Modulation of host immunity by tick saliva

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Abstract

Next generation sequencing and proteomics have helped to comprehensively characterize gene expression in tick salivary glands at both the transcriptome and the proteome level. Functional data are, however, lacking. Given that tick salivary secretions are critical to the success of the tick transmission lifecycle and, as a consequence, for host colonization by the pathogens they spread, we thoroughly review here the literature on the known interactions between tick saliva (or tick salivary gland extracts) and the innate and adaptive vertebrate immune system. The information is intended to serve as a reference for functional characterization of the numerous genes and proteins expressed in tick salivary glands with an ultimate goal to develop novel vector and pathogen control strategies.

Graphical abstract

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Conflicts of interest

The authors declare that no conflicts of interest exist.
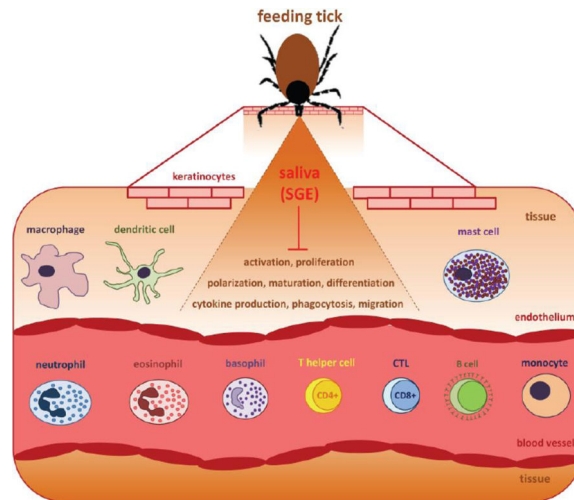
Introduction

Ticks are obligatory blood-feeding arthropods that belong to the subclass Acari, order Ixodida, and three families: Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttallielidae. Soft ticks feed repeatedly for minutes to hours, while hard ticks usually stay attached to their hosts and feed for several days or even weeks, but only once in each life stage [1, 2]. The amount of blood ingested is species and life-stage specific, with females of some tick species increasing their volume up to 200 times by the end of blood feeding [3].

Ticks are important vectors that transmit a wide range of pathogens. The most common tick-borne pathogens are viruses and bacteria, but fungi, protozoa, and helminths can also be transmitted [4]. Clinically and epidemiologically, the most important tick-borne diseases are: tick-borne encephalitis (TBE), caused by the TBE virus; Lyme disease, caused by spirochetes belonging to the *Borrelia burgdorferi sensu lato* complex in Europe and *B. burgdorferi sensu stricto* in the USA; tick-borne spotted fever, caused by *Rickettsia spp.*; anaplasmosis, caused by *Anaplasma spp.*; and babesiosis, caused by *Babesia spp.* protozoa [5, 6]. Pathogens have different life cycles, but the transmission usually begins with a tick biting an infected vertebrate host and pathogen uptake by the tick in the blood meal. Pathogens, e.g. *Borrelia spp.* spirochetes then stay in the midgut and wait until next feeding, which triggers their proliferation and migration through the midgut wall to haemocoel and, ultimately, to the salivary glands. Moreover, spirochetes interact with some midgut and salivary components that induce Borrelia proliferation or increase their infectious potential [7]. When the tick bites its next vertebrate host, pathogens are transmitted via tick saliva. In some tick species the pathogens are transmitted transovarially from the female to laid eggs, thus keeping the level of prevalence in the tick population [8]. Tick saliva has been shown to facilitate pathogen transfer to the vertebrate host by virtue of its pharmacological properties, including modulation of the vertebrate immune system [9–11]. Moreover, tick saliva

contains toxins belonging to families also found in venomous animals, such as spiders or snakes, and that can induce paralysis and other toxicoses [12].

To secure uninterrupted blood uptake, ticks suppress and evade the complex physiological host immune and homeostatic responses that are raised against them. Hemostasis, which includes coagulation, vasoconstriction, and platelet aggregation, is the first innate host defense mechanism against the mechanical injury caused by intrusion of tick mouthparts into the host skin. This early vertebrate host response further includes complement activation and inflammation, with the host inflammatory response including, among other factors, rapid leukocyte infiltration after skin injury [13]. Keratinocytes, endothelial cells, and resident leukocytes such as mast cells, dendritic cells, and macrophages make immediate contact with tick saliva or the tick hypostome and are activated. Pro-inflammatory chemokines and cytokines including interleukin-8 (IL-8), tumor necrosis factor (TNF), and IL-1β are released to recruit neutrophils and other inflammatory cells to the area of tick infestation [14]. Following tick feeding, there is activation of both the cellular and humoral branches of vertebrate adaptive immunity [15]. Activated memory T and B cells (in the case of secondary infestation) amplify the host inflammatory response to ticks by releasing specific cytokines and producing antibodies that target tick salivary or mouthpart-derived antigens to activate complement or sensitize mast cells and basophils [9, 14, 15]. The strength and specificity of the host immune response and its effect on tick physiology depend on the host and tick species, the host's health, and its genotype [16]. The same is true for tick defense mechanisms, since both tick salivary components and host immune mechanisms have been co-evolving. As a result, the tick-host interaction can be considered an "arms race" between the new defense mechanisms developed by the host and the evasion strategies developed by ticks [17]. As an adaptation to blood feeding, ticks secrete a complex mixture of immunomodulatory substances in their saliva that suppress both innate and adaptive host immune responses that can cause pain, itch, blood flow disruption in the tick feeding cavity, or even direct damage to the tick, thereby subverting tick rejection and death [18–20]. Despite the specificity of tick salivary component targets, there is also redundancy at the molecular, cellular, and functional level [9, 13]. The richness and diversity of tick salivary compounds have been established in several transcriptomic studies over the last fifteen years and, more recently, by next generation sequencing (NGS) studies.

The rapid developments in NGS and proteomics are reflected in the recent progress made in tick research, in which several transcriptomic and proteomic studies have been published over the last few years. These studies represent a rich data source that provides the basis for functional studies and investigation of gene expression dynamics during tick feeding and different physiological states. For instance, significant differences in the salivary proteome of partially and fully engorged female *Rhipicephalus (Boophilus) microplus* ticks have been described [21]. More recently, a transcriptomic study described over 800 immuno-proteins in *Amblyomma americanum* saliva during 24–48 hours of feeding [22]. A transcriptomic analysis of *Dermacentor andersoni* salivary glands resulted in over 500 singletons and 200 clusters in which a number of sequences with similarity to mammalian genes associated with immune response regulation, tumor suppression, and wound healing were identified [23]. By

combining transcriptomic and proteomic approaches, nearly 700 proteins were identified in *D. andersoni* saliva after two and five days of feeding, from which 157 were postulated to be involved in immunomodulation and blood feeding [24]. Schwarz and colleagues performed a comprehensive study of *Ixodes ricinus* salivary and midgut transcriptomes and proteomes and found that the transcriptomic and proteomic dynamics did not 100% overlap in different tick tissues [25]. A recent study by Kotsyfakis and colleagues characterized transcriptional dynamics in the *I. ricinus* female and nymph salivary glands and midguts at various feeding time points [26], and established that some gene families show stage- and time-specific expression, possibly via epigenetic control. In addition, the genes encoding secreted proteins exhibited a high mutation rate, possibly representing a mechanism of antigenic variation, and analysis of the midgut transcriptome revealed several novel enzymes, transporters, and antimicrobial peptides [26]. A transcriptomic analysis of *Amblyomma maculatum* salivary glands revealed almost 3500 contigs with a secretory function [27]. Another sialome (salivary gland transcriptome) of *Amblyomma* ticks was published by Garcia and colleagues [28]: the authors analyzed samples from *A. triste* nymphs and females, *A. cajennese* females, and *A. parvum* females and focused on putative transcripts encoding anticoagulants, immunosuppressants, and anti-inflammatory molecules. A further study characterized *A. americanum* nymph and adult proteomes and compared the data with other *Amblyomma* species [29]. A *Rhipicephalus pulchellus* tick sialome study revealed differences between males and females [30], with the sequences identified used for a preliminary proteomic study to identify 460 male and over 2000 female proteins. A sialomic study was also performed in *Haemaphysalis flava* that revealed tens of thousands of genes, some of which were putative secreted salivary proteins thought to be involved with blood feeding and ingestion [31]. A *Rhipicephalus sanguineus* salivary proteome showed recycling of host proteins and their secretion back into the host [32]. Lewis and colleagues used a transcriptomic approach to characterize immunogenic *I. scapularis* salivary proteins present after 24 hours of feeding [33]; these appeared to be involved in tick feeding even before the majority of pathogens could be transmitted.

In addition to analysis of secreted tick salivary proteins, tick-feeding lesions on the host have been analyzed by high-throughput and histological methods. Recently, the feeding lesion of *D. andersoni* was described in detail together with microarray analysis of host gene expression dynamics, thereby characterizing the inflammatory infiltrate at the feeding site and the changes occuring in the epidermal and dermal compartments near the tick [34, 35]. The skin lesions examined from rats infested by *Ornithodoros brasiliensis* showed edema, muscle degeneration, and hemorrhage [36], with the rats themselves presenting with a bleeding tendency and signs of toxicosis. *O. brasiliensis* salivary gland homogenates delayed wound healing and had anti-proliferative or even cytotoxic activity on cultured epithelial cells [37]. An analysis of skin-draining lymph nodes in goats repeatedly infested with *A. cajennese* nymphs revealed an increased number of antigen presenting cells (APCs) such as B lymphocytes, macrophages, and dendritic cells [38]. A skin lesion from a human infested with female *Amblyomma testudinarium* was characterized by an inflammatory infiltrate and an eosinophilic cement in the center of the lesion [39]. Feeding lesions from rabbits injected with salivary gland extract (SGE) collected from *R. sanguineus* ticks after 2, 4, and 6 days of feeding showed signs of inflammation, especially at day four [40],

suggesting that molecules present in *R. sanguineus* SGE have high immunogenicity and that immune reaction raised against SGE is stronger than the immunomodulatory action of *R. sanguineus* salivary effectors.

Such high-throughput studies in both ticks and hosts and complemented with histological information and detailed characterization of salivary components have made a valuable contribution to our knowledge of the dynamic processes occurring at the tick-host interface. However, experiments with saliva or SGE highlight the complexity of host modulation by the tick *in vivo*. Characterizing individual salivary components can help link specific pathophysiological events to particular molecules to provide a complete picture of tick-host interactions. In this review, we focus on the immunomodulatory actions of whole tick saliva or salivary gland extracts (SGE) rather than the effects of the individual salivary components, since these are reviewed elsewhere [13, 41, 42].

The role of tick saliva in modulating host hemostasis and complement

Ticks have developed various mechanisms to counteract the hemostatic responses of the host so that they can successfully feed on blood for many days [13, 19]. Serine proteases are key players in host hemostasis and, therefore, are specifically targeted by the wide range of serine protease inhibitors present in tick saliva. The net result is that the physiological balance between host proteases and endogenous anti-proteases is impaired. Tick salivary secretions also contain vasodilators, platelet activation inhibitors, and coagulation modulators, as reviewed elsewhere [14, 43, 44].

Complement is a cascade of proteolytically-activated components that eventually leads to the creation of pores in the walls of microbes, leading to their destruction. There are three main complement activation pathways: classical, alternative, and lectin; the central reaction in all pathways is the conversion of complement component C3 to C3a and C3b [45, 46]. The inhibition of the host alternative complement pathway is crucial for tick feeding and, indeed, the saliva of several *Ixodes* species inhibits this pathway [47, 48]. In an *in vitro* study, the ability of tick saliva to counteract complement activity varied according to the animal species source of serum, with specificity shown toward the most common hosts for each *Ixodes* species [49]. Several anti-complement molecules have been identified to date; however, a detailed description is beyond the scope of this review. Further information about the role of complement in tick-host interactions can be found in the reviews by Schroeder and colleagues [50] or Wikel [14].

Innate immunity and tick saliva

Innate immune responses against tick feeding involve the activation of resident immune cells that initiate and promote the local inflammatory response as a reaction to skin damage. The resident leukocytes are macrophages, Langerhans cells (LCs), mast cells, or innate lymphoid cells, and pro-inflammatory mediators are also released by endothelial cells and keratinocytes [51]. These mediators and complement components are chemotactic for circulating inflammatory cells including neutrophils and monocytes.

Interaction of macrophages and monocytes with tick saliva

Macrophages are APCs as well as cytokine and chemokine producers [52]. They can be further divided into two different subpopulations: (i) bone marrow-derived hematopoietic macrophages, which circulate as monocytes and, after extravasation at the site of inflammation, differentiate into pro-inflammatory [53] or alternatively-activated macrophages [54] and (ii) tissue-resident macrophages of yolk sac origin that are found in many organs including the skin; the latter tend to be more immune-modulatory [55]. These macrophage subpopulations differ with respect to cytokine production, receptor expression, and their overall effect on any subsequent immune response [54, 56, 57].

Numerous interactions have been identified between macrophages, tick saliva or SGE, and pathogens, suggesting that they play a major role in host defenses against ticks and tick-borne infectious agents. The effects of saliva or SGE on macrophages are summarized in Figure 1. *I. ricinus* SGE inhibited superoxide and nitric oxide (NO) production by *Borrelia afzelii*-activated macrophages, which led to the inhibition *of Borrelia* killing in a murine host [58]. *I. ricinus* SGE also reduced phagocytosis of *B. afzelii* spirochetes by murine macrophages and inhibited IFN-γ- and *B. afzelii*-stimulated TNF production by macrophages [59]. It was recently shown that *I. ricinus* saliva could induce the production of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2) in splenocytes [60]. MCP-1 attracts monocytes, and MIP-2 is a chemokine secreted by monocytes and macrophages that is chemotactic for neutrophils.

Similarly, *I. scapularis* saliva inhibited TNF, IL-1β, IL-6, and IL-12p40 production by murine bone marrow-derived macrophages (BMDMs) after stimulation with lipopolysaccharide (LPS) or *A. phagocytophilum*. It was further reported to inhibit IL-8 secretion by human peripheral blood mononuclear cells (PBMCs) after TNF stimulation [61] and NO synthesis upon LPS stimulation [62].

Incubation with SGE isolated from *R. microplus*, a tick of veterinary importance, resulted in diminished expression of the co-stimulatory molecules CD80, CD86, CD40, and CD69 on the surface of bovine macrophages after 24 hours of LPS stimulation, which was accompanied by a decrease in TNF, IFN-γ, and IL-12 production [63]. Conversely, CD86 expression was increased in the murine macrophage cell line RAW 264.7 in response to *R. microplus* SGE and LPS but not SGE alone. Furthermore, SGE had no effect on CD40 and CD80 expression [64]. However, both bovine primary macrophages and murine macrophage cell line displayed an increase of CD86 expression after 6 hours incubation with LPS and SGE. [64]. These partially contradictory observations may be attributed to the host specific response. The difference may origin also from altered signaling in immortalized cell line, as CD86 upregulation was shown to be at least partially dependent on the ERK1/2 pathway and may, therefore, promote polarization of the immune response towards a less pro-inflammatory Th2 profile (see below) [64]. In another study, *R. sanguineus* saliva diminished NO production by IFN-γ-activated macrophages and thus impaired *Trypanosoma cruzi* killing. The authors suggested that decreased NO production was due to a saliva-induced cytokine imbalance, leading to decreased NO synthase activity [65]. Similar to the results with primary macrophages, SGE from *Rhipicephalus appendiculatus*

affected cytokine production by the murine macrophage cell line JA-4. SGE from *R. appendiculatus* inhibited the transcription of IL-1α, IL-10, and TNF after macrophage stimulation with LPS. NO production was also lower, in accordance with the similar effect observed with *I. ricinus* saliva [58, 66].

Dermatocentor variabilis saliva has been shown to impair phagocytosis and alter gene expression in the murine macrophage cell line IC-21, as well as increase basal and platelet-derived growth factor (PDGF)-stimulated macrophage migration and the expression of the Th2-specific cytokines IL-4 and IL-10 [67].

The tick salivary component prostaglandin E_2 (PGE_2) subverted macrophage secretion of proinflammatory mediators and was able to recruit fibroblasts to heal tick-bite wound [68]. In addition to PGE_2 from tick saliva, the saliva of *D. variabilis* upregulated PGE_2 secretion in IC-21 murine peritoneal macrophages and reduced secretion of the pro-inflammatory mediators CCL5, TNF, and soluble TNF receptor I (sTNFRI) via a PGE_2-dependent mechanism mediated by cAMP [68].

In summary, the tick saliva of various tick species inhibits the pro-inflammatory activities of macrophages, supporting a major role for macrophages in anti-tick defenses.

Dendritic cells and tick saliva

Dendritic cells (DCs) are APCs and are part of the innate immune system. After immature (unstimulated) DCs recognize and phagocytose pathogens, they mature and migrate to draining lymph nodes where they present antigens derived from the processed pathogen to CD4+ T cells, which subsequently launch an adaptive immune response. Thus, DCs initiate host adaptive immunity via presentation of pathogenic antigens. Two DC states exist: an immature form present in skin or mucosae and a mature form in lymphoid tissues. Langerhans cells (LCs) are a specialized resident cell type found in the vertebrate skin. Similar to macrophages, LCs have two origins and share many properties with macrophages [69]; therefore, they are sometimes considered to be a subtype of tissue macrophage [57]. Immature DCs primarily have an antigen uptake and presenting function, while mature DCs effectively stimulate T cells but have limited phagocytic activity. Several studies suggest that there are interactions between tick saliva and DCs [70–72]. For a review of the interactions between DCs, tick saliva, *and Borrelia*, see [73].

Oliveira and colleagues studied the effect of *R. saguineus* saliva on DC migration and function, and found that tick saliva reduced immature DC migration toward macrophage inflammatory proteins MIP-1α and MIP-1β but not MIP-3β [74]. Tick saliva also inhibited the chemokine RANTES by reducing expression of its surface receptor CCR5 [74]. DC maturation was impaired via toll-like receptor (TLR) signaling [75]. However, the inhibition of migration was limited to immature DCs. DC maturation and differentiation was inhibited in the presence of *A. cajennese* saliva [76]; in this study, the DCs showed reduced expression of CCR5 and CCR7 and, therefore, diminished migration toward the corresponding chemokines. Furthermore, tick saliva polarized cytokine production toward a Th2 phenotype. The authors suggested that most of the observed effects were due to the presence of PGE_2 in tick saliva [76]. *I. scapularis* saliva has displayed various effects on

bone marrow-derived DCs: it inhibited TNF and IL-12 production upon stimulation of different TLRs, in particular TLR-2, TLR-4, or TLR-9 [77], and the DC's ability to stimulate antigen-specific CD4+ proliferation and IL-2 production was also suppressed [77]. LC-deficient mice induced Th1 responses after *I. scapularis* infestation, demonstrating the requirement for LCs in attenuating tick-mediated Th1 responses in regional lymph nodes [78].

CD40 or TLR3, 7, and 9 ligation impaired DC maturation, and *I. ricinus* saliva inhibited DC migration *in vivo* and antigen presentation [79]. *I. ricinus* saliva has also been shown to impair Th1 and Th17 polarization in DCs [79] and activation of specific CD4+ T lymphocyte subsets by *Borrelia*-exposed DCs [80]. In the latter study, *I. ricinus* saliva decreased DC phagocytosis of *B. afzelii*. Interestingly, *I. ricinus* saliva inhibited DC production of both Th1 cytokines (TNF and IL-6) and the Th2 cytokine IL-10 after 48 hours (but not 24 hours) of incubation with *B. afzelii* [80]. *I. ricinus* saliva also impaired DC maturation and production of TNF and IL-6 in response to infection with TBE virus [81]. Lieskovská and Kopecký studied the signaling pathways activated in DCs via TLR-2 ligand and *B. afzelii* in the presence of tick saliva [82]; upon both types of activation, the NF-κB and phosphatidylinositol-3-kinase (PI3K)/Akt pathways were inhibited by *I. ricinus* saliva. When activated by *Borrelia* spirochetes, TNF levels decreased in DCs due to selective suppression of ERK1/2, Akt, and NF-κB as a result of tick saliva mimicking the native inhibitors. Tick saliva also attenuated IFN-β production, and IFN-β triggered signal transducer and activator of transcription-1 (STAT-1) activation [83]. A summary of the known interactions between DCs and tick saliva is shown in Figure 2.

Mast cells and tick saliva

Mast cells serve as sentinel cells and reside in many tissues. They are divided into two main types based on the presence of mast cell-specific proteases: connective tissue mast cells, which produce both tryptase and chymase (MC_{TC}), and mucosal mast cells, which produce only tryptase (MC_T) [84]; skin mast cells are of the first type. Upon exposure to pathogens or other stimuli, activated mast cells degranulate and release a variety of pre-stored mediators including vasoactive compounds, serine proteases, histamine, and cytokines. Activated mast cells also secrete newly synthesized mediators to recruit more inflammatory cells [85].

The immunological importance of mast cells in tick-host interactions remains unclear. Mast cell numbers increase after secondary or subsequent tick infestations, but remain unchanged during primary tick infestations [86–88]. The number of degranulated mast cells is also significantly higher after repeated tick infestations. Mast cell-deficient mice have been shown to develop some resistance to *D. variabillis* after repeated exposure, similar to wild type mice [89]. On the other hand, mast cell-deficient mice were not resistant to *Haemaphysalis longicornis*, with tick resistance re-established after mast cell injection [90, 91]. Such differences might be due to species-specific host responses or other unknown factors. Highly tick-resistant zebuine cattle breeds have more dermal mast cells than taurine breeds [92]. F2 crossbreeds of these two cattle were resistant to *R. microplus* infestation, with feeding with *R. microplus* larvae inducing significant increases in dermal mast cell

numbers. Mast cells are major producers of the inflammatory mediator histamine, and ticks can affect histamine actions by either binding histamine via histamine-binding lipocalins [93, 94] or by promoting its secretion via histamine release factor [95], further evidence of the ambiguous role for mast cells in tick feeding responses. One explanation for histamine binding followed by its release can be explained by the need to suppress inflammatory responses at the early stage of feeding, followed by an increased need for vascular permeability during the rapid engorgement phase of tick feeding.

Granulocytes and tick saliva

Granulocytes are bone marrow-derived myeloid leukocytes that contain granules in their cytoplasm. The granulocyte group consists of three major cell types: basophils, eosinophils, and neutrophils [96].

Basophils and tick saliva

Basophils are IgE-activated granulocytes that, unlike tissue-resident mast cells, circulate in the blood. They play a critical role in the IgE-mediated development of chronic allergic reactions and inflammation [97, 98], and they can also promote polarization towards Th2 responses by IgE-independent antigen presentation in mice [99, 100]. Basophils are recruited to a tick-feeding site and accumulate in the host skin during second and consequent (but not primary) tick infestation, where they act as important tick rejection factors [101, 102]. After migration to the site of injury, basophils degranulate and release mediators such as histamine to reject ticks in a host reaction known as cutaneous basophil hypersensitivity [103]. Similar to mast cells, histamine release from basophils can be mediated by tick histamine release factor binding [95]. Several studies have confirmed the role of basophils in acquired immunity against ticks in mice [102, 104, 105]. Basophils expressing the immunoglobulin Fc receptor were found to be responsible for antibody-mediated acquisition of *H. longicornis* resistance [102], with selective basophil ablation by diphtheria toxin leading to loss of resistance to *H. longicornis* feeding in subsequent tick infestations [102]. Basophils appear to play a non-redundant role in antibody-mediated acquired immunity against ticks [102].

As noted above, *I. ricinus* saliva increased MCP-1 production by stimulated splenocytes [60]. MCP-1 is a potent basophil activator that triggers their degranulation and histamine release [106].

Basophils can cause cutaneous basophilia, a mechanism of tick resistance [104, 105]. The susceptibility or resistance of cattle to ticks (*R. microplus*) was associated with the number of basophils at the feeding site, with skin biopsies from tick-resistant breeds contain significantly more basophils than biopsies from susceptible breeds [107–109].

Eosinophils and tick saliva

Eosinophils are mainly present in mucosal areas in contact with the external environment such as the gut or lung mucosae. Their circulating levels are relatively low in healthy organisms, but increase during allergic reactions or parasitic infections [46]. Eosinophils

produce cytokines, chemokines, and other mediators, some of which (e.g., indoleamine 2,3 deoxygenase; IDO) induce apoptosis and inhibit T cell proliferation [110, 111]. Eosinophils are also rich in granules that contain cytotoxic effectors such as eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin, or major basic protein, which can cause mast cell (and probably also basophil) degranulation [112]. Finally, eosinophils are an important source of inflammatory and tissue repair-related molecules such as the transforming growth factors TGF-α and TGF-β1 and the extracellular matrix glycoprotein tenascin [113, 114].

Repeated exposure to both soft and hard tick species raised eosinophil levels at the feeding site in many host species including cattle [115, 116], dogs [117], guinea pigs [118, 119], rabbits [86], mice [88], woolless lambs [120], rats [36], capybaras [121], and even anteaters and armadillos [122]. The relationship between eosinophil number and tick resistance is not clear. Similar to mast cells, the susceptibility or resistance to ticks in cattle was associated with the number of eosinophils (and basophils) at the feeding site. Cattle breeds with more eosinophils (*Bos taurus indicus*, Nelores breed) appeared to be more resistant to *R. microplus* feeding than the *Bos taurus taurus* Holstein breed with fewer eosinophils [107]. In contrast, the tick count on Nguni and Bonsmara cattle was positively correlated with the eosinophil count in skin biopsies from tick feeding sites, while the correlation was negative in the case of mast cells and basophils [109].

Ticks inhibit the chemokine-mediated attraction of eosinophils to tick feeding sites. SGE from many tick species blocked CCL3, CCL5, or CCL11 (eotaxin) eosinophil chemoattractant activity [123–126].

Neutrophils and tick saliva

Neutrophils are granulocytes with both phagocytosis and degranulation roles. They are highly motile cells and they have a relatively short lifespan. Neutrophils play an important role in the early stages of vertebrate immune homeostasis, such as during acute inflammation, but they also play a role in some chronic inflammatory diseases. Neutrophils are generally activated by pathogens and secrete effectors and mediators that promote inflammation by recruiting other leukocytes, and they also directly kill pathogens by releasing their granules [46, 127]. They can also phagocytose and kill pathogens intracellularly [127].

Tick saliva modulates a local cutaneous immune response at the tick feeding site almost immediately after tick attachment, as shown by gene expression analysis of skin biopsies taken at several time points after the initiation of *I. scapularis* nymph feeding [128]. The expression of neutrophil-specific chemokines (CXCL1 and 5) was induced as early as 12 hours after tick attachment to the host [128]. Neutrophil abundance in the skin was high during the first tick infestation compared to other cell types but decreased during subsequent tick infestations of the same host [120, 129]. It is unknown whether the absence of neutrophils affects resistance of the host to ticks.

Saliva or SGE from soft and hard ticks have been shown to attenuate neutrophil functions such as recruitment by interfering with the neutrophil chemoattractants CXCL8 (IL-8) or

CCL3 [123, 124, 126, 130, 131]. In one study, *I. ricinus* saliva significantly decreased neutrophil reactive oxygen species (ROS) production [132]. In contrast, the formation of neutrophil extracellular traps (NETs), which are formed by extrusion of neutrophil DNA and can retain and kill bacteria, was not affected by saliva [132]. *I. scapularis* (published as *Ixodes dammini*) saliva inhibited granule release and neutrophil infiltration and had an inhibitory effect on neutrophil phagocytosis of *B. burgdorferi* [133]. *I. scapularis* saliva also reduced polymorphonuclear leukocyte (PMN) adhesion by downregulating β2-integrin expression and signaling, which decreased pro-inflammatory functions of PMNs [134]. Finally, SGE from *R. microplus* inhibited neutrophil phagocytic activity in cattle [135]. These data show that tick saliva inhibits several pro-inflammatory neutrophil properties that are deleterious to tick feeding but does not affect anti-bacterial NET formation, suggesting that tick salivary activity is specific. The effects of tick saliva and SGE on neutrophils are illustrated in Figure 3.

T and B lymphocytes and tick saliva

Adaptive immunity relies on a wide range of antigen receptors (with varying antigen recognition specificities), which are clonally distributed in two types of lymphocytes: T cells and B cells. The induction of a specific immune response is only possible when a foreign antigen is recognized by the corresponding receptor. This first recognition signal is consolidated by the interaction of co-stimulatory molecules on T or B cells with those on APCs - such as dendritic cells or macrophages – that belong to the innate immune system. In this way, links are made between the cell populations that play dominant roles in the two branches of vertebrate immunity [136].

T cells are produced in the bone marrow from lymphoid progenitors and differentiate in the thymus. Mature T cells then migrate to the peripheral lymphoid tissues; they also circulate throughout the body [46]. Two major T cell subpopulations are recognized based on the co-receptor molecule expressed at the cell surface: CD4+ (T helper cells) and CD8+ T cells (which develop into cytotoxic T lymphocytes, CTLs). According to the secreted cytokine profile, CD4+ T helper cells can be further divided into several subpopulations that have different roles in immune responses [137], with Th1 and Th2 populations the most thoroughly studied in tick-host interactions thus far. Th1 populations are associated with host cellular and inflammatory responses, and Th2 populations with host humoral responses against ticks [138, 139]. Figure 4 illustrates how tick saliva and SGE influence T and B cell functions.

In 1985, *I. scapularis (dammini)* tick saliva was shown to inhibit IL-2 production by T lymphocytes, with PGE$_2$ proposed to be responsible for this effect [140]. Urioste and colleagues confirmed diminished IL-2 levels in the presence of *I. scapularis* saliva, and showed profoundly inhibited splenic T cell proliferation in response to stimulation with concanavalin A (ConA) or phytohemagglutinin in the presence of saliva [62]; however, they disproved the PGE$_2$ hypothesis, providing evidence that IL-2 is in fact inhibited by a proteinaceous salivary component. Later, in 2001, an unknown salivary component from *I. scapularis* was reported to bind IL-2 and inhibit T lymphocyte proliferation [141].

The inhibition of lymphocyte proliferation by SGE has also been reported in other tick species such as *I. ricinus*, *Amblyomma variegatum*, and *R. appendiculatus*, with species- and sex-specific differences shown for the effects of tick salivary gland antigens on human lymphocyte proliferation [142]. *I. ricinus* SGE suppressed isolated B cell proliferation and IL-10 production in response to LPS. CD69 activation marker expression on both activated T and B cells was also reduced [143]. *I. ricinus* saliva inhibited splenic T cell proliferation in response to ConA, and both SGE and saliva reduced the responsiveness of T cells draining to lymph nodes and sensitized splenic T cells [144]. The same observation was made with naïve splenic T cells [145]. T lymphocytes from mice infested nine days previously with *I. ricinus* nymphs displayed suppressed responses to ConA stimulation compared to cells from naïve mice [145]. In contrast, the lymph node cell response to LPS was increased in infested mice compared to naïve mice [145]. The authors attributed the observed effect to increased B lymphocyte numbers or activity [145]. On the other hand, soluble salivary gland antigens derived from female *I. ricinus* ticks stimulated lymph node T cells from mice infested with *I. ricinus* larvae or nymphs, but not those infested with *Amblyomma hebraeum* nymphs [146]. A 65 kDa protein fraction (IrSG65) isolated from the salivary glands of partially fed *I. ricinus* females induced specific T cell proliferation in lymph node cells obtained from mice infested with *I. ricinus* nymphs [146]. Feeding of *I. ricinus* nymphs on BALB/c mice revealed that CD4+ T cells were more abundant than CD8+ cells [147], which changed from 2:1 upon primary tick infestation to 7:1 in tertiary tick infestation. The ratio of CD3+ and CD4+ T lymphocytes was identical in *I. ricinus* infested and control mice [148].

D. andersoni SGE reduced ConA-induced proliferation of T cells [149, 150]. *R. microplus* feeding on cattle decreased the T lymphocyte percentage in peripheral blood lymphocytes (PBLs) [151], with the B lymphocyte percentage only lowering after repeated heavy infestations [151]. *R. microplus* saliva also suppressed PBL responsiveness to phytohemagglutinin [151]. *R. sanguineus* feeding on dogs suppressed ConA, phytohemagglutinin, and pokeweed mitogen-induced lymphocyte responses [152]. In the same study, SGE also suppressed all mitogen-stimulated blastogenic responses of lymphocytes from healthy dogs *in vitro*. Feeding of the *Haemaphysalis bispinosa* and *Hyalomma anatolicum anatolicum* ticks on sheep resulted in reduced circulating T lymphocyte counts as tick feeding progressed [153]. The authors showed that depletion of CD8+ populations and increased CD4+ T cell levels accounted for the observed effects [153]. Feeding of these two ticks also suppressed *in vitro* proliferation of T cells isolated from the tick-infested animals [153]. The CD4+/CD8+ and B/T lymphocyte ratios were increased in all sheep during infestation with either *H. bispinosa* and *H. anatolicum anatolicum* [153]. Interestingly, reduced CD4/CD8 T cell ratios were observed in skin biopsies taken at primary and secondary infestation with *H. anatolicum anatolicum* ticks on sheep compared to healthy skin biopsies [154].

B cells also originate from lymphoid progenitors in the bone marrow [46]. Their further differentiation involves migration from the bloodstream to the spleen, where they develop into mature B cells. Mature B cells circulate between the spleen and lymph nodes. The role of B cells lies in the surface expression and secretion of immunoglobulins upon activation

[155]. In immunity against ticks, B cells produce specific antibodies against tick salivary and gut antigens.

Both primary and secondary infestations of sheep with *H. anatolicum anatolicum* ticks caused a significant increase in circulating B lymphocytes over several days [153]. In dogs, *R. sanguineus* SGE was shown to suppress total immunoglobulin and IgA (but not IgM) production by PBLs *in vitro* upon activation with LPS or pokeweed mitogen [156]. It has also been observed that anti-BSA IgG and IgM levels decreased in mice immunized with BSA during *I. ricinus* feeding [148]. However, anti-BSA IgG and IgM production was not decreased when BSA was injected prior to tick infestation. Interestingly, this study did not demonstrate a shift towards the Th2-type immune response when anti-BSA IgG1 and IgG2a antibody levels were compared between mice groups [148]. It was later shown that total IgG and IgM antibody levels were not reduced in animal sera by tick infestation, anti-BSA antibody production was not delayed, and memory cell formation did not appear to be inhibited by tick saliva [157]. Tick saliva did not affect memory B cell production of either anti-BSA IgG or IgM [157].

Experiments with tick saliva or SGE have shown polarization of the immune response from Th1 to Th2 branches by suppression of Th1 and upregulation of Th2 cytokines in both mice and humans. This polarization leads to an attenuated inflammatory response, which is beneficial for tick survival and feeding [15, 158]. Briefly, saliva or SGE inhibited secretion of IL-2, IL-12, TNF, and IFN-γ. In contrast, IL-4, IL-6, and IL-10 secretion was stimulated [66, 139, 159]. IL-10-specific neutralizing antibodies abrogated the suppressive effect of *I. ricinus* SGE on IFN-γ production [160]. IL-1α secretion was inhibited in JA-4 macrophage cells exposed to *R. appendiculatus* SGE [66]. In contrast, and in spite of their pro-inflammatory properties, IL-1α and IL-1β production was increased by Th1 lymphocytes and splenocytes after treatment with *I. ricinus* SGE [161, 162]. This can be explained by the fact that IL-1 can also act as a co-stimulator for Th2 lymphocyte proliferation. One of the mechanisms described for the action of *I. ricinus* saliva involves a negative effect on DCs, which then prime naive CD4+ T cells to induce Th2 cell differentiation *in vitro* and *in vivo* [71].

Feeding of *D. andersoni* decreased expression of two integrins, leukocyte function-associated antigen-1 (LFA-1) and very late activation-4 (VLA-4), by lymphocytes [163]. The same effect was achieved by exposing tick-naïve mouse lymphocytes to both *D. andersoni* saliva and SGE [163]. Infestation with *D. andersoni* nymphs or intradermal administration of female or male tick SGE increased IL-4 and IL-10 transcript levels in the draining lymph nodes and skin of the host [164]. Intracellular IL-4 levels were significantly increased in CD4+ T cells [164], and increased IL-4 levels were also observed during *I. scapularis* nymph feeding or by intradermal application of SGE [165]. Primary *I. scapularis* infestation on mice was characterized by late induction of an innate immune response and by inhibition of proinflammatory Th17 immunity. During secondary tick infestation, a mixed Th1/Th2 response was elicited [35].

Ticks have evolved various ways to circumvent adaptive immunity. Their saliva inhibits lymphocyte proliferation to reduce immune responses. Furthermore, ticks actively direct the

immune response towards the Th2 arm that favors their feeding. The immunosuppressive properties of tick saliva also include inhibition of antibody production by B cells that could damage tick mouthparts and activate other cells or complement. The effects of tick saliva and SGE on lymphocytes are illustrated in Figure 4.

Natural killer cells and tick saliva

Despite their lymphoid origin, natural killer (NK) cells are part of the innate immune system [46]. Their main function is microbial or tumor cell killing and the regulation of endothelial cell, dendritic cell, and macrophage interactions with T lymphocytes [166]. SGE from female *Dermatocentor reticulatus* ticks that fed for 3–6 days on mice decreased human NK cell activity, while SGE from unfed or one day-fed ticks had no effect. Weaker activity was reported for SGE from *A. variegatum* and *Haemaphysalis inermis* ticks [167, 168], and NK cell cytotoxicity was suppressed after treatment with *I. ricinus* SGE [169].

Conclusions

Tick saliva clearly contains numerous different pharmacologically-active molecules that affect various immune cell populations and facilitate tick feeding. In this "systems biology" era, the effects of tick saliva described in this review can help in the design of experiments to discover specific salivary molecules that account for those effects. Although molecular biology and biochemical methods such as transcriptome and proteome analyses have provided excellent information about the genes expressed in the salivary glands of different tick species, the number of identified and functionally characterized salivary molecules remains limited. Ultimately, the goal is to fully uncover the complexity of how ticks modulate the host immune system so that this information can be used to pioneer the development of novel control strategies for ticks and tick-borne diseases and aid drug discovery.

Acknowledgements

List of abbreviations

Akt	protein kinase B
BMDMs	bone marrow-derived macrophages
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCL	chemokine (C-C motif) ligand
CCR	C-C motif receptor
CD	cluster of differentiation
ConA	concanavalin A
CTL	cytotoxic T lymphocytes
CXCL	chemokine (C-X-C motif) ligand
DC	dendritic cell
ERK	extracellular signal-regulated kinase
IDO	indoleamine 2,3 deoxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRAK	interleukin-1 receptor-associated kinase
LC	Langerhans cell
LFA-1	leukocyte function-associated antigen-1
LPS	lipopolysaccharide
MC	mast cell
MCP	monocyte chemotactic protein
MIP	macrophage inflammatory protein
NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa light chain-enhancer of activated B cells
NK	natural killer
NO	nitric oxide
PBL	peripheral blood leukocytes
PGE$_2$	prostaglandin E$_2$
PI3k	phosphatidylinositol-3 kinase
PMNs	polymorphonuclear lymphocytes

RANTES	regulated upon activation, normal T cell expressed and secreted
ROS	reactive oxygen species
SGE	salivary gland extract
STAT	signal transducer and activator of transcription
sTNFRI	soluble TNF receptor I
TGF	transforming growth factor
Th	helper T cell
TLR	toll-like receptor
TNF	tumor necrosis factor
VLA-4	very late activation-4

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Significance

We overview all the known interactions of tick saliva with the vertebrate immune system. The provided information is important, given the recent developments in high-throughput transcriptomic and proteomic analysis of gene expression in tick salivary glands, since it may serve as a guideline for the functional characterization of the numerous newly-discovered genes expressed in tick salivary glands.

Highlights

- Tick salivary secretion is important in the interaction of ticks with the vertebrate hosts and the pathogens they transmit.

- Recent Systems Biology approaches have characterized massively gene expression in tick salivary glands.

- An important function of tick salivary secretion is to modulate vertebrate innate and adaptive immunity.

- We overview the available literature about the immunomodulatory properties of tick saliva.

- Our review may serve as a guideline for the discovery of the genes that mediate the specific function of tick saliva.
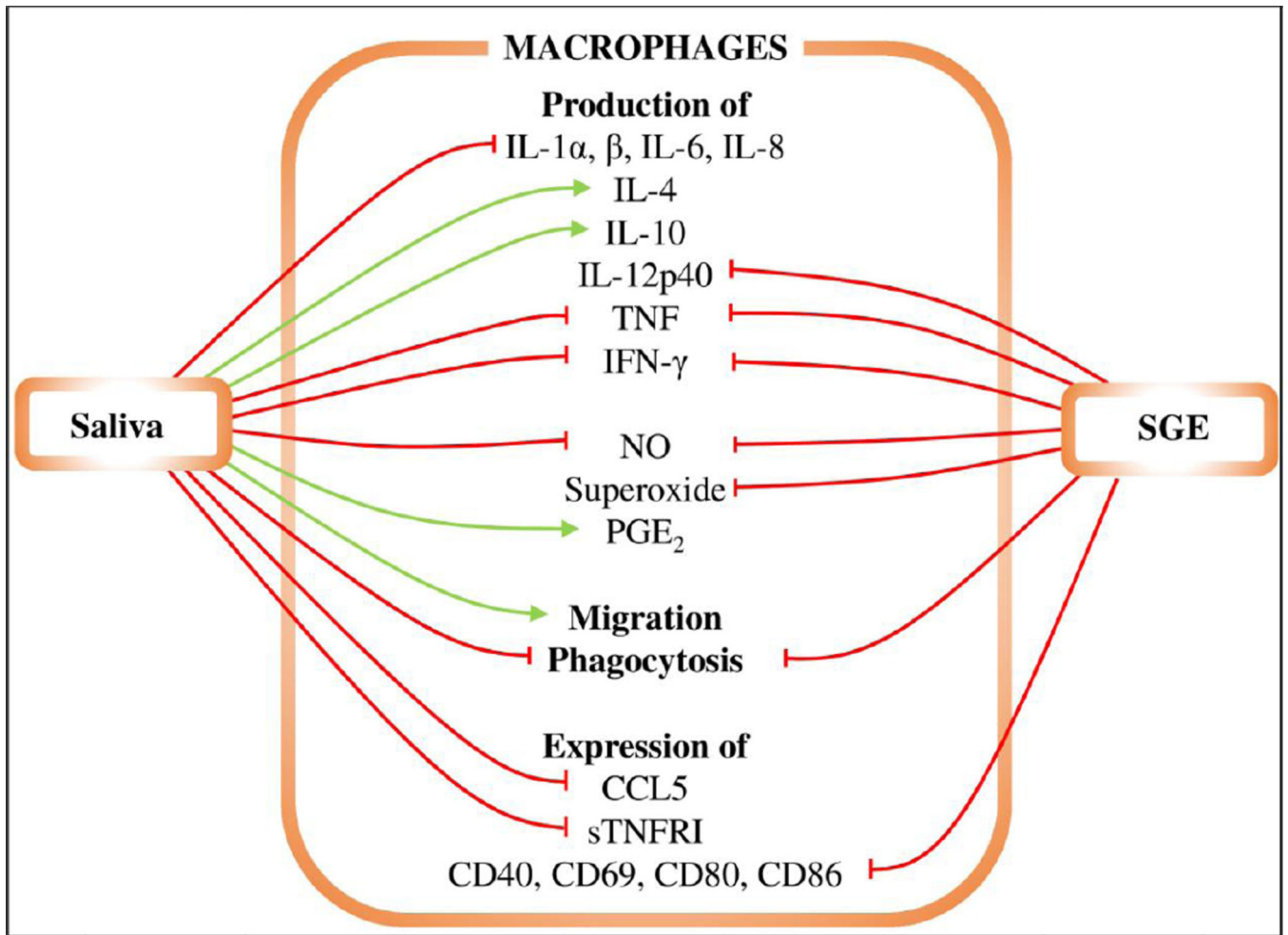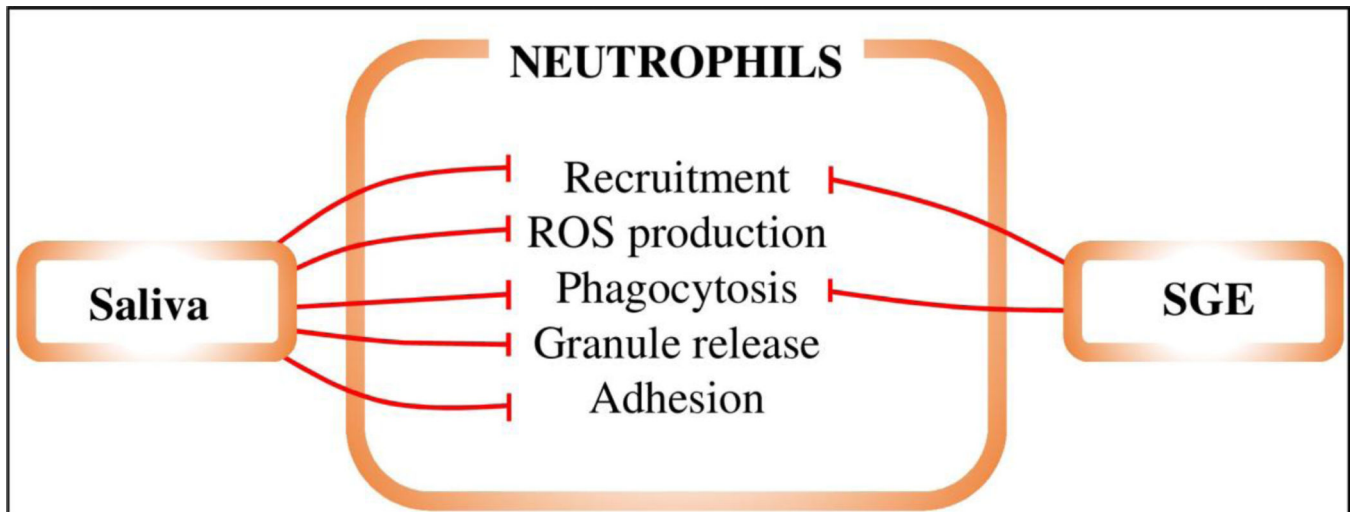
Figure 1. The effects of saliva and SGE on macrophages

Red lines represent inhibition, green lines enhancement. Tick saliva inhibits production of IL-1α, IL-1β, IL-6, IL-8, TNF, IFN-γ, NO, superoxide, and CCL5, as well as expression of sTNFRI and phagocytosis. Tick saliva increases production of IL-4, IL-10, and PGE_2 and macrophage migration. Tick SGE inhibits production of IL-12p40, TNF, IFN-γ, and NO, expression of CD40, CD69, CD80, and CD86, and phagocytosis.
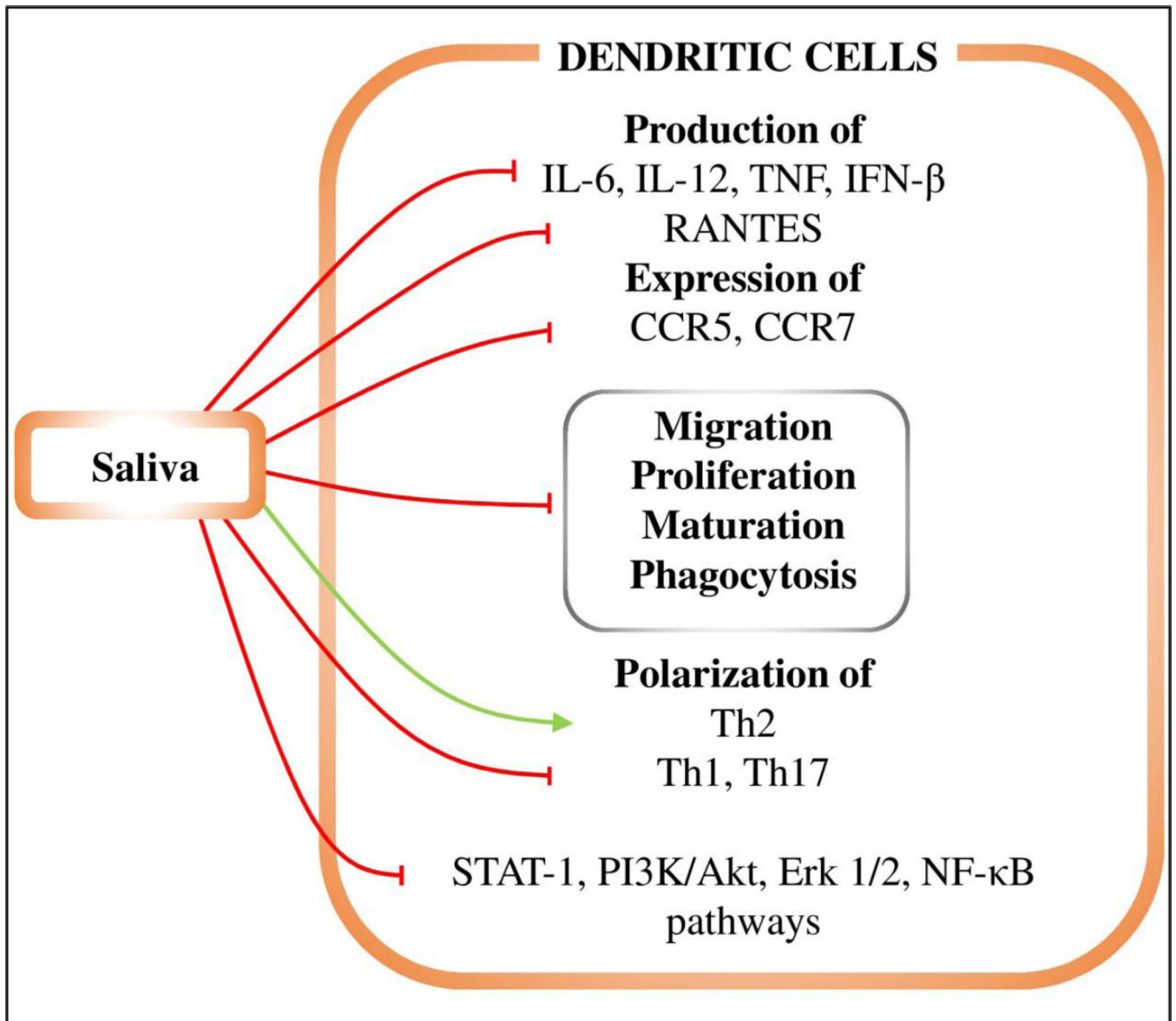
Figure 2. The effects of saliva on dendritic cells
Red lines represent inhibition, green lines enhancement. Tick saliva inhibits production of
IL-6, IL-12, TNF, IFN-β, and RANTES cytokines. It also inhibits expression of CCR5 and
CCR7, DC migration, proliferation, maturation, and phagocytosis, and STAT-1, PI3K/Akt,
Erk1/2, and NF-κB signaling pathways. The saliva induces Th2 polarization while
suppressing Th1 and Th17 differentiation.

Figure 3. The effects of saliva and SGE on neutrophils
Red lines represent inhibition, green lines enhancement. Tick saliva inhibits neutrophil recruitment, phagocytosis, adhesion, granule release, and production of ROS.
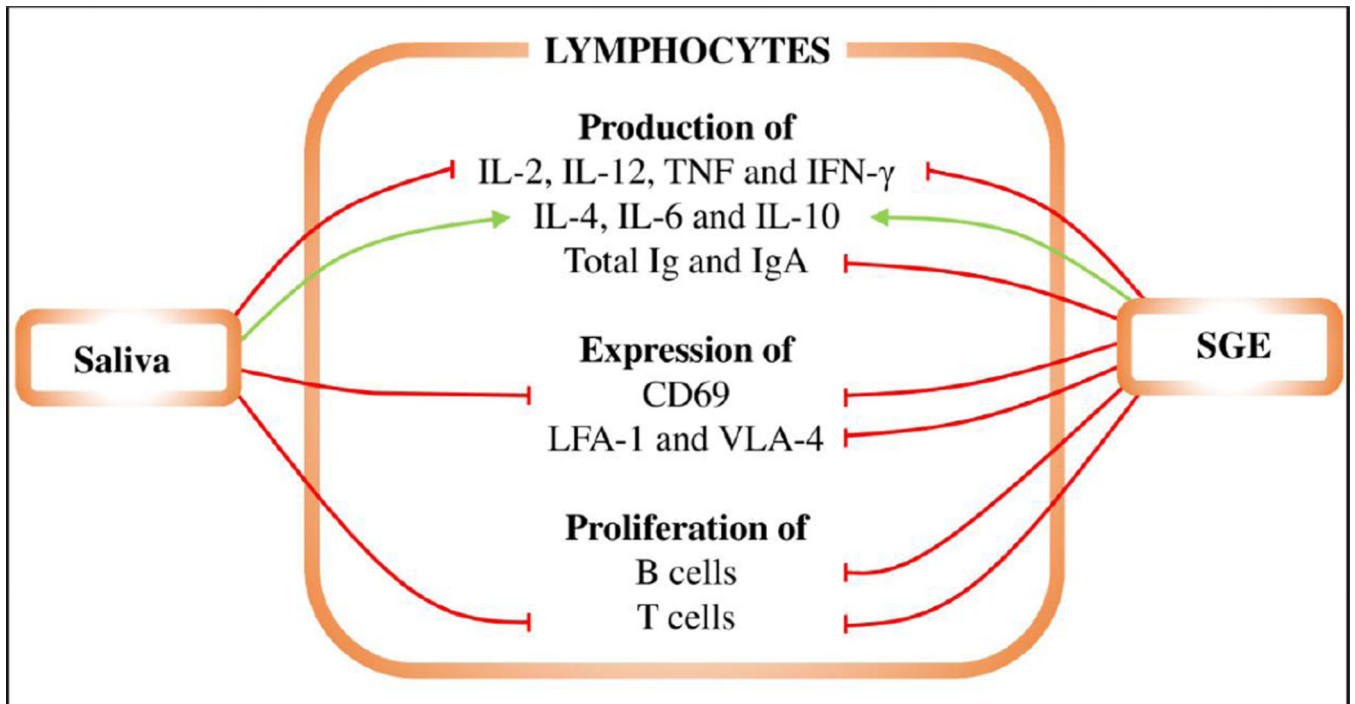
Figure 4. The effects of saliva and SGE on B and T lymphocytes
Red lines represent inhibition, green lines enhancement. Tick saliva inhibits T cell proliferation, CD69 expression, and production of IL-2, IL-12, TNF, and IFN-γ by lymphocytes. In contrast, it increases production of IL-4, IL-6, and IL-10. Tick SGE has the same effects as tick saliva and, furthermore, suppresses LFA-1 and VLA-4 expression, proliferation of B cells, and total Ig and IgA production.

Table 1

The effects of tick saliva, SGE, or feeding on immune cell populations.

Macrophages			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
Dermatocentor variabilis	saliva	impaired phagocytosis and altered gene expression, stimulation of migration	[67]
		stimulation of PGE_2 production, inhibition of cytokine production	[68]
Ixodes ricinus	SGE	inhibition of superoxide and NO production	[58]
		inhibition of phagocytosis and TNF production	[59]
Ixodes scapularis	saliva	inhibition of cytokine production	[61]
		inhibition of NO production	[62]
Rhipicephalus appendiculatus	SGE	inhibition of cytokine and NO production	[66]
Rhipicephalus microplus	SGE	altered surface molecule expression, inhibition of cytokine production	[63, 64]
Rhipicephalus sanguineus	saliva	inhibition of NO production	[65]
Dendritic cells			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
Amblyomma cajennese	saliva	inhibited maturation and differentiation; reduced migration due to decreased expression of receptors; polarization towards Th2 cytokines	[76]
Ixodes ricinus	saliva	inhibited maturation, migration and antigen presentation; blocked Th1 and Th17 polarization	[79]
		inhibited proliferation, phagocytosis and cytokine production	[80]
		impaired maturation and cytokine production	[81]
		inhibition of signaling pathways	[82, 83]
Ixodes scapularis	saliva	inhibition of proliferation and cytokine production	[77]
Rhipicephalus sanguineus	saliva	reduced migration, maturation and cytokine production	[74, 75]
Basophils			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
Amblyomma cajennense	feeding	increased amount of basophils in feeding cavity	[121]
Amblyomma dubitatu	feeding	increased amount of basophils in feeding cavity	[121]
Eosinophils			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
soft and hard ticks	feeding	increased amount of eosinophils in feeding cavity	[36, 88, 120–122]
hard ticks	SGE	inhibition of attraction to the feeding site	[123, 124]

Macrophages			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
Ixodes ricinus	saliva	basophil activation via MCP-1 released from splenocytes	[60]
Neutrophils			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
soft and hard ticks	SGE	anti-IL-8 activity	[123, 130]
Amblyomma americanum	SGE	altered dynamics of chemokine activity	[125]
Ixodes ricinus	saliva	decrease in ROS production	[132]
Ixodes scapularis	saliva	inhibition of granule release, infiltration, phagocytosis	[133]
		reduced adhesion of polymorphonuclear leukocytes	[134]
Rhipicephalus appendiculatus	SGE	altered cytokines mRNA production by peripheral blood leukocytes	[170]
Rhipicephalus microplus	SGE	inhibition of phagocytosis	[135]
Lymphocytes			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
soft and hard ticks	saliva, SGE	polarization of the immune response towards Th2 via cytokines	[66, 71, 139, 159, 161, 162, 171, 172]
Amblyomma variegatum	SGE	inhibition of lymphocyte proliferation	[142]
Dermacentor andersoni	SGE	reduced T cells proliferation	[149, 150]
		reduced Th1 cytokine production	[173, 174]
	saliva, SGE, feeding	inhibition of integrin expression	[163]
	SGE, feeding	increased IL-4 and IL-10 levels	[164]
Haemaphysalis bispinosa	feeding	reduction in T lymphocyte count and proliferation, increased CD4+/CD8+ ratio	[153]
Hyalomma anatolicum anatolicum	feeding	reduction in T lymphocyte count and proliferation, increased CD4+/CD8+ ratio, increase in circulating B lymphocyte count	[153]
Ixodes ricinus	SGE	inhibition of lymphocyte proliferation	[142]
		suppression of B cell proliferation, inhibition of IL-10 production, reduction of markers on the surface of T and B cells	[143]
	saliva	inhibition of T cell proliferation	[144]
		induction of Th2 differentiation of CD4+ T cells via dendritic cells	[71]
	feeding	increased CD4+/CD8+ ratio	[147]
		inhibited proliferation and responsiveness	[145]
		reduced amount of specific Ig against antigen, no change in total Ig amount	[148, 157]
Ixodes scapularis	saliva	inhibition of IL-2 production by T cells, inhibition of splenic T cell proliferation	[62, 140, 141]
	feeding	inhibition of Th17 immunity, priming of a mixed Th1/Th2 response during secondary infestation	[35]

Macrophages			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
	SGE, feeding	increased IL-4 levels	[165]
Rhipicephalus appendiculatus	SGE	inhibition of lymphocyte proliferation	[142]
Rhipicephalus microplus	feeding	decreased T and B lymphocyte percentage among PBLs	[151]
	saliva	decreased PBL responsiveness to phytohemagglutinin	[151]
		inhibition of the blastogenic response of mononuclear cells	[175]
Rhipicephalus sanguineus	feeding	suppressed response to mitogens	[152]
	saliva	suppressed response to mitogens	[152]
	SGE	suppressed Ig production by PBL	[156]
NK cells			
Tick	**Saliva/SGE/feeding**	**Effect**	**Reference**
Amblyomma variegatum	SGE	decreased NK cell activity	[168]
Dermatocentor reticulatus	SGE	decreased NK cell activity	[167]
Haemaphysalis inermis	SGE	decreased NK cell activity	[168]
Ixodes ricinus	SGE	suppression of NK cell cytotoxicity	[169]