The effects of mosquito saliva on dengue virus infectivity in humans.

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Arboviruses such as Dengue, Chikungunya, and Zika viruses represent a major public health problem due to globalization and propagation of susceptible vectors worldwide. Arthropod vector-derived salivary factors have the capacity to modulate human cells function by enhancing or suppressing viral replication and, therefore, modify the establishment of local and systemic viral infection. Here, we discuss how mosquito saliva may interfere with Dengue virus (DENV) infection in humans. Identification of saliva factors that enhance infectivity will allow the production of vector-based vaccines and therapeutics that would interfere with viral transmission by targeting arthropod saliva components. Understanding the role of salivary proteins in DENV transmission will provide tools to control not only Dengue but also other arboviral diseases transmitted by the same vectors.

Addresses
[1] Laboratory of MIVEGEC, UMR 224 IRD/CNRS/UM1, Montpellier, France
[2] Environment and Infectious Risks Unit, Pasteur Institute, Paris, France

Corresponding author: Missé, Dorothée (dorothee.misse@ird.fr)

Introduction

Mosquitoes are blood-feeding arthropods and, therefore, vectors of causative agents of important emerging infectious diseases. They are able to transmit several types of micro-organisms associated with vector borne disease, that cause more than 1 million deaths each year (WHO, 2016). Dengue virus (DENV) is the most important arbovirus in humans, infecting about 390 million individuals globally, with up to 96 million symptomatic infections. DENV infection results in a spectrum of illnesses, ranging from a flu-like disease (dengue fever) to dengue hemorrhagic fever (DHF) that can progress to dengue shock syndrome (DSS) and death. There are over 500 000 cases of DHF/DSS reported worldwide each year, 25 000 of which lead to death, mostly among young children under the age of 15 [1]. Epidemics with a high frequency of DHF/DSS continue to expand geographically in Asia and South America, highlighting the need to control the spread of this disease [2]. To date, there are no vaccines or effective treatments available for DENV infection.

DENV is a single-stranded RNA virus belonging to the *Flaviviridae* family. The four dengue serotypes (DENV-1 to DENV-4) are transmitted to humans by the *Aedes* (*Ae.*) mosquitoes species (*Ae. aegypti* and *Ae. albopictus*), which are spreading throughout the world due to climate change and globalization [3]. A dramatic increase in urbanization has also created ideal conditions for increased transmission of mosquito-borne dengue disease. Outbreaks of DENV have generally been restricted to resource-limited areas of tropical and subtropical latitudes, in which 40% of the global population live. However, due to the increasing range of the DENV vectors, the virus could spread outside of these regions [4].

Understanding the transmission cycle of DENV is important for the control of the spread of this disease. The cycle is initiated when pathogen-containing fluids are ingested by the vector from an infected vertebrate during a blood meal. Once the virus crosses the midgut barrier and has replicated in the mosquito, it reaches the salivary glands which leads to high levels of viral titers in the saliva of infected arthropods [5–7]. An infected mosquito will then transmit the virus to a host by delivering most of the viral content through the saliva into both the epidermis and dermis where resident and migratory cells encounter the pathogen [8••].

Though it is known that the DENV contained in salivary glands is closely associated with mosquito saliva, little is known about the function of the *Aedes* mosquito's salivary proteins and their role in the transmission of the virus.

Mosquito saliva helps to successfully obtain a blood meal [9] and could also contribute to an optimal transmission of the pathogen by modulating the host immune response [10]. It is as yet unknown, however, how salivary proteins may affect the human immune system during the transmission of the virus.
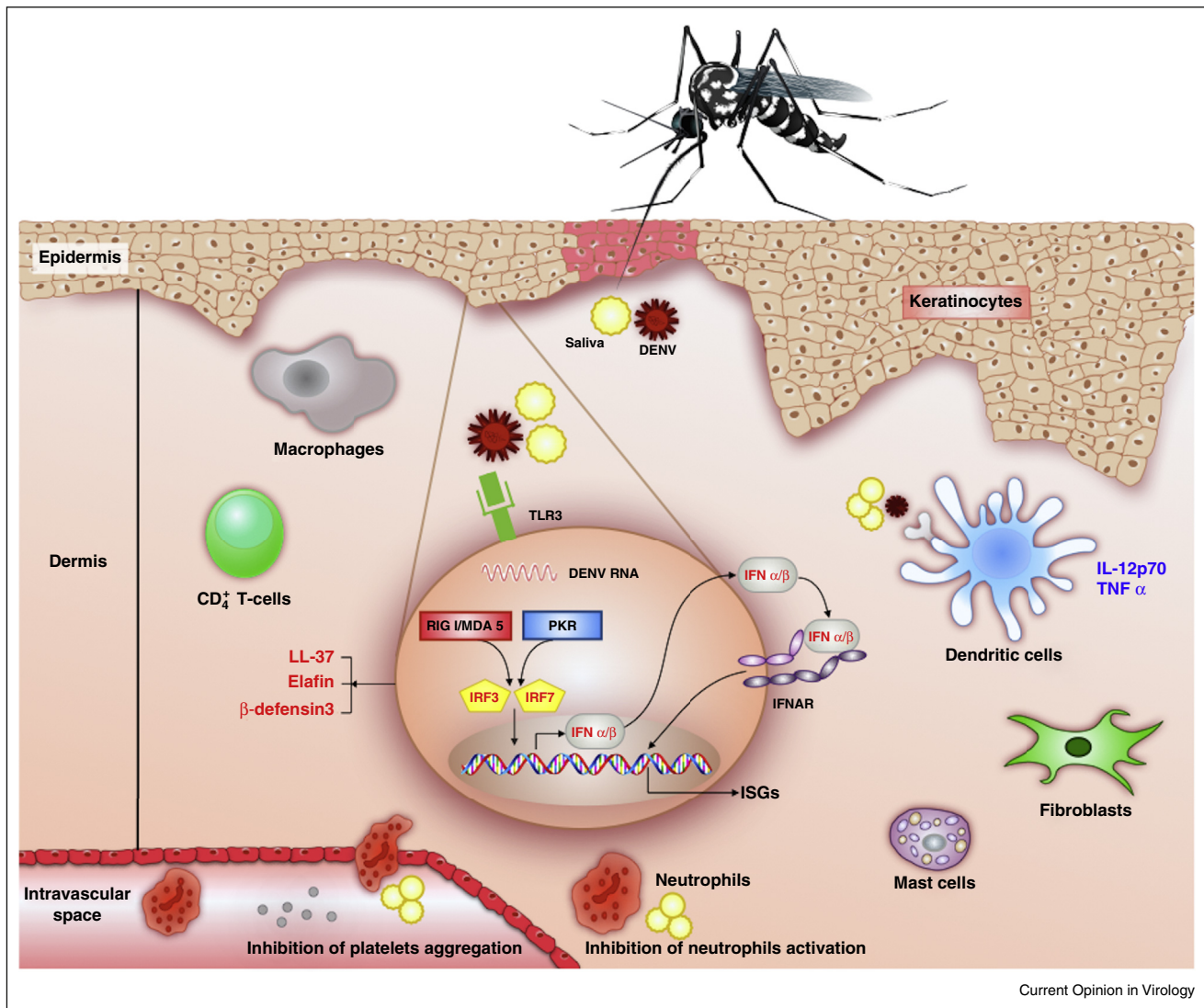
This review will summarize the effect of mosquito saliva and salivary proteins on the transmission of DENV in humans.

Human innate immune response against DENV

Human skin cells represent the first physical and immunological barrier after being infected by DENV through a mosquito bite [8••]. Skin cells may contribute to the pro-inflammatory and antiviral microenvironment that is established in the early stages of DENV infection. Pattern recognition receptors (PRRs) such as membrane-bound toll like receptors (TLRs), cytoplasmic helicases (retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5)) and protein kinase R (PKR) are the first lines of defense in the immune recognition of DENV [11–13]. These PRRs trigger signaling cascades that induce the activation of transcription factors including interferon regulatory factors (IRF3 and IRF7). This will lead to the production of type I interferons (IFN-α/β) resulting in the expression of hundreds of interferon-stimulated genes (ISGs) with various levels of antiviral activity [14]. TLR3, RIG-I, MDA-5 and PKR are induced during DENV infection as well [15]. DENV infection of human fibroblasts and keratinocytes induces the production of antimicrobial peptides such as LL37, β-defensin, RNase 7 and S100 family members, that are essential components of the innate immune response (Figure 1) [16,17]. Dendritic cells (DCs) are sentinels of the immune system that

Figure 1

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Schematic representation of the impact of *Aedes aegypti* saliva on the human antiviral response to DENV. DENV infection of human epidermal keratinocytes in the presence of *Aedes aegypti* mosquito saliva affects the immune response by decreasing the production of type I interferons (IFN-α/β), the transcriptional regulation of IRF3 and IRF7 and the expression of the antimicrobial peptides β-defensin, LL37 and Elafin which increases viral replication. Concurrently, the presence of saliva during DENV infection of human DCs enhances the production of IL-12p70 and TNF-α, resulting in a decrease of viral replication.

detect pathogens at sites of entry such as the skin. Following this initial contact with the virus, DCs mature and migrate to the draining lymph nodes, becoming effective stimulators of T cell responses [18]. In addition to the ability of DCs to control infections directly via their innate immune functions, these cells can also become infected [19]. Hence, DENV was shown to infect epidermal Langerhans cells (LCs) in healthy human skin explants *in vitro* [20,21]. Another cell type that plays an important role in host defense against viruses through its capacity to modulate both innate and adaptive immune responses is the mast cell [22–24]. Following infection with DENV mast cells have been shown to produce a spectrum of chemokines that are important for the trafficking of natural killer cells, monocytes and T cells [25]. The RNA interference (RNAi) pathway has also been shown to exert antiviral activity against DENV. The knockdown of Dicer, Drosha, Argo1, and Argo2 proteins of the RNAi pathway during DENV infection of mammalian cells results in increased DENV replication [26]. However, DENV has developed several ways to directly inhibit cellular signaling cascades by evading innate antiviral responses [27].

Modulation of vertebrate immune response by mosquito saliva

It has been shown in the literature that mosquito saliva is able to modify the host immune response, although the underlying molecular mechanisms are unclear as yet. Results from initial studies, carried out with C3H/HeJ mice on which *Ae. aegypti* fed for a blood meal, showed a significant down regulation of IFN-γ production while that of (interleukin (IL) IL-4 and IL-10) was up-regulated [28]. Further studies explored the effect of *Aedes* saliva or salivary proteins on the transmission of arboviruses. One study reported that viral replication of Rift Valley Fever Virus, as well as the pathogenesis associated with infection with this virus was strongly enhanced by factors contained in saliva and salivary gland extract (SGE) of *Aedes* mosquitos [29]. Similarly, in the context of DENV infection, co-infection with *A. aegypti* saliva, saliva proteins or SGE was also reported to boost viral replication [17,30,31••,32••,33•].

Pathological investigation in fatal human cases revealed spleens with significant destruction of the germinal centers and atrophy of lymphoid follicles, which may be associated with a decrease in the frequency of T cells [34]. Infection of AG129 mice with dengue serotypes triggered gross pathology and histopathological changes in spleens [35]. And in a study using murine splenocytes, a reduction of T-cell and splenocyte proliferation as well as cytokine production (Th1 (IFN-γ) and Th2 (IL-10)) was observed in the presence of *Aedes* SGE [36]. Production of IFN-γ was also observed in inbred A/J mice infected intravenously with DENV-2 [37]. The effects of *Ae. aegypti* saliva on DENV infection in human keratinocytes have also been investigated. Both the saliva and recombinant saliva proteins were associated with an increase of mRNA level in the cells and a decrease of type I IFNs, IRF-3 and IRF-7 (Figure 1) [17,31••].

It has furthermore been shown that SGE of *Ae. aegypti* inhibits the release of tumor necrosis factor alpha (TNF-α) from rat mast cells, but has no effect on antigen-induced histamine secretion [38]. A previous study demonstrated that *Ae. aegypti* SGE also does not affect the viability of a fetal skin-derived DC line, nor the production of IL-12 by these cells [39]. Consequently, *Ae. aegypti* SGE has no effect on the basal expression of IFN-β by DCs, but instead decreases the production of this cytokine in West Nile virus-infected cells [40]. There is reportedly no effect of *Ae. aegypti* SGE on the maturation and function of mice dendritic cells [41]. On the other hand, Ader *et al.* found that *Ae. aegypti* saliva had an effect on myeloid DCs by inhibiting their susceptibility to infection by DENV and by enhancing their production of both IL-12p70 and TNF-α (Figure 1). Moreover, presensitization of DCs with saliva prior to infection enhanced inhibition of DENV replication [42]. Variations in the results of these two observations may thus be due to the origin of the DCs, that is, whether they belong to the mouse or human lineage. Together, these observations suggest that *Ae. aegypti* saliva is involved in the regulation of both innate and adaptive immunity in vertebrate hosts.

Aedes mosquito saliva proteins involved in DENV infectivity

Since *Ae. aegypti* saliva can affect the transmission and the establishment of arboviruses in humans [8••], much effort has been devoted to characterize the sialome and sialotranscriptome of this vector [43]. The salivary gland protein profile from the *Ae. aegypti* mosquito was evaluated using a proteomic approach and approximately one hundred proteins were identified in this organ [44]. *Ae. aegypti* saliva contains many pharmacologically active proteins that can facilitate blood feeding, pathogen transmission and modulate host immune responses [45–47] (Table 1). A functional genomic and proteomic analysis of salivary glands of *Ae. aegypti* mosquitoes, identified four proteins that strongly enhance DENV replication in human keratinocytes: serpin-like protein (AT), adenosine deaminase (AD), a serine protease with a MW of 34 kDa and a putative secreted protein (VA) [31••]. All of these proteins have been shown to down regulate the expression of IRF-7, but only the 34 kDa protein was able to inhibit the transcriptional expression of IRF-3, resulting in a stronger abrogation of type I IFN production [31••]. These results are in line with a study showing that the combined action of IRF-3 and IRF-7 is required for efficient control of viral replication [14]. The mechanisms by which AT, AD and VA increase DENV infection in keratinocytes have not yet been studied. The 34 kDa

Table 1

Identified proteins in *Aedes aegypti* and *Aedes albopictus* saliva

Name family	Gene Bank accession number	Activity	DENV infectivity	Immunomodulation	Reference
Apyrase					
68 kDa	AAEL006347	Hydrolase	ND	ND	[46,47,56]
AD	AAEL005672	Adenosine deaminase	Enhanced	Suppressed IRF7, IFN type-I and AMP	[31••,47,55]
D7					
D7L1	AAEL006417	Odorant binding	ND	ND	[56]
D7s2	AAEL006423	Odorant binding	ND	ND	[44,51,56]
D7L2/37kDA	AAEL006424	Odorant and biogenic amines binding	ND	ND	[44,46,51]
Serine protease					
34 kDa	Q1HRW0_AEDAE	Serine protease	Enhanced	Suppressed IRF7, IRF3, IFN type-I and AMP	[47,31••]
CLIPA3	AAEL005718	Serine-type endonuclease	Enhanced	Not changed Th1 and Th2 expression	[48•]
Aegyptin					
30 kDa salivary gland allergen Aed a 3	AAEL010235	Collagen binding	Inhibited	Increased GM-CSF, IFN-γ, IL-5, and IL-6	[32••,44, 46,47,49]
Serpin-like protein					
serpin-4	AAEL002704	Serine protease inhibitor	ND	ND	[44]
SRPN26	AAEL003182	ND	ND	ND	[56]
Antithrombin (AT)	Q1HRTV7_AEDAE	Thrombin inhibitor	Enhanced	Suppressed IRF7, IFN type-I and AMP	[31••]
Vitellogenin-B	AAQ92367	Lipid transporter	ND	ND	[44]
Vitellogenin-C	AAQ92366	Lipid transporter	ND	ND	[44]
SGS1	AAV28546	ND	ND	ND	[44,47]
34 kDa	AAL76018, Q8T9VI_AEDAE	ND	ND	ND	[44,47]
Venom Allergen (VA)	Q8T9U5_AEDAE	ND	Enhanced	Suppressed IRF7, IFN type-I and AMP	[31••]
Cecropin					
3.8 kDa	AAEL000598	Antibacterial activities	Inhibited	ND	[5,55]
Sialokinin	AAEL000229	Vasodilatory	ND	ND	[28,56]

ND: not determined.

protein also decreases the expression of antimicrobial peptides in the context of DENV infection, which may also explain the increase in viral replication in human keratinocytes. Another study also identified a serine protease, CLIPA3, responsible for DENV transmission enhancement [48•]. This protein is known for breaking down the extracellular matrix, which may lead to cell migration at the inoculation site.

Also, certain saliva factors may enhance, while others may inhibit, virus infectivity due to the complex nature of mosquito saliva. *Aegyptin* is a collagen-binding protein that inhibits platelet aggregation and a member of the GE-rich 30 kDa antigen family present in *Ae. aegypti* saliva [49]. Mice inoculated with *Aegyptin*, show a decrease in DENV titers, both at the inoculation sites and in the circulation, as well as increased serum concentrations of GM-CSF, IFN-γ, IL-5, and IL-6, as compared to mice that were inoculated with DENV alone. These results suggest that *Aegyptin* impacts DENV infectivity through activation of

the immune response [32••]. Reducing the abundance of *Aegyptin* through transgene-mediated dsRNA interference results in prolonged probing time and less successful uptake of a blood meal [50•]. It has also been shown that a cecropin-like peptide in the salivary glands of *Ae. aegypti* decreased DENV infectivity in the C6/36 *Ae. albopictus* cell line [5].

Among the proteins up-regulated in DENV infected *Ae. aegypti* SGE, D7 proteins were shown to inhibit platelet aggregation, vaso-constriction and inflammation [51]. A 36 kDa member of the D7 protein family was found to be associated with dengue disease severity [46]. The role of D7 proteins in DENV transmission, however, remains largely unknown. Another major protein found in *Ae. aegypti* saliva, Apyrase, which inhibits ADP-dependent platelet aggregation during blood-feeding and prevents neutrophils activation [52], has also been largely uncharacterized in its role in DENV infection. SAAG-4 protein and sialokinin demonstrated the capacity to modulate the

ratio of Th1/Th2 cytokine production by suppressing and simultaneously enhancing the expression of IFN-γ and IL-4, respectively, outside the context of viral infection [28,53]. The role of these proteins in DENV transmission remains to be studied.

Table 1 provides a list of saliva proteins that were identified in several studies. However, further investigation is needed to better understand their effect on the DENV infectivity in humans. It is of note that, even if all major epidemics of DHF have occurred only in areas where *Ae. aegypti* is found, *Ae. albopictus* is also a competent vector for DENV and has the ability to adapt to diverse environmental conditions in both tropical and temperate regions [54]. To date, there have been no studies on the effect of salivary proteins of this vector in the transmission of DENV.

Conclusion

Arboviral diseases have become a great concern due to the expanding geographical range of many mosquito species. Unpredictable outbreaks of arboviral diseases, such as the recent expansion of ZIKA virus, highlight the need to control the spread of their vectors. DENV is arguably the most important arbovirus causing disease in the world today, and despite decades of research, a vaccine is not yet available. Increased understanding of the activity of saliva proteins on DENV infectivity could provide novel targets that may aid in the development of vector-protein based vaccines and therapeutics.

Acknowledgements

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