CD 8+ Rep 1
CD 8+ Rep 2
CD 8+: APC

Figure 4.5: Apoptosis of CD4+ and CD8+ human PBMCs is observed with increased dosages of *A. aegypti* SGE. PBMCs labeled with CD4+ and CD8+ fluorescent Ab were incubated with SGE for 2 h, resuspended in annexin binding buffer and incubated with annexin V-FITC 3.5 μl/100 μl annexin binding buffer. Cells were washed with annexin binding buffer, and 5 μl of 50 μg/ml PI was added. PECy5 and APC Ab fluorescence was first gated (A), and these cells were analyzed for PI+ and Annexin+ Ab. All cells were analyzed with a FACSCalibur (Becton Dickinson) within 1 h of staining. Cell compensation in all experiments was performed using FlowJo.

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CHAPTER 5

CONCLUSIONS

Extensive research has established that saliva of sandflies (15, 20, 29) and ticks (2, 4, 14, 31, 40) contain components that modulate both innate and cell-mediated immune effector functions. In contrast, despite it's importance as a vector of viral diseases, little is known about the effects of *Aedes aegypti* saliva (3, 10), or indeed of any mosquito species, on host immune effector cell's functions. As vector-borne pathogens are transmitted to a host in the presence of saliva, the immunomodulatory effects of saliva may be crucial to the infection process.

When anti-hemostatic proteins are secreted into the host during vector feeding, the host's immune system responds to the proteins as antigens. Initially an innate response is elicited, often including inflammation, which then stimulates an acquired immune response (31). Two important components of the innate immune system are the complement system and phagocytic cells, which include neutrophils and macrophages. Initially, antigen is endocytosed by phagocytic cells, and/or opsonized by antibodies. Phagocytic cells detect, engulf, and destroy pathogens. Macrophages present peptide fragments complexed with major histocompatibility class (MHC) Class II- to CD4+ T-cells, which then up-regulates adaptive immunity. Additionally, Langerhan's cells as well as macrophages, present in the dermis, take up antigen and migrate to the lymph node. Once in the lymph nodes, the Langerhan's cells mature into dendritic cells and also present MHC Class II-bound antigen to naive T-cells.

Upregulation of naive T-cells leads to clonal expansion of antigen-specific T-cells via the autocrine release of Interleukin (IL)-2. In addition, dendritic cells, macrophages, and most other nucleated cells sample cytosolic proteins (including proteins derived from intracellular pathogens), and present peptides derived from these proteins, complexed with MHC Class I, to CD8+ T-cells. Activated CD8+ T-cells are thus able to recognize host cells presenting non-self antigens, and kill them by cytolytic mechanisms.

The major inducer of Th1 cells is the cytokine IL-12, produced by dendritic cells and macrophages. Th1 cells are the major responders to intracellular pathogens. The Th2 response is activated by allergens and helminth infection; Th2 cells differentiate in the presence of IL-4. This induces chronic T-cell stimulation and little macrophage activation. Th1 cells are characterized by the production of the macrophage activating cytokine interferon gamma (IFN-γ) and IL-2, which stimulates T-lymphocyte proliferation. Th2 cells are defined by the production of IL-4, IL-5, IL-6, IL-10, and IL-13; the latter two cytokines inhibit activation of macrophages and have anti-inflammatory activity (25). Th1 and Th2 cells have opposing roles in determining how the immune system will respond to an antigen (25).

To model the normal route of CD4+ T-cell stimulation, where salivary antigens are processed and displayed in complex with MHC class II by antigen-presenting cells (APCs) for interaction with the T-cell receptor (TCR), I used DO11 transgenic mice whose TCRs have been genetically fixed to respond only to interaction with OVA peptide. To eliminate the need for APC presentation of antigen to T-cells and B-cells, we also used the mitogens Concanavalin A (Con A), or *Escherichia coli* lipopolysaccharide (LPS), to non-specifically stimulate T-cells or B-cells, respectively.

Initially, I examined the effect of *A. aegypti* SGE on host immune functions using a mouse model. Phagocytosis and MHC Class I and II expression by macrophages were dose-dependently decreased. However, nitric oxide production was unaffected by saliva. Decreased phagocytosis could be expected to reduce the ability of macrophages to clear pathogens from an infection site. Further, inhibition of phagocytosis and decreased MHC I and II expression will lead to a reduction in interactions with T-cells. Stimulated macrophages incubated with *A. aegypti* saliva, even at very low concentrations, had decreased pro-inflammatory cytokine secretion, but low doses of saliva did not inhibit IL-10 secretion. In particular, the Th1-inducing cytokine IL-12 was markedly dose-dependently decreased, which will lead to a bias favoring a Th2 response following any macrophage/Th0 interactions that do occur. This would result in a reduction in CD4+ T-helper cells that otherwise would secrete IFN-γ and stimulate macrophage activation and phagocytosis.

Similar effects on macrophage function have been ascribed to the sandfly immunomodulatory peptide maxadilan (22). Effects of this peptide involve an indomethocin-sensitive increase in intracellular prostaglandin E_2 (PGE_2). Other studies have highlighted a role for cAMP in regulating TNF-α secretion from macrophages (1, 21). The effect of *Aedes aegypti* SGE differs in that it is independent of cAMP- or PGE_2-dependent signaling pathways.

Splenocytes contain T-lymphocytes, antigen presenting cells (APCs), as well as other important immune cells, and thus may serve as a model for immune responses that allows for cell-cell interactions. Low doses of *A. aegypti* saliva dose-dependently decreased proliferation and secretion of the Th1 cytokines IL-2 and IFN-γ by antigen-

stimulated mouse splenocytes, which was consistent with the reduced IL-12 secretion seen in SGE-treated macrophages. The reduction in T-cell proliferation could be due to the reduced secretion of IL-2 in this system. However, slightly higher concentrations of SGE inhibited secretion of both Th1 and Th2 cytokines. Inhibition of both proliferation and cytokine secretion also occurred with mitogen-stimulated splenocytes, indicating that saliva directly inhibits T-cell functions, in addition to its disruptive effects on APC-T-cell interactions. Inhibition of lymphocyte effector function involved modulation of viable T-cells at low SGE concentrations, and decreased viability at higher concentrations. An SGE-induced loss of viability also accounts for the inhibition of B-cell proliferation following stimulation with LPS. In contrast to the T-cells and B-cells, dendritic cells and macrophages were not killed by SGE even at high concentrations. From this data, *A. aegypti* saliva seems to suppress the adaptive immune response in these naive mice, by inhibiting both Th1 and Th2 cytokine secretion, and T-cell and B-cell proliferation. The decrease in T-cell proliferation and T-cell viability may be expected to greatly impact cell mediated immunity by decreasing the number of T-cells that are available to interact with APCs and secrete cytokines such as IFN-γ.

Study of salivary modulation of human effector cell functions is required, because humans and not mice are the primary host of *A. aegypti*. Therefore, I investigated if saliva modulated human peripheral blood mononuclear cell (PBMC) effector functions. As was the case with mouse splenocytes, PBMCs are complex mixtures of CD4+ and CD8+ T-cells, APCs, B-cells, neutrophils, and other immune cells, which allows for cell-cell interaction to occur. Saliva decreased PBMC proliferation, and more specifically CD4+ and CD8+ T-cell division, in a dose-dependent manner, but without a loss of cell

viability. Secretion of IL-2 was decreased, which could explain the reduction in T-cell proliferation. However, secretion of IL-12 increased 40-fold over the range of SGE concentrations tested (which proves the viability of the APCs even at high SGE concentrations), and IFN-γ and TNF-α secretion was neither inhibited nor stimulated. Taken together these results are more consistent with a pro-inflammatory response than with a Th1 response. A similar mixed message applies to Th2 responses: IL-10 secretion was modestly increased at low SGE concentrations and decreased at higher levels, IL-5 and IL-13 were decreased, and IL-4 levels were unaffected at any SGE concentration. The decrease in IL-13 and, at higher SGE levels, IL-10, is consistent with an inflammatory response to *A. aegypti* saliva, but normal IL-4 levels could allow a significant Th2 character to the net immune response. In fact, Chen et al (9) have reported high levels of IgG2, typical of Th2 responses in humans, as well as elevated IL-4 and reduced IFN-γ, in response to mosquito challenge in sensitized patients.

The response of human neutrophils, dendritic cells, and monocytes to *Aedes* saliva also supports the finding of an inflammatory response. In monocytes, IL-10 secretion was elevated by low concentrations of SGE, but higher concentrations were inhibitory. Neutrophils and dendritic cells responded to saliva exposure with a dose-dependent decrease in IL-10 secretion. Both IL-6 and TNF-α were increased at low SGE concentrations and dose-dependently decreased with higher SGE concentrations in both monocytes and neutrophils. TNF-α secretion was strongly inhibited in dendritic cells. These results are again consistent with an overall pro-inflammatory response to challenge with *A. aegypti* saliva.

Two very different pictures emerge when one compares the overall effect of *A. aegypti* saliva on human and mouse immune cell functions. Although SGE inhibited proliferation of both mouse splenocytes and human PBMS, the human cells were about 50-fold less sensitive compared to the mouse cells. In the mouse, saliva inhibited secretion of Th1 cytokines at low concentrations, but slightly higher concentrations inhibited secretion of both Th1 and Th2 cytokines through a mechanism that also decreased T-cell viability. On the other hand, *A. aegypti* saliva produced a mixed pro-inflammatory/Th2 response in human PBMCs and isolated cells. Most strikingly, *A. aegypti* saliva produced a strong dose-dependent decrease in IL-12 secretion from mouse splenocytes and macrophages, whereas there was a marked dose-dependent increase in IL-12 secretion from human PBMCs.

To explain these results, one must take into consideration the individuals from whom cells were obtained. Mouse splenocytes were isolated from genetically identical, immune naive mice that had no prior exposure to mosquito antigen. In contrast, PBMCs were isolated from humans that have greatly differing genetic make-up, and a wide exposure to different antigens including many types of mosquito saliva, specifically including *A. aegypti*. These differences between the two types of test subjects may significantly contribute to the type of immune response that will be elicited by *A. aegypti* SGE. Human CD4+/CD8+ T-cell proliferation was slightly stimulated with low SGE concentrations, and at higher concentrations this stimulation may have partially offset any inhibition, so that a net reduction in T-cell proliferation was only evident with the highest SGE concentrations. This stimulation likely was a consequence of the presence of salivary antigen-specific CD4+ and CD8+ T-cell clones in the human PBMCs. Effects

on cytokine secretion (such as IFN-γ) from T-cells might also have been influenced by the presence of saliva-stimulated T-cell clones in the human PBMCs. An increased pro-inflammatory response will lead to the migration of memory and clonally activated T-cells to the bite site. On the other hand, in immunologically naive mice, *A. aegypti* saliva will not elicit an antigen specific CD4+/CD8+ T-cell proliferative or cytokine response.

There is also considerable evidence for innate species-specific differences between mouse and human responses to *A. aegypti* saliva. In particular, secretion of IL-12 was inhibited in mice and stimulated in human cells. This cytokine is secreted from antigen-presenting cells, especially dendritic cells and macrophages. As these cells are not stimulated in an antigen-specific manner, the different cytokine responses must reflect innate differences between mouse and human macrophages rather than acquired memory. It is possible that these differences reflect different responses to glycosylation (potentially mannose) patterns on salivary proteins, mediated by Toll-like receptors (TLRs) or pattern recognition receptors such as the mannose receptor (36). Mouse and human monocytes also had different patterns of secretion of IL-6, IL-10, and TNF-α, which reinforces the idea of intrinsic differences between these two species. As humans are a natural host for *A. aegypti*, and mice are not, one might speculate that these differences result from a coevolved response to saliva of this mosquito. This data does indeed make one cautious about using mice or other species as models for humans. However, the use of mice and other animals remains a necessity, especially for determining *in vivo* interactions between various cell types and between immune cells and pathogens.

Given the presence of immunomodulatory activity in the saliva of a wide range of blood feeding arthropods, including sandflies (18, 28, 30, 33), blackflies (10-12), ticks (2, 4, 14, 31, 40), and now mosquitoes, it is reasonable to propose that this activity confers a fitness benefit to the arthropod. Experimental evidence for this is strongest in the ixodid ticks. William Trager in 1939 conducted experiments using natural and unnatural hosts (white footed mice and guinea pigs respectively) of the tick *Dermacentor variabilis*. Tick larvae feeding on naïve guinea pigs had a 50% tick mortality rate; and almost all the larvae that fed on previously exposed guinea pigs died. Edema and subsequent basophil infiltration at the bite site has been implicated as the main mechanism underlying tick rejection by unnatural hosts (35, 38, 39). Additionally, those ticks that do survive feeding have decreased fitness reflected in their decreased ability to molt (38, 39). Histamine and the alternative complement pathway both seem to have roles in these tick-rejection reactions. Guinea pigs, treated with histamine antagonists had decreased rejection of feeding ticks (5-7, 38). Guinea pigs that are deficient in complement had a decreased ability to reject feeding ticks compared to wild type animals (8). The guinea pig's immune cells present at the bite site seem to overwhelm any salivary immunomodulators and cause a reduction in tick fitness by reducing blood feeding (26). Conversely natural hosts such as the white footed mouse mentioned above do not develop the ability to reject *D. variabilis,* even after multiple episodes of tick feeding. Ticks have immunomodulators that have evolved with these natural hosts. For example, saliva of the adult tick *Ixodes scapularis* contains components that inhibit T-cell proliferation (27), and neutrophil aggregation (26). However, *I. scapularis* saliva does not contain a histamine antagonist. Histamine is an

important mediator in guinea pig anaphylactic reactions, but not in human reactions which utilize leukotrienes (1). Additionally, the white footed mouse contains almost no histamine rich basophils (1). From this experimental evidence, it is reasonable to hypothesize that histamine may be a key factor in tick rejection by guinea pigs but not the mice (21, 22). These results suggest that tick saliva has coevolved with the immune response of the natural but not unnatural hosts. As a result, saliva contains immunomodulators that inhibit the specific immune response of host vertebrates, but those immunomodulators may be ineffective against different responses in non-host species.

In contrast to the experiments with ticks, there is no direct evidence for an increased fitness advantage due to salivary immunomodulators in the fast feeders such as *A. aegypti*. The biology of the two arthropods in terms of blood-feeding is vastly different. Ticks spend days on the same host, allowing ample time for a host response and making the presence of immunomodulators vital for their survival. On the other hand, fast feeders such as mosquitoes or sandflies spend only minutes on the host obtaining a blood meal, which would seem to be insufficient time for the host to respond immunologically. As well, mosquitoes and sandflies have a large geographic range to find hosts to feed on, so repeated feeding on the same host is not likely. Under these circumstances it is difficult to understand the selective benefit underlying the salivary immunomodulation. However, fast feeders do imbibe immune cells from the host while blood-feeding. Under some circumstances these host immune cells may be harmful for the insect's fitness; therefore salivary immunomodulators may be necessary to counteract these cells. For example, macrophage phagocytosis, important in both

innate and acquired immunity, is greatly decreased by *A. aegypti* SGE. If macrophages are present as part of the blood meal, phagocytosis and subsequent damage to the blood-feeding insect may ensue; therefore inhibition of the macrophage's effector functions could increase insect fitness.

Evidence in support of this hypothesis is seen in a study by Foy et al (13). These researchers vaccinated mice with a midgut cDNA library from blood fed females, or with cDNA encoding specific midgut mucins. These mice produced a Th1 response to *Anopheles* mosquito feeding, which significantly increased mosquito mortality and decreased egg production in survivors. However, when mice were boosted with midgut protein, they had a shift toward a Th2 type immune response, and mosquito mortality was no longer observed. Mosquitoes that fed on mouse sera alone did not have increased mortality, suggesting that the cDNA-induced mosquitocidal immunity was cell mediated. This experiment points to the fact that the mosquito midgut is a crucial area for interactions between insect factors, like saliva, and host cells, such as T-lymphocytes and macrophages. As well, this experiment indicates that a Th1 response can be toxic to mosquitoes, and the effect is due to interactions between the mosquito and the host immune system that occur in the mosquito gut rather than during the relatively fast feeding bout. As some saliva (about 0.2 SGP, D.E. Champagne, unpublished data) is reingested with the blood meal, it seems plausible that the effects of *A. aegypti* SGE on lymphocyte and phagocyte function could effectively inhibit the potentially toxic effects of these cells in the mosquito midgut. In addition, a fitness benefit may follow from the bias towards a Th2 response to mosquito saliva, as this type of response was not toxic to mosquitoes in the Foy (13) study.

Experimental evidence that may directly answer the question of the benefit of salivary immunomodulators to fitness is needed. A first step to any such experiment would require the identification of the *A. aegypti* salivary immunomodulator that inhibits T-cell proliferation and cytokine secretion. Once the specific protein, or a cDNA clone, is in hand it would be possible to manipulate concentrations of the protein in saliva or salivary gland extracts, using specific antibodies to neutralize or immunoprecipitate the protein, or RNAi-based approaches. One could then compare feeding success, egg production, and survivorship of mosquitoes with or without the salivary immunomodulator. As a first step towards this goal, I characterized the immunomodulator as a relatively large protein or protein complex, approximately 387 kDa, based on sensitivity to boiling, protease digestion, and gel filtration chromatography.

Pathogen transmission by blood-feeders may be enhanced by salivary immunomodulators. Transmission of *Borrelia burgdorferi*, the Lyme Disease pathogen, is enhanced by saliva of its vector, the tick *Ixodes scapularis,* and this enhancement is reversed by restoring a normal cytokine profile to tick-bitten hosts (41, 42). Similarly, transmission of *Leishmania* to immunologically naive mice is enhanced by saliva from sandfly vectors (24). This dissertation has shown that *A. aegypti* SGE modulates many different types of immune cells, including T-and B-lymphocytes, macrophages, neutrophils, dendritic cells, and monocytes, in a manner likely to favor pathogen transmission. Decreased phagocytosis and MHC presentation is likely to impair the ability of macrophages to remove pathogens and activate acquired immune responses. Saliva modulated both stimulated and unstimulated cells as well as mitogen and

antigen-specific responses of CD4+ T-cells. Specifically, a strong Th1 response, which includes the CD4+ and CD8+ T-cells, is needed for protection against intracellular viral infections, including dengue and yellow fever (23, 34). Although protective immunity against many of these vector borne diseases requires a Th1 response, salivary immunomodulators polarize the response towards a Th2 cytokine environment, which may favor pathogen transmission at least in the early stages. The specific role of the immunomodulatory activity in pathogen transmission by *A. aegypti* should be a fertile area for future research.

In *Anopheles* mosquitoes, monocytes/macrophages and polymorphonuclear granulocytes are able to phagocytize *Plasmodium* gametocytes for several hours after ingestion in the mosquito midgut, resulting in reduce numbers of oocysts (16, 17, 19, 32). It is probable that saliva, ingested with the blood meal as described above, inhibits these phagocytic cells in the *A. aegypti* midgut. However, the *Anopheles/Plasmodium* experiments suggest that inhibiting the salivary immunomodulator may increase activity of phagocytic cells in the blood meal, resulting in a reduction in the number of pathogens that survive to infect the mosquito.

A cDNA vaccine strategy, previously described for a sand fly salivary immunomodulator by Valenzuela (37), might be a good route to follow for an anti-*A. aegypti* immunomodulatory vaccine. Mice would be injected with a cDNA vaccine containing a non-functional variant of the immunomodulator, designed to result in high titers of neutralizing antibodies against the salivary factor. In the sandfly experiment, inoculated mice were protected against *Leishmania* infection transmitted by sandfly bite. Protection was seen in the form of a DTH response as well as an intense humoral

response. Similar protection against yellow fever or dengue virus infection could be observed upon anti-*A. aegypti* immunomodulatory vaccination.

In conclusion, this dissertation has clearly established the presence of a potent immunomodulatory effect of *A. aegypti* saliva on mammalian immune effector cells. Further investigation is needed on the *in vivo* mammal response to SGE. This will help to determine the extent to which the use of cell cultures are is applicable to the whole-animal response. We still need to isolate, purify, and characterize the immunomodulator. Ultimately, these studies are likely to contribute to a fuller understanding of disease transmission by this notorious vector species, and suggest novel approaches for mitigating its impact on humanity.

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