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Mosquito biting modulates skin response to virus infection

Marieke Pingen[1*], Michael A. Schmid[2*], Eva Harris[3], and Clive S. McKimmie[1✉]

[1] Virus Host Interaction Team, Section of Infection and Immunity, Leeds Institute of Cancer and Pathology, University of Leeds, Leeds LS9 7TF, UK

[2] Laboratory of Virology and Chemotherapy, Rega Institute for Medical Research, Department of Immunology and Microbiology, University of Leuven, Leuven, Belgium

[3] Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, Berkeley, California, United States of America

*Authors contributed equally

✉Correspondence: c.s.mckimmie@leeds.ac.uk (Clive S McKimmie)

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Abstract

Mosquito-borne infections are increasing in number and are spreading to new regions at an unprecedented rate. In particular, mosquito-transmitted viruses, such as those that cause Zika, dengue, West Nile encephalitis and chikungunya,, have become endemic or caused dramatic epidemics in many parts of the world. *Aedes* and *Culex* mosquitoes are the main culprits, spreading infection when they bite. Importantly, mosquitoes do not act as simple conduits that passively transfer virus from one individual to another. Instead, host responses to mosquito-derived factors have an important influence on infection and disease, aiding replication and dissemination within the host. Here, we discuss the latest research developments regarding this fascinating interplay between mosquito, virus and the mammalian host.

Mosquito-borne viruses constitute an increasing threat to human and animal health

Pathogens transmitted by vectors such as flies, snails, ticks and mosquitoes constitute a profound and growing health burden, causing more than 1 billion cases and 1 million deaths annually, according to the World Health Organisation (http://www.who.int/mediacentre/factsheets/fs387/en/). Increasing globalisation, migration and changing land use are allowing more opportunities for the spread of infections. In addition, a warming planet is enlarging the geographic range of endemic viruses and their vectors; including arboviruses, which are spread by arthropod vectors. Of concern, the frequency and magnitude of arboviral epidemics has increased in both established and new geographic areas. Globally, up to 400 million people are infected each year by dengue virus, and many millions more by arboviruses that cause epidemics of e.g. Zika, yellow fever and chikungunya [1-4], of which the day-biting *Aedes aegypti* mosquito is the primary vector. The economic burden of these diseases is enormous, with the global annual cost of dengue alone estimated at US$8.9 billion [5], while chikungunya is commonly associated with long-term detrimental sequelae, as reflected in disability adjusted life years [6]. The recent and continuing pandemic of Zika is particularly concerning due to its association with severe congenital birth defects following infection of pregnant women [7] and Guillain-Barré Syndrome in adults [8]. No effective antiviral treatments are available for arbovirus-associated diseases and only a few effective vaccines exist.

Arboviruses are genetically highly diverse and represent one of the largest virus groups, with more than 600 members, of which at least 80 are known human pathogens [9]. Most medically important arboviruses transmitted by mosquitoes are found in three distinct families; *Flaviviridae*, which includes dengue (DENV), Zika (ZIKV), yellow fever (YFV), and West Nile (WNV) viruses; *Togaviridae*, which includes chikungunya (CHIKV), Semliki Forest (SFV) and Venezuelan equine encephalitis (VEEV) viruses; and *Bunyaviridae*, which includes La Crosse virus. Depending on the virus, infection can result in a diverse range of severe manifestations that include arthritis, encephalitis, or vascular leakage leading to shock [10-12]. This heterogeneity, combined with our inability to accurately predict the nature and timing of future epidemics, makes developing and stockpiling virus-specific drugs and vaccines very challenging [13].

Despite their considerable diversity, arboviruses share a common attribute: transmission via the skin at the site of the arthropod bite. In the case of infected mosquitoes, virus is transmitted to the mammalian host as they probe the skin for a blood meal and deposit saliva [14,15]. Local virus replication in the skin represents a key stage of infection, which is followed by rapid dissemination to the blood and tissues remote from the bite. Importantly, mosquito-derived factors deposited at the bite site, and the resulting local host immune response, play an important role in determining the severity of viral infection [16-21].

This review describes the current state of knowledge regarding early cutaneous events during arbovirus transmission and discusses how localized immune responses to vector-derived components influence infection outcome. Modulation of parasite transmission by host responses to mosquito bites is also briefly discussed (Text Box 1).

Mosquito-derived factors augment systemic arbovirus pathogenesis

The ability of mosquito-sourced factors to augment arbovirus infection has been established in a variety of experimental systems [9,15]. Together, these data show that arboviruses inoculated via a mosquito bite or accompanied experimentally by mosquito saliva or salivary gland extracts (SGE) (Text Box 2) induce more rapid viraemia, higher pathogen load, and greater morbidity compared to needle inoculation in the absence of mosquito-derived factors (Table 1). Although different models for delivery of vector-derived salivary factors may yield similar results, care needs to be taken when comparing these approaches (discussed in Text Box 2). Thus, mosquito-derived factors appear to influence infection by modulating events at the inoculation site, as delivery of saliva via a mosquito probing for blood vessels or via needle inoculation at sites distal from the site of virus infection do not augment infection [18,22,23].

Mosquito bite enhancement of WNV infection and mortality has been studied in mice. Following transmission of WNV via infected *Culex* mosquitoes, needle inoculation of WNV mixed with SGE, or needle inoculation of WNV alongside bites by uninfected mosquitoes (“spot feeding”), WNV disseminates more rapidly and to higher levels to the central nervous system and causes higher mortality compared to inoculation with WNV alone [20,22,23]. SGE acts in a dose-dependent manner, with as little as 0.01 μg being able to increase infection [23].

Similarly, infection of mice with DENV by spot feeding [24] or via DENV-infected *Ae. aegypti* mosquitoes [25] augments systemic DENV infection compared to infection with virus alone. These studies were performed in either mice deficient in interferon (IFN) signalling (*Ifnar*$^{-/-}$) or humanized mice, since DENV does not replicate efficiently in immune-competent mice as it fails to suppress the murine IFN response. When co-inoculated into the footpad of *Ifnar*$^{-/-}$ mice, mosquito SGE increased DENV titers in lymph nodes draining the site of inoculation [26]. Similarly, spot feeding increased DENV titers at peak viremia in mice lacking IFN regulatory transcription factor IRF3/7 [24,25]. Mosquito-derived factors also prolonged viremia and exacerbated disease, including fever and thrombocytopenia, in humanized mice [17]. A complication of dengue pathogenesis is that serotype cross-reactive antibodies that stem from a prior DENV infection can enhance disease severity during a secondary infection with a different DENV serotype. In this case, non-neutralized DENV-antibody complexes enhance virus uptake and infection of Fcγ receptor-bearing target cells [27]. A recent study showed that intradermal inoculation of *Ae. aegypti* SGE together with DENV exacerbates pathogenesis only in the presence of enhancing antibodies [21]. Vector-derived factors can thus synergize with adaptive immune memory responses that cross-react among DENV serotypes to enhance disease severity. Consequently, pre-clinical testing of improved vaccine candidates or therapies against dengue need to consider the mosquito vector as well as enhancing antibodies that may be present in individuals after a prior natural exposure or vaccination.

The ability of *Ae. aegypti* mosquito bites and saliva to enhance the systemic course and clinical outcome of infection with other arboviruses including SFV, bunyamwera virus, CHIKV, Rift Valley fever virus and Cache valley virus has also been demonstrated in mice [16,18,28,29]. SFV is a model arbovirus that is genetically related to CHIKV, disseminates efficiently in immunocompetent mice, and has been engineered to express a variety of markers that make it a useful tool for investigating host responses to arbovirus infection [16,30]. SFV delivered via mosquito bite augmented virus replication, dissemination and mortality. Enhancement of virus infection was rapid, resulting in several orders of magnitude higher viral titer in some tissues within 24 hours. Interestingly, otherwise avirulent strains of SFV and bunyamwera virus were only able to disseminate efficiently *in vivo* from skin when inoculated via mosquito bite, demonstrating that these viruses require a mosquito bite to establish systemic infection [16]. In comparison, strains of SFV and WNV that are highly virulent in laboratory mice do not require a mosquito bite to

disseminate from skin and cause disease, although mosquito bites do accelerate their pathogenesis [16,22]. However, these virulent strains, which are consistently lethal in mice, do not model natural human arbovirus infection particularly well, as human mortality is low for most arboviruses. Taken together, these studies demonstrate that genetically distinct arboviruses make use of common mosquito-derived factors to augment their transmission to, and replication within, the mammalian host. Although viruses have co-evolved with the blood feeding strategies of their arthropod vectors, it is not yet clear if bite enhancement of infection is serendipitous or an evolved strategy on the part of the virus. Either way, an appreciation of how arthropods modulate cutaneous responses to infection is crucial for understanding arbovirus transmission and pathogenesis.

Cutaneous immune response to mosquito bites and arbovirus infections

To determine how mosquito bites enhance virus infection, we first need to consider separately how the skin responds to bites and to virus infection. Natural infection with arboviruses elicits at least three distinct host responses: to bite trauma, to mosquito saliva and to virus. Here, we summarize the current knowledge about early cutaneous immune responses to mosquito bites and mosquito saliva and how this differs from host responses to virus infection.

Cutaneous responses to mosquito bites

While seeking a blood meal, mosquitoes probe for blood vessels in the dermis with their probiscus, continuously depositing saliva, and imbibe blood once a blood vessel is pierced [14,31]. Saliva contains many biologically active components, including molecules that enhance leukocyte influx [16,21,32,33], and in addition contains a complex bacterial microbiota [34] that may also be inflammatory [35]. Trauma associated with arthropod bites induces local inflammation, and salivary protein(s) activate immune processes locally and possibly more distally in the draining lymph node [33,36,37].

Chemokine-mediated recruitment of leukocytes to mosquito bites. Mosquito bites elicit a rapid cutaneous response that includes expression of cytokines [16,36] and degranulation of mast cells [38]. Inflammatory chemokines (chemotactic cytokines) are expressed at sites of damage or

146 infection and control the entry and positioning of leukocytes within tissues [39]. Chemokines that
147 attract neutrophils are expressed particularly highly following a bite, resulting in a rapid influx of
148 these cells [16,21,37,38]. In other models of inflammation, neutrophils have been shown to
149 undertake a range of important anti-microbial functions and promote the influx of additional
150 leukocytes [40]. Following a mosquito bite, mast cell degranulation may be a necessary first step
151 mediating neutrophil recruitment, as mast cell-deficient mice failed to upregulate the key
152 neutrophil chemoattractant CXCL2 [36]. Bite-infiltrating neutrophils express high levels of the key
153 pro-inflammatory cytokine IL-1β and are important for coordinating inflammatory responses, as
154 neutrophil-deficient mice exhibit significantly reduced expression of some innate immune genes in
155 the skin, including chemokines that attract CCR2-expressing inflammatory myeloid cells [16], some
156 of which can differentiate into dendritic cells (DCs) [21]. Mosquito biting and SGE can also induce
157 the expression of T-cell associated cytokines, most notably IL-10 [16,41]. In summary, mosquito
158 bites induce a multi-step recruitment of leukocytes that begins with mast cell degranulation and
159 neutrophil recruitment, followed by an influx of monocytes.
160 Considerable insight into host responses to arthropod saliva has also been gained by
161 studying tick feeding [32]. In contrast to mosquitoes, ticks spend many days probing the skin and
162 preparing the bite site. The prolonged feeding time and associated risk of immune rejection of ticks
163 has driven the evolution of a powerful set of molecules to suppress host immunity. Tick saliva has
164 numerous immunomodulatory properties, including those that blunt chemotactic responses via a
165 family of proteins called Evasins [42,43]. Evasins bind with high affinity to inflammatory
166 chemokines, thus functioning as highly effective suppressors of leukocyte recruitment. In
167 comparison, there is no evidence that mosquitoes express salivary proteins with similar immune-
168 suppressing activity.
169
170 *Mosquito saliva promotes extensive cutaneous edema*. The swelling associated with a mosquito bite
171 is an obvious symptom; however, the mechanisms involved are still poorly defined. Quantification
172 of bite edema by measuring the extent of plasma leakage into the skin has demonstrated that
173 edema is both rapid and robust [16,21]. Mosquito saliva contains components that facilitate
174 efficient blood feeding, including vasodilation of blood vessels and inhibition of blood clotting
175 [32,44]. Importantly, SGE in the absence of bite trauma can not only induce endothelial

permeability in the skin of mouse ears, but can also directly disrupt the barrier function of human endothelial cells *in vitro* in the absence of virus or other cell types [21]. In addition to these direct effects, mosquito probing also causes tissue trauma and inflammation. This includes histamine release from mast cells [38] and neutrophil influx, which are both key regulators of vascular permeability and edema. Indeed, depletion of neutrophils prior to mosquito biting partially suppresses bite edema [16]. Together, this suggests that bite edema is due to a combination of direct action of mosquito saliva on endothelial cells and coagulation pathways and indirect activation of host immune responses.

Effect of pre-existing immunity to vector saliva. Inflammatory reactions to mosquito bites can vary dramatically between individuals. A history of prior exposure to mosquito bites and genetic predisposition to hypersensitivity may explain this variation [33,45]. Furthermore, those who live in *Aedes*-infested regions for many years can also gain tolerance to bites, which limits adaptive immune responses to bites [46]. In two separate studies, bite-experienced mice did not demonstrate significant differences in their susceptibility to arbovirus infection in the presence of mosquito bites [16] or mosquito SGE [22] compared to bite-naïve mice, despite the fact that mice exhibited either elevated IFN-γ responses to bites and high titers of SGE-specific antibodies respectively. However, these experiments were performed in C57BL/6 mice that are refractory to allergy. In comparison, BALB/c mice generate strong Th2 responses to various antigens [47] and, when repeatedly bitten by uninfected mosquitoes, demonstrated exaggerated cutaneous immune responses to further biting, including expression of the Th2-associated cytokine IL-4 [48]. Critically, these bite-experienced mice exhibit increased susceptibility to WNV infection when inoculated in the presence of SGE as compared to bite-naïve mice. Furthermore, passive transfer of sera from SGE-inoculated mice was also able to confer increased susceptibility to WNV infection with SGE [33]. Thus, IL-4 associated hypersensitivity to bites may prove to be a good indicator for predisposition to arbovirus infection.

Cutaneous innate immune responses to virus infections

Infection of skin-resident cells. Arbovirus infection of the skin is a critical stage of infection during

which the virus must quickly replicate and disseminate before adequate antiviral innate immune responses are activated (Text Box 3). When probing for blood vessels, infected mosquitoes deposit the majority of virus directly into extracellular spaces of the dermis [14,49,50]. *Culex* mosquitoes, for example, deposit >99% of WNV into the skin at a median dose of ~10^5 plaque forming units, while the 0.1% of virus that directly enters the bloodstream is rapidly inactivated or cleared [14]. Following infection with SFV, the majority of virus in the blood by 24 hours was derived from the inoculation site and draining lymph node [16]. Furthermore, the importance of viral replication at the mosquito bite site for dictating the subsequent systemic course of infection has also been demonstrated by studies that have surgically removed this site post-inoculation, e.g., for St. Louis encephalitis virus [50], Rift Valley fever virus [49], or DENV [21]. The protective effect of removing the site of transmission was lost at later time-points, which coincides with virus dissemination to other tissues [49,50].

Cellular targets for arbovirus infection are not well defined following natural transmission via mosquitoes. Extensive work using needle-inoculated virus in the absence of mosquito-derived factors has demonstrated that WNV and some alphaviruses can infect fibroblasts and DCs [51-54], while DENV mostly infects DCs and macrophages [55-57]. For DENV, replication within DCs and macrophages is particularly important [58-60]. Together, this suggests that infection of hematopoietic cells in addition to cutaneous fibroblasts is an important aspect of several arbovirus infections in the absence of mosquito bites.

Arbovirus infection recruits leukocytes to the skin. In contrast to mosquito bites, arbovirus infection by needle in the absence of mosquito factors results in only modest neutrophil recruitment to the skin, especially when inoculated in small volumes using hyper-thin needles that mimic transmission by mosquitoes [16,21]. The anti-viral function of neutrophils in skin during is not well understood [61]. However, recent work has shown that neutrophils can guide the migration of anti-viral $CD8^+$ T cells during the later adaptive immune response [62] and release anti-viral extracellular traps [63]. Nonetheless, a clearly defined role for neutrophils in coordinating cutaneous innate anti-viral responses to arboviruses is lacking, and indeed neutrophils are dispensable for the induction of skin IFN responses following SFV infection [16]. Following intraperitoneal inoculation with WNV, neutrophils are recruited to the peritoneum and worsen outcome of infection. In contrast, neutrophils may have a protective role during later stages of infection by encephalitic arboviruses,

such as SFV or WNV [16,64]. Together, these data suggest a biphasic role of neutrophils in arbovirus pathogenesis, initially contributing to virus replication and spread and later supporting virus clearance.

Monocytes are innate immune cells found in the blood that are recruited to sites of inflammation via signals that primarily involve the chemokine receptor CCR2 [65]. DENV and WNV infection in the skin leads to the recruitment of monocytes to the dermis and differentiation to monocyte-derived DCs. DENV can replicate in dermal monocytes and DCs [55-57,66], while a variety of arboviruses can replicate in DCs [52,54,56,67,68]. The effect that DC infection by arboviruses has on the systemic course of infection is currently a matter of active research (see text box 4).

How do mosquito bites enhance arbovirus infection?

Mosquito bites and the saliva that is deposited in the skin may enhance arbovirus infection through a number of mechanisms, including host inflammatory responses to mosquito bites [16]; saliva-induced edema [16,21]; enzymatic activity of saliva components [26]; and immune suppression/subversion by saliva [28,37,41,69].

Inflammatory responses to mosquito bites augment arbovirus infection. Host inflammatory responses to mosquito bites have been shown to have a defining effect on the systemic course and clinical outcome of SFV or bunyamwera virus infection [16]. Bite-recruited neutrophils coordinate a cutaneous inflammatory response that facilitates the entry of inflammatory myeloid cells. Some of these infiltrating cells and skin-resident macrophages become infected and generate infectious virus progeny. In the absence of CCR2-dependent inflammatory myeloid cell influx, bites were unable to enhance virus infection [16]. Suppression of bite inflammation by therapeutic depletion of neutrophils or by inhibition of the IL-1β pathway was also able to suppress bite enhancement of virus infection. Interestingly, structurally unrelated pro-inflammatory molecules that induce gene expression profiles similar to bites (e.g., supportive of early neutrophil influx and absence of type I IFNs) were also able to enhance SFV infection [16]. As such, bite-induced inflammation may be an attractive target for strategies that aim to prevent or limit arbovirus infection, as they constitute a common element of all mosquito-borne infections. It will be important to determine whether these findings, which primarily used model arboviruses in mice, also apply to human pathogens.

Along the same lines, significant insights have emerged from studies using DENV and SGE that parallel the findings with SFV [21,55]. While proteins from mosquito saliva can bind to DENV and decrease infectivity *in vitro* [70], only the combined presence of SGE and enhancing antibodies in mice significantly increased DENV infection of dermal $CD11b^+$ classical DCs and macrophages and enhanced mortality [21]. Furthermore, mosquito SGE boosts the migration of DCs from the skin to draining lymph nodes and may augment pathogenesis by facilitating virus dissemination or skewing immune responses. However, preliminary experiments have not yet detected significant differences in the activation or proliferation of $CD4^+$ or $CD8^+$ T cells *in vivo* by SGE [21]. Additionally, SGE activation of DCs theoretically could affect the generation of memory T cells that protect or enhance pathogenesis during subsequent DENV infections. Future studies are needed to determine the link between early effects of mosquito saliva on skin DCs and subsequent pathogenesis.

The vascular response to mosquito saliva enhances infection and virus dissemination. The dynamics of virus dissemination from the site of inoculation is an important determinant of pathogenesis, a process that is augmented by SGE in the case of DENV infection [26]. In addition, removal of the inoculation site 4 hours post-infection does not alter the systemic course of infection when co-inoculated with SGE; a finding that may relate to the potent effects of SGE on vascular function [21]. Other than facilitating virus dissemination, vascular permeability may also increase the entry of enhancing antibodies, and thus DENV infection of DCs and macrophages in the dermis or entry of monocytes into the skin [21]. Enhancing antibodies that cross-link Fcγ receptors on mast cells may further increase mast cell activation and endothelial permeability [71]. Future studies are needed to determine the combined effect of virus-specific antibodies and mosquito saliva on mast cell activation and endothelial permeability.

Can mosquito bites suppress immune responses to virus? Immune suppression by mosquito-derived factors has also been suggested to account for the observed enhancement of concurrent virus infection. In particular, suppression of type I IFN function by mosquito saliva is currently being investigated [16,24]. It should be noted that suppression of IFN signalling is unlikely to solely account for the boosting of DENV infection, as saliva increased DENV infectivity in the absence of type I IFN responses [21,26]. Suppression or subversion of T cell responses to virus by saliva has also

been hypothesized. Indeed, the presence of mosquito saliva was linked to higher expression of Th2 cytokines [41] and dysregulation of IL-10 expression [36,37]. Further, recombinant IL-4 can enhance DENV infection of human dermal cells *in vitro* [57]. It should, however, be noted that virus enhancement by mosquitoes in bite-naïve mice occurs too quickly for adaptive immune components to play a significant role. In addition, mosquito bites can also enhance infection in mice that lack adaptive immune responses [16], suggesting that modulation of infection likely occurs via alternative mechanisms.

Concluding remarks

The unexpected rise of Zika illustrates once again that mosquito-transmitted viruses cause epidemics for which we are unprepared. Due to the unpredictable nature of outbreaks, great genetic heterogeneity of arboviruses, and continuous territorial expansion of their vectors, further research in this area should be a major priority (see Outstanding Questions). Recent insights have highlighted the importance of the early events following transmission of virus to their mammalian hosts. The local response to the mosquito bite, which includes increased vascular permeability, edema, inflammation and recruitment of virus-susceptible cells, unwittingly promotes a beneficial niche for arbovirus replication [16,21]. This profound enhancing effect on initial viral replication and subsequent dissemination underlines the need to use models that incorporate mosquito-derived factors.

Many aspects of the early immune response in the skin to mosquito bites and arbovirus infection remain poorly understood. Nonetheless, it is becoming clear that targeting common denominators could be a promising novel strategy to limit infection with multiple arboviruses. Improved understanding of cutaneous immune responses will aid the identification of such targets. Possible strategies include targeting the immune pathways that are inadvertently beneficial for arboviruses, such as recruitment of additional susceptible cells, or improving the antiviral response in the skin. Pan-viral treatments would be particularly beneficial in regions where multiple arboviruses circulate concurrently, especially as it is hard to determine which virus is being most commonly transmitted; patients are diagnosed based on clinical symptoms that are often overlapping for distinct viruses [72]. In addition, lab-based diagnostics are either absent or take too long to meaningfully impact case management, particularly in resource-poor settings. We suggest that it is now appropriate to

explore whether vaccines or medicines that either target common mosquito-sourced factors or common aspects of viruses can be protective/efficacious. One such approach could involve vaccines that target mosquito saliva components. Mosquitoes have evolutionary diverged from their last common ancestor in their Nematocera suborder over 100 million years ago, resulting in at least 76 families of salivary genes of which most are species specific, such as those that have evolved to inhibit blood clotting [73]. Vaccines that target *Culex* salivary proteins have already been preliminarily explored and shown to have some protective effects against *Culex*-transmitted WNV [74], although such vaccines will have to be carefully designed to avoid worsening host inflammatory responses to bites [75]. The US National Institutes of Health has just announced the initiation of a Phase 1 clinical trial to explore a 'universal' mosquito-borne disease vaccine that targets components in the vector saliva. Alternatively, as there is serological overlap within some arbovirus families, vaccines could be designed to raise a broadly neutralising antibody response to multiple related viruses [76], while being cautious as immune cross-reactivity with e.g. dengue or Zika viruses bears the theoretical risk of antibody-dependent enhancement of infection [77]. In conclusion, early events during arbovirus transmission are understudied but have already begun to highlight the possibility of new strategies that aim to prevent or treat mosquito-borne virus infections.

Figure 1

Local immune response after transmission of virus with mosquito saliva into the dermis.

Mosquito saliva and virus trigger mast cell degranulation (1), which increases the permeability of blood vessels (2) and leads to leakage of plasma into the skin that causes edema (3). The virus first infects stromal cells (such as fibroblasts) as well as dendritic cells (DCs) or macrophages (MΦs) that reside in the dermis (4). Mosquito bite trauma, saliva, and infection induce inflammation that leads to the recruitment of neutrophils (5), which secrete additional attractants to recruit monocytes (6). Monocytes differentiate to inflammatory DCs and MΦs that can become targets for a second wave of virus infection (7). At the same time, resident dermal DCs migrate along lymphatic vessels to skin-draining lymph nodes to induce adaptive immune responses (8). Also, virus rapidly drains to lymph nodes (9). This virus dissemination may be accelerated via the saliva-induced plasma leakage into the skin and contribute to exacerbation of disease severity (10) after spread to the brain, liver, lung and/or other organs.

Text box 1. Commonalities between mosquito-transmitted viruses and arthropod-transmitted parasites

Extensive literature on the effects of sandfly saliva on the transmission of *Leishmania* parasites pioneered the field of vector-derived factors in human infectious diseases [78]. Similar to *Ae. aegypti* mosquitoes [16], bites from sandflies enhance recruitment of neutrophils to the site of *Leishmania* infection [79]. Here, neutrophils serve as a "Trojan horse" reservoir for *Leishmania* replication and enhanced infection [79,80]. Vaccination with specific sandfly-derived components can either protect rodents [81-83] or make them more susceptible to subsequent *Leishmania major* challenge in the presence of salivary gland extract [84]. Interestingly, injection with autoclaved *L. major* parasites protected against challenge with *L. major* via needle inoculation, but not against challenge with sandfly-transmitted parasite, due to the recruitment of neutrophils by the sandfly bite [85]. *L. major*-infected monocytes at sand-fly bites can instead differentiate into DCs to support protective Th1-type $CD4^+$ T cell responses [86].

371 Studies that examined whether mosquito salivary components directly modify *Plasmodium*
372 infection in malaria are more controversial. Some have demonstrated that prior sensitization to
373 uninfected mosquitoes or their saliva confers protection against infection [87], while other data
374 suggest transmission via infected mosquitoes is more efficient than needle inoculation [88]. More
375 recently, mosquito saliva was shown to have no detectable effect on *Plasmodium* infection in mice
376 [89]. Perhaps more important is the observation that passage of the malarial parasite through
377 mosquitoes appears to attenuate virulence in mice. In this study, mosquitoes were shown to
378 modify the biology of the parasite, resulting in altered mammalian host immune responses to
379 infection that rendered the infection less virulent [90].

380 **Text Box 2. Models for vector-derived factors**. The effects of mosquito saliva on the mammalian
381 host have been studied by either using live mosquitoes to bite mice or injecting purified mosquito
382 saliva. Saliva can be obtained from mosquitoes by either forced salivation into a capillary collection
383 device [17] or from homogenization of dissected salivary gland tissue [22]. Salivary gland extracts
384 likely contain additional compounds (e.g., from disrupted cells) that are not included in secreted
385 saliva. In comparison, artificially collected saliva from mosquitoes differs qualitatively from saliva
386 injected into the skin during probing for blood vessels [91]. Saliva obtained via forced salivation is
387 nonetheless able to enhance infection when co-inoculated by needle with SFV [16]. Alternatively,
388 feeding of non-infected mosquitoes and subsequent needle inoculation of virus at the same site (
389 "spot-feeding") enables delivery of arboviruses at a defined dose [16,22]. However, virus infection
390 of mosquitoes may modulate gene expression in salivary glands that could also affect transmission
391 [92]. To compensate for this, virus and saliva can be delivered to the skin via infected mosquitoes,
392 but such an approach cannot easily control for the amount of virus delivered.

393 **Text Box 3. Type I Interferons (IFNs) are important for anti-viral responses**
394 Following detection of virus by evolutionary-conserved, germline-encoded sensors of infection
395 [93,94], cells express highly potent antiviral type I IFNs. Type I IFNs bind to a common receptor
396 expressed on most cells that induces the expression of several hundred IFN-stimulated genes (ISGs)
397 [95,96], which makes them highly refractory to virus infection [97] and additionally recruits
398 leukocytes [98]. In the absence of a functioning type I IFN system, mice succumb rapidly to infection
399 with arboviruses such as SFV [99] and WNV [100]. Less work has been done to specifically study

400 cutaneous IFN responses to arbovirus infection. Cutaneous cells can express a variety of anti-viral
401 immune meditators following arbovirus infection *in vitro* [101] or *in vivo* [16,24]. However, the
402 cellular and molecular basis by which they are activated and coordinated in the skin is not well
403 understood.
404
405 **Text box 4. Dissemination of virus from skin to draining lymph nodes**
406 The mechanism by which arbovirus disseminates to draining lymph nodes is currently the subject
407 of research and debate. Virus may disseminate from the skin as free virus in lymph fluid, or
408 alternatively may also disseminate within infected cells, such as DC which are highly migratory.
409 Dermal DCs act as sentinels of infection and migrate from the skin to draining lymph nodes when
410 activated by inflammation or infection, including infection with arboviruses [102,103]. Arbovirus
411 infection of dermal DCs could lead to amplification of virus, suppress priming of adaptive immune
412 responses, and/or facilitate virus dissemination to draining lymph nodes as they migrate. In the
413 case of DENV infection of mouse skin, infected DC migrate to the draining lymph node [56].
414 However, DC migration may not be the primary route of virus dissemination, as virus spreads to
415 draining lymph nodes very quickly compared to DCs. Infection of macrophages in lymph nodes
416 occurs within 6 hours following intradermal inoculation, and DC activation and migration takes far
417 longer [60,102,104]. In addition, following infection of mice with alphaviruses such as SFV and
418 CHIKV, animals exhibit high titre viremia by 24 hours post infection [16,30,53,105], suggesting virus
419 disseminates quickly from the inoculation site. Consequently, most virus is likely carried from the
420 skin passively by draining lymph fluid. Nevertheless, DC that migrate from infected skin or reside in
421 lymphoid organs likely play an important role in inducing adaptive immune responses; a process
422 that may be hindered by arbovirus infection. Indeed, DENV for example, has developed strategies
423 to suppress the function of infected DCs in activating adaptive immune responses, as recently
424 reviewed [66].

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654 Table 1: Summary of publications that study the effects of mosquito bites on arbovirus infection *in vivo.*

Virus (strain) or parasite species	Vertebrate host	Mosquito	Inoculation	Inoculation site	Viraemia	Viral dissemination	Pathology	Ref
West Nile virus (NY lineage I cDNA clone)	Five-week-old, female C57BL/6	*C. tarsalis*	10^5 PFU by needle or 1 infected bite	needle > bite (n=1/2) needle = bite (n=1/2)	Infected bite > needle	Infected bite > needle	Infected bite = needle	[22]
	Five-week-old, female C3H/HeN		10^5 PFU by needle ±bite	12h resting > bite 24h bite > resting	Bite > resting	Bite > resting	Infected bite = needle	
	Five-week-old, female C57BL/6		10^5 PFU needle ±SGE	n.d.	SGE > no SGE	n.d.	n.d.	
West Nile virus (NY lineage I cDNA clone)	Six/seven-week-old, female C57BL/6	*C. tarsalis*	10^5 PFU by needle ±bite	n.d.	Bite > resting	n.d.	n.d.	[23]
			10^5 PFU needle ±SGE	n.d.	SGE > no SGE	n.d.	n.d.	
WNV (114)	Female, 4-week-old, Swiss Webster mice	*Ae. aegypti*	10^2 or 10^4 PFU by needle ± bite	n.d.	Bite > resting	Bite > resting	Earlier morbidity with bite	[20]
			10^4 PFU by needle ± 1 SGE	n.d.	n.d.	n.d.	Earlier morbidity with SGE	
West Nile virus (NY99)	6-week old female C57BL/6	*C. tarsalis*	$5x10^2$ PFU by needle ± 1 SGE	SGE > no SGE	n.d.	n.d.	n.d.	[26]
Dengue (DENV2, TH-36)	15-week old mixed gender $Ifnar^{-/-}$ C57BL/6	*Ae. aegypti*	10^7 genomes by needle ± 1 SGE	SGE = no SGE	SGE = no SGE	SGE > no SGE	n.d.	
Dengue (DENV-2, 1232)	IRF3/7 $^{-/-/-}$C57BL/6	*Ae. aegypti*	$6.7x10^4$ PFU by needle ± bite	Resting = bitten	D3+4: Biting > resting D1,2,5,6: biting = resting	n.d.	n.d.	[24]
Dengue (DENV-2, 1232)	IRF3/7 $^{-/-/-}$C57BL/6	*Ae. aegypti*	10^5 PFU by needle or infected bites	n.d.	Infected bite > needle	n.d.	n.d.	[25]
Dengue (DENV-2, K0049)	NOD.Cg-*Prkdc*^{scid} *IL2rg*^{tm1Wjl}/SzJ (NSG), newborn radiated and transplanted with $3x10^5$ purified cord blood $CD34^+$ cells, both genders used at 6-8 weeks	*Ae. aegypti*	9 $\log_{10}$ RNA copies by needle or 4-5 infected mosquitoes bites	n.d.	Peak equal, duration infected bite > needle	n.d.	Erythema index: Bite > injection Temperature: Needle > bite Thrombocytopenia: delayed after bite-infection	[17]
			9 $\log_{10}$ RNA copies ± 5 mosquitoes' saliva	n.d.	Peak: bite = SGE = needle Duration: bite = saliva > needle	n.d.	Erythema index: Bite > SGE = needle Temperature: Needle = saliva > bite Thrombocytopenia d14: Needle = saliva= bite	

Dengue (DENV2, D220)	Ifnar$^{-/-}$ C57BL/6	*Ae. aegypti*	10^5 PFU by needle ± 1 SGE, as first infection or under ADE conditions	First and ADE: SGE > no SGE	n.d.	n.d.	Morbidity: first infection: SGE = no SGE ADE: SGE > no SGE	[21]
Chikungunya (DRDE-06)	2-3 day old Swiss albino	*Ae. aegypti*	$2.5x10^4$ PFU by needle ± bites	8h bite > resting (other time points bite = resting)	2-6dpi: Bite > resting	4dpi: bite = resting 6dpi: Bite > resting	Biting > resting	[28]
Semliki forest virus (SFV4 and SFV6)	C57BL/6	*Ae. aegypti*	$2.5x10^2$ PFU SFV6 or 10^3 PFU SFV4 by needle ± mosquito bites	Bite > resting	Bite > resting	Bite > resting	Bite > resting	[16]
Bunyamwera virus			10^4 PFU SFV4 by needle ± mosquito bites	Bite > resting	Bite > resting	Bite > resting	n.d.	
Rift Valley fever virus (ZH548)	C57BL/6	*Ae. aegypti*	10^3 PFU by needle ± 1 SGE	n.d.	SGE > no SGE	SGE > no SGE	SGE > no SGE	[29]
		Ae. vexans	10^3 PFU by needle ± 1 SGE or mosquito bite	n.d.	n.d.	n.d.	SGE = bite > no bite	
			10^3 PFU by needle ± 1 SGE	n.d.	n.d.	n.d.	SGE = no SGE	
Cache Valley virus	Outbred 6-week old ICR mice	*Ae. triseriatus*	$3.2x10^6$ $TCID_{50}$ by needle in resting, mosquito bites or with 2 SGE	n.d.	Bite > resting = SGE	n.d.	No morbidity observed	[18]
		Ae. aegypti	$3.2x10^6$ $TCID_{50}$ by needle ± mosquito bites	n.d.	Bite > resting = SGE	n.d.	n.d.	
		C. pipiens	$3.2x10^6$ $TCID_{50}$ by needle ± mosquito bites	n.d.	Bite > resting = SGE	n.d.	n.d.	
Vesicular stomatitis virus (New Jersey)	IRC mice, 3 days old	*Ae. triseriatus*	3 $\log_{10}$ $TCID_{50}$ by needle injection or 1 infected bite		n.d.	Injection = bite	Injection = bite	[19]
	IRC mice, 3 weeks old				No viraemia. Antibody seroconversion: Injection < bite	n.d.	Low morbidity (n=1/30)	
	IRC mice, retired breeders				No viraemia. Antibody seroconversion: Injection < bite	n.d.	No morbidity	
P. berhei (NK65)	Female outbread CD-1 mice	*A. stephensi*	$2\text{-}5x10^4$ parasites by needle (i.v.) or 5 or 10 infected mosquito bites	n.d.	Infection rate: bite > injection	n.d.	n.d.	[88]
P. berhei (NK65)	C57BL/6	*A. stephensi*	$2x10^2$ parasites i.v. or $2x10^3$ parasites i.d. ± 0.5 SGE	n.d.	Infection rate: SGE = injection	n.d.	n.d.	[89]
P. yoelii (17NXL)	BALB/c		5 parasites i.v. or 10 parasites i.d. ± 0.5 SGE	n.d.	Infection rate: SGE = injection	n.d.	n.d.	

Abbreviations

SGE: salivary gland extract, number indicates how many mosquitoes; i.d.: intradermal injection; i.v.: intravenous injection; ADE: antibody-dependent enhancement; n.d.: not determined; Ae: *Aedes*; A: *Anopheles*; C.: *Culex;* P: *Plasmodium*