Host Responses to Sandfly Salivary Factors Enhance the Severity of Toscana Virus Infection

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I confirm that the work submitted is my own and that appropriate credit has been given where reference has been made to work of others.

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Abstract

Arbovirus infections have increasingly become a profound burden on public health. Inflammatory response to mosquito bites and the saliva deposited, aids the replication of arboviruses. In this thesis, we developed a new *in vivo* model that mimics natural transmission by sandfly vectors, to define whether saliva from sandflies enhances vertebrate host susceptibility to Toscana virus (TOSV), an important and growing cause of infectious neurological disease. We established new quantitative PCR assays, and plaque assays to define TOSV titre. New flow cytometry panels and genetically modified TOSV were utilised to interrogate the cellular tropism of TOSV in skin, and how this was modified by sandfly saliva/bite. Our results show a statistically significant increase in virus RNA in mouse tissues, such as skin, when TOSV is co-inoculated with sandfly salivary factors, compared to inoculation with TOSV alone. Higher quantity of virus RNA was also associated with an increase in the number of clinical signs, including atypical neurological signs and inflamed foot joints. These findings suggest that the enhancement effect of sandfly saliva is a general pro-viral mechanism, not limited to specific vector species. We found virus infection by sandfly saliva/bite was more inflammatory with increased cytokine expression. We identified dermal fibroblasts as crucial for TOSV replication and our flow cytometry analysis suggested that sandfly saliva/bites alter fibroblast biology, promoting a more primitive state. We suggest that while this is an aspect of normal wound healing, it simultaneously increases their capacity for TOSV replication. Salivary microbiota was dispensable for its ability to enhance TOSV infection, suggesting that the sandfly encoded salivary factors are instead responsible. A better understanding of how sandfly saliva increases host susceptibility to arbovirus infection will provide new insights into this crucial intersection of virus, vector and host. Such insights can aid development of an urgently needed vaccine.

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Abbreviations

A		
	µg	Microgram
	µl	Microlitre
	Abx	Antibiotics
	ADE	Antibody-dependent enhancement
	AIDS	Acquired immunodeficiency syndrome
	AME	Acute meningitis and encephalitis
	APC	Allophycocyanin
	APCs	Antigen-presenting cells
	AWERB	Animal Welfare Ethical Review
B		
	BALB/c	Bagg Albino
	BBB	Blood-brain barrier
	BCR	B-cell receptor
	BFV	Barmah Forest virus
	BHK	Baby Hamster Kidney
	BM	Bone marrow
	bp	Base pair
	BSA	Bovine serum albumin
	BSL	Biosafety laboratory
	Bti	*Bacillus thuringiensis israelensis*
	BTV	Bluetongue virus
	BUNV	Bunyamwera Virus
	BV	Brillian violet
	BALB/c	Bagg Albino
C		
	C	Capsid
	C57BL/6	C57 black 6
	CARDs	Caspase recruitment domains
	CCHFV	Crimean-Congo Hemorrhagic Fever
	CCL	CC-chemokine ligand
	CD	Cluster of Differentiation
	cDC	Conventional DC
	cDNA	Complementary DNA
	CHIKV	Chikungunya Virus
	CHPV	Chandipura virus
	CL	Cutaneous Leishmaniasis
	CLP	Common lymphocyte progenitor
	CNS	Central nervous system
	CO2	Carbon Dioxide
	COVID-19	Coronavirus Disease-19
	CPE	Cytopathic effect
	CSF	Cerebrospinal fluid
	Ct	Cycle threshold
	CTD	C-terminal regulatory domain
	CTL	Cytotoxic T-cells
	CVF	Cache valley virus
	CXCL	Chemokine (C-X-C motif) ligand
	CXCR	CXC-chemokine receptor
	Cy7	Cyanine 7
D		
	DAMPs	Damage-Associated Molecular Patterns
	DCs	Dendritic cells
	DDCs	Dermal dendritic cells
	DDT	Dichlorodiphenyltrichloroethane
	DEET	N,N-diethyl-meta-toluamide
	DENV	Dengue Virus
	DETCs	Dendritic epidermal T cells
	dH2O	Distilled water
	DMEM	Dulbecco's Modified Eagle Medium
	DNA	Deoxyribonucleic acid
	DNTPs	Deoxynucleotide triphosphates
	dpi	days post infection
	dsDNA	Double-stranded DNA
E		
	E. coli	*Escherichia coli*
	E1	Envelope Protein 1
	ECSA	East/Central/South African
	EDTA	Ethylenediaminetetraacetic Acid
	EEEV	Eastern equine encephalitis virus
	EGF	Epidermal growth factor
	ELISA	Enzyme-Linked Immunosorbent
	ER	Endoplasmic reticulum
	EU/ml	Endotoxin units per millilitre
F		
	FACS	Fluorescence-activated cell sorting
	FBS	Foetal Bovine Serum
	FCS	Forward scatter
	FDA	Food and Drug Administration
	FITC	Fluorescein isothiocyanate
	Flt3L	FMS-like tyrosine kinase 3 ligand
	FMO	Fluorescence Minus One
	FO	Follicular zone
	FSC-A	Forward scatter area
	FSC-H	Forward scatter height
G		
	GBS	Guillain-Barré syndrome
	GPCRs	G-Protein Coupled Receptors
H		
	H&E	Hematoxylin and Eosin
	HBSS	Hanks balanced saline solution
	hi	High
	HIV	Human immunodeficiency viruses

I	HIVDR	HIV Drμ Resistance
	hpi	hours post infection
	HSC	Hematopoietic precursor cells
	HUVECs	Human umbilical vein endothelial cells
	Hz	Hertz
	i.c.	Intracranial
	i.d.	Intradermal
	i.p.	Intraperitoneal
	ICTV	International Classification for Taxonomy of Viruses
	IECs	Intestinal epithelial cell
	IELs	Intraepithelial T lymphocytes
	IFA	Immunofluorescence Assay
	IFN	Interferon
	ifnar-/-	Interferon alpha and beta receptor subunit 1 null allele
	IFNAR-I	Interferon alpha and beta receptor subunit 1
	Ig	Immunoglobulins
	IIFA	Indirect immunofluorescence
	IL	Interleukin
	ILCs	Lymphoid cells
	ILN	Inguinal lymph node
	int	Intermediate
	IntDen	Integrated density
	IRFs	Interferon regulatory factors
	IRS	Indoor residual spraying
	ISG	Interferon stimulated gene
	ISREs	Interferon-stimulated response elements
	ITNs	Insecticide-treated nets
J		
	JAK	Janus kinase
	JEV	Japanese encephalitis virus
K		
	kb	Kilobase
L		
	L/D	Live/Dead
	LACV	La Crosse Virus
	LAL	Limulus amebocyte lysate
	LC	Long COVID
	LCs	Langerhans cells
	LGP-2	Laboratory of genetics and physiology-2
	LLINs	Long-lasting insecticidal nets
	LN	Lymph node
	lo	Low
	LPS	Lipopolysaccharide
	LRRs	Leucine-rich repeats
	Lu.	*Lutzomyia*
	Ly-6C	Lymphocyte antigen 6 complex, locus C
	Ly-6G	Lymphocyte antigen 6 complex, locus G
M		
	MACS	Magnetic activated cell sorting
	MAPKs	Mitogen-activated protein kinases
	MASV	Massilia virus
	MAVS	Mitochondrial antiviral signalling protein
	MAYV	Mayaro virus
	MCL	Muco-Cutaneous Leishmaniasis
	M-CSF	Macrophage colony-stimulating factor
	MDA-5	Melanoma differentiation-associated 5
	MEM	Temin's Modified Eagle's Medium
	MERS-CoV	Middle East respiratory syndrome
	MERTK (Mer)	Mer Tyrosine Kinase
	MHC-II	Major histocompatibility complex class II
	ml	Millilitre
	mNGS	Metagenomic next-generation sequencing
	moDCs	Monocyte-derived dendritic cells
	MOI	Multiplicity of infection
	mRNA	Messenger RNA
	MyD88	Myeloid differentiation primary response 88
	MZ	Marginal zone
N		
	N	Nucleoprotein
	NAPs	Nucleic acid polymers
	NETs	Neutrophil extracellular traps
	ng	Nanogram
	NIH	National Institutes of Health
	NK	Natural killer
	NLRs	Nucleotide-binding oligomerization domain (NOD)-like receptors
	nm	Nanometre
	ns	non-significant

	Ns	Nonstructural
	NTC	non-template control
	NTDs	Neglected tropical diseases
O		
	ONNV	O'nyong'nyong virus
	ORF	Open reading frame
P		
	Pam3CSK4	Pam3CysSerLys4
	PAMPs	Pathogen-Associated Molecular Patterns
	PB	Pacific Blue
	PBMCs	Peripheral blood mononuclear cells
	PBS	Phosphate buffered saline
	PBSA	Phosphate buffered saline + BSA
	PC	Panniculus carnosus
	PCR	Polymerase Chain Reaction Release
	pDC	Plasmacytoid DC
	PE	Phycoerythrin
	Pen	Penicillin
	PFA	Paraformaldehyde
	PFU	Plaque forming units
	Ph.	*Phlebotomus*
	PKR	Protein kinase R
	PLN	Popliteal lymph node
	POWV	Powassan virus
	pr/M	Premembrane/membrane
	PRRs	Pattern recognition receptors
	PSG	Promastigote secretory gel
	PTV	Punta Toro virus4
Q		
	qPCR	Quantitative Polymerase Chain Reaction Release
R		
	RdRp	RNA-dependent RNA polymerase
	RIDL	Release of Insects carrying Dominant Lethal transgenes
	RIG-I	Retinoic acid-inducible gene I
	RLRs	RIG-I-like receptor family
	RNA	Ribonucleic acid
	RNP	Ribonucleoprotein
	ROS	Reactive oxygen species
	RPM	Revolutions per minute
	rRNA	Ribosomal RNA
	RRV	Ross River Virus
	Rsad2	Radical-SAM-domain-containing-2
	RT	Reverse transcription
	rTOSV	Recombinant TOSV
	RT-qPCR	Real time polymerase chain reaction
	RVFV	Rift Valley Fever Virus

S		
	s.c.	Subcutaneous
	Sca-1	Stem Cell Antigen 1
	SFV	Semliki Forest Virus
	SGE	Salivary Gland Extract
	SINV	Sindbis virus
	SIV	Simian immunodeficiency virus
	SLE	St. Louis encephalitis
	SSC	Side scatter
	ssRNA	Single-stranded RNA
	STAT	Signal transducer and activator of transcription
	STING	Stimulator of interferon genes
	Strep	Streptomycin
T		
	TBEV	Tick-borne encephalitis virus
	TBP	Tata binding protein
	TCR	T-cell receptor
	TIR	Toll–IL-1 receptor
	TLR	Toll-like receptor
	Tm	Melting temperature
	TM7	Seven-span transmembrane domain
	TNF	Tumour necrosis factor
	TNFSF	Tumor necrosis factor superfamily
	TOSV	Toscana Virus
	TPB	Tryptose Phosphate Broth
	TRAFs	Tumor necrosis factor receptor-associated factors
	TRAM	TRIF-related adaptor molecule
	Tregs	Regulatory T-cells
	TRIF	TIR-domain-containing adapter inducing interferon-β
U		
	U	Units
	UBL	Ubiquitin-like protein
	US	United States
	USD	United states dollar
	USUV	Usutu Virus
	UTR	Untranslated regions
	UV	Ultraviolet
V		
	VEEV	Venezuelan equine encephalitis virus
	Vero	African Green Monkey
	VL	Visceral Leishmaniasis
	VP	Vesicle packets
	vRNA	Viral ribonucleic acid
W		
	w/v	weight/volume

	WEEV	Western equine encephalomyelitis virus
	WHO	World Health Organization
	WNV	West Nile Virus
	WT	Wild type
Y		
	YFV	Yellow Fever Virus
Z		
	ZIKV	Zika virus

CHAPTER 1: INTRODUCTION

1.1 BRIEF INTRODUCTION OF EMERGING AND RE-EMERGING INFECTIOUS DISEASES

The term “disease” generally attributes to an abnormal status that can be observed in either the structure or function of a portion or the entire body, and each disease is described as having distinct underlying causes. Internal and physiological factors, including age and the body's genetic makeup, may cause them and are limited to individuals. However, “infectious diseases” are defined as illnesses resulting from pathogens' invasion of a host and have the potential to be transmitted to other individuals. These external pathogens can be bacteria (e.g., *E. coli*), parasites (e.g., malaria), prions or viruses (e.g., yellow fever virus).

The terminology “emerging and re-emerging diseases” was formally introduced by Joshua Lederberg, Robert B. Shope, and Mary Wilson in 1987. They described these terms as either “an infectious disease whose incidence had increased over the preceding two decades” or one “which had the potential to increase in the near future” (Joshua Lederberg, Robert E. Shope et al., 1992). Since their official designation, these terms have garnered acceptance within the scientific community. These infectious diseases are either novel to humans (newly emerging e.g., from a non-human animal) or have previously only affected limited populations. Interestingly, the emergence of new infectious disease and their impact on health, was thought to have been diminishing for much of the latter half of the 20th century. However, in recent decades they have rapidly increased in incidence, or they have only recently been recognised as distinct diseases (emerging and re-emerging) or have appeared in new geographic locations (re-emerging) (Nii-Trebi, 2017).

Perhaps one of the best examples is the Human immunodeficiency viruses (HIV), a well-known emerging infectious pathogen. Acquired immunodeficiency syndrome (AIDS) was first recognised as a new disease in the 1980s, which is caused by HIV. Here, HIV strains gained the ability to pass to humans because of transmission and recombination of Simian Immunodeficiency Virus (SIV) strains, which only infect primates in nature (Sharp and Hahn, 2011; Wegner et al., 2022). It is now estimated that 40.4 million individuals have died from AIDS-related illnesses (UNAIDS, 2022). Regarding emerging infectious diseases, coronaviruses [severe acute respiratory syndrome (SARS-CoV-1)], Middle East Respiratory Syndrome (MERS-CoV)] have caused public health problems and increased their incidence in humans in the past two

decades (Morens et al., 2020; Giovanetti et al., 2023). Importantly, Coronavirus Disease-19 (COVID-19), caused by infection with SARS-CoV-2, has emerged and become a pandemic, leading to at least over 6 million deaths since 2019 (World Health Organization (WHO), 2024). Measles serves as a notable example of a re-emerging infectious disease. Measles disease is caused by *Measles morbillivirus.* Despite a notable reduction in reported measles cases globally from 2000 to 2016, attributed mainly to vaccination efforts, there was a significant surge in cases between 2016 and 2019, reaching levels not seen since 1996 (Hübschen et al., 2022).

Emerging or re-emerging infectious diseases are not only a recent phenomenon. Notable historical examples include some of the most catastrophic pandemics in human history. This includes the Black Death in the 14th century, which caused approximately 30% of the European population's deaths (Seifert et al., 2016) and the 1918 influenza pandemic, known as the Spanish flu, which resulted in an estimated 50 million deaths worldwide (Johnson and Mueller, 2002).

Importantly, over 60% of human pathogens are zoonotic in origin and constitute a significant portion of aetiological agents that lead to pandemics (Rahman et al., 2020). Infectious pathogens continuously evolve and adapt to their environment, influenced by evolutionary forces. Adaptation to a new host species is considered one of the first and most important process for the emergence and re-emergence of infectious. Once adapted, these pathogens must be able to disseminate between individuals of the host populations. Several factors contribute to the emergence and transmission of the infectious diseases. Such factors involve growing human populations, globalisations, urbanization, socioeconomic states (Morse, 2004; Spernovasilis et al., 2022). Table 1.1 provides a summary of the major factors contributing to emergence and includes some examples of infections that have recently emerged or re-emerged. However, it should be noted that there are no sharp boundaries between the factors and their examples.

Major factors	Examples of specific factors	Examples of disease emergence
Human Populations and Demographics	Due to overpopulation, human-driven land use changes and urbanisation	2014 Ebola outbreak in West Africa (Obeng-Kusi et al., 2024) Zika virus (ZIKV) outbreak is closely linked with the development of urban areas (Gubler et al., 2017)
Ecological and Environmental	Climate change (various environmental changes, including but not limited to rising sea levels and increased global temperatures), can alter the ecological range of vector habitats	Autochthonous cases of chikungunya due to the expanding geographical area of *Aedes albopictus* mosquito in France in 2014 (Roiz et al., 2015; Delrieu et al., 2023)
Globalisation	Increase rate of international travel, trade	COVID-19 has spread from its initial reported city to numerous countries across the globe through global travel (Lin et al., 2020)
Political and Social Determinants	Migration, Conflicts, Poverty and Social Inequality	Ebola re-emerged in the Democratic Republic of Congo due to several attacks on health centres and healthcare workers because of the ongoing civil war (Marou et al., 2024)
Natural Disasters	Earthquakes, flooding	ZIKV cases in Ecuador promptly increased after a 7.8-magnitude earthquake (Topluoglu et al., 2023)
Bioterrorism	Potential bioterrorist agents (such as various haemorrhagic fever viruses, smallpox)	Smallpox outbreaks were often intentionally used on populations who do not have immunity to it (Barras and Greub, 2014; Green et al., 2019)

Table 1.1: Main Determinants in Emerging and Re-emerging Infectious Diseases with Examples.

In some cases, vaccines and laboratory tests have often been developed in a remarkable and timely manner, e.g., with recent estimates suggesting 67% of the world's population has been vaccinated with a complete primary series of COVID-19 vaccines. Nonetheless, the pandemic still maintains its negative influences, including economically, on mental health, and Long COVID (LC) (IMF, 2020; WHO, 2023; Cruickshank et al., 2024)

There is sadly no vaccine for HIV/AIDS. Indeed, with 39 million people living with the disease, this virus continues to be an immense global health issue worldwide (WHO, 2022). There is yet no cure for HIV infection, but effective HIV prevention, treatment, and care does exist. However, the World Health Organization's (WHO) latest HIV Drug Resistance (HIVDR) report suggested that dolutegravir, an antiretroviral medication, has encountered increased HIV resistance (WHO, 2024).

In summary, although we have partial treatment (e.g., vaccines, antivirals) against many infectious agents, as discussed above, there is one group of emerging infectious diseases for which there is an urgent unmet need to identity and develop new medicines, those caused by arthropod-borne viruses. Indeed, when we evaluate this situation regarding these infections, we find that we have no specific treatments, and few have proven effective vaccines (Barrett, 2017). Together this emphasises the importance of scientific research in deepening our understanding of any new emerging or re-emerging pathogens, with particular emphasis on arthropod-borne pathogens; especially those that result in novel treatments and medicines.

1.2 AN OVERVIEW OF ARTHROPOD-BORNE PATHOGENS

1.2.1 Arthropod-borne Viruses (Arboviruses)

Arthropod-borne virus, also known as arbovirus, is a term that defines viruses that have a blood-feeding arthropod and at least one vertebrate in their life cycle. They can be transmitted by any arthropod vector such as sand flies, mosquitoes, ticks, and biting midges (Gubler, 2002). Following the taking of a blood meal from an infected vertebrate host, viruses must pass through several cellular barriers, and in doing so evade the arthropods' immune system, comprising RNAi, inducible signalling pathways, cellular immunity, melanisation, and ROS (Cheung et al., 2022). From the gut, they infect gut epithelium cells and disseminate to the salivary glands. Infected arthropods then can transmit arboviruses to humans or other vertebrates during biting (Franz et al., 2015; Perveen et al., 2023).

Many of these viruses have an enzootic lifecycle, which involves continuous transmission between a nonhuman vertebrate host and an arthropod vector (Musso et al., 2022). Arboviruses circulate in nature in an enzootic sylvatic cycle involving e.g., rodents, birds, or nonhuman primates as reservoir hosts. Occasionally, transmission to humans occurs through incidental bites by vectors from this cycle (e.g., WNV). In some cases, arboviruses may spread to domestic animals, that may act as amplifying hosts,

leading to a rural epizootic cycle (e.g., JEV and pigs). Additionally, certain arboviruses can adapt to infect humans directly, transitioning to an urban epidemic transmission cycle (e.g., DENV) (Figure 1.1) (Weaver and Barrett, 2004).

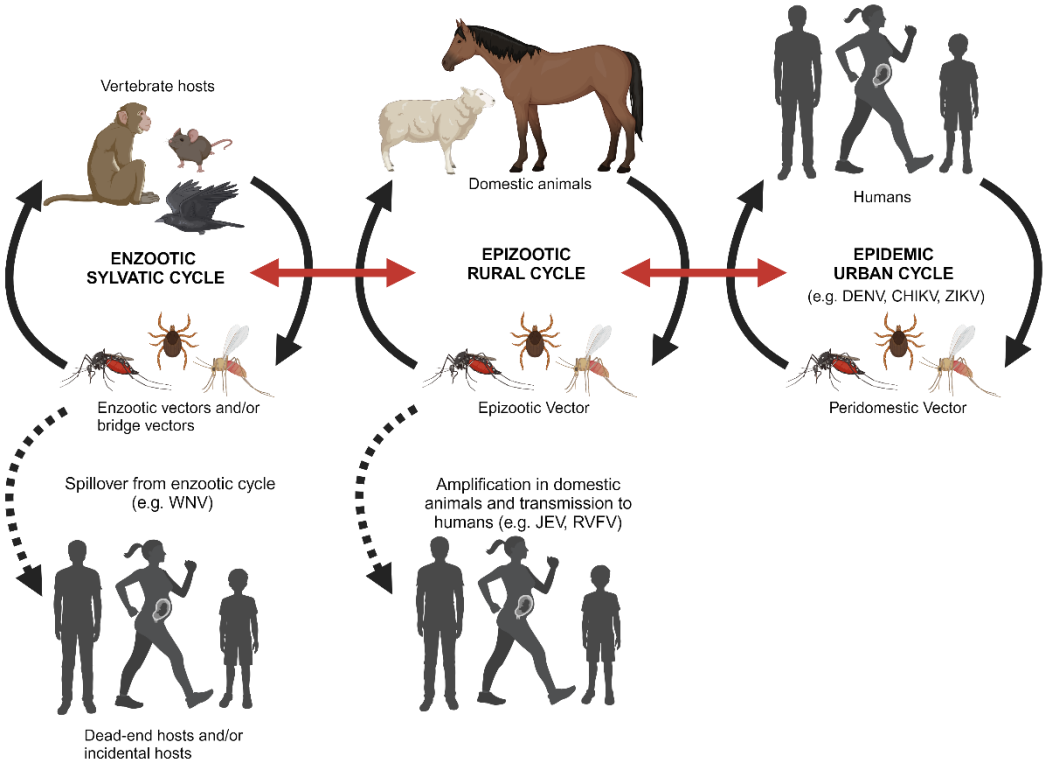


Figure 1.1: Arboviruses Transmission Cycles

Arboviruses are transmitted and maintained through various transmission cycles, including the enzootic sylvatic cycle, the epizootic rural cycle, and the epidemic urban cycle. The enzootic cycle occurs in natural hosts, such as birds or non-human primates, within sylvatic habitats. However, spillover events from these cycles can result in human infections, where humans serve as dead-end or incidental hosts—an example being the spillover of West Nile virus (WNV). The transmission may involve enzootic or bridge vectors. Certain arboviruses, like Japanese encephalitis virus (JEV) and Rift Valley fever virus (RVFV), can undergo further amplification in domestic animals, leading to human infections through an epizootic rural cycle. Additionally, many arboviruses, including Chikungunya virus (CHIKV), Dengue virus (DENV), and Zika virus (ZIKV), have adapted to be transmitted directly between humans and peridomestic vectors, sustaining transmission in urban settings. Created with BioRender.com.

Arboviruses can be passed across generations of arthropod vectors through the common and effective process known as vertical transmission. In addition to this, venereal transmissions and horizontal oral transmissions between susceptible arthropods also may occur (Ciufolini et al., 1989; Bente et al., 2013; Mueller and Cao-Lormeau, 2018).

Arboviruses encompass a wide array of viruses from at least eight taxonomic families, with over 500 identified viruses, 150 of which are recognised as causing diseases in humans (Madewell, 2020). Many arboviruses are characterised as single positive-

stranded RNA *(Togaviridae* and *Flaviviridae* virus families), whilst the majority of the segmented negative-stranded RNA arboviruses belong to the class *Bunyaviricetes* (Young, 2018).

Such arboviruses are present in tropical and sub-tropical areas and have been expanding their global distribution in recent years (Figure 1.2) (Wu et al., 2019; Socha et al., 2022; Mapalagamage et al., 2022). For example, West Nile virus (WNV), which belongs to the genus *Flavivirus* and is transmitted by mosquitoes, is also categorised as a re-emerging infection. WNV was first isolated in 1937 and is a well-known pathogen in the Old World (commonly found in Africa, Europe, and the Middle East). However, the virus reached the New World in the 1990s and subsequently became endemic in the Americas (Lanciotti et al., 1999; Gould et al., 2017).

Arbovirus infections are mostly asymptomatic (Scott C. Weaver et al., 2018). However, they can cause disease, from mild, temporary influenza-like symptoms, to disease that adversely affects daily life such as arthritis (e.g., O'nyong-nyong fever, chikungunya), or life-threatening diseases such as haemorrhagic fevers (e.g., dengue, yellow fever) and encephalitis (e.g., Toscana virus (TOSV), JEV) (Weaver and Barrett, 2004; Higgs and Vanlandingham, 2016; Brasil and Gabaglia, 2019).

WNV and Zika virus (ZIKV) infections also cause neurological involvement. For instance, ZIKV can cause congenital malformation, microcephaly, in foetuses and new-borns(Miner and Diamond, 2017). In addition to this, Guillain-Barré syndrome (GBS) as a neurological sequela in adults whose were diagnosed with ZIKV infection (Sánchez et al., 2021). Furthermore, WNV is also neuroinvasive and can cause West Nile encephalitis or meningitis (David and Abraham, 2016). TOSV is the only sandfly-borne *Phlebovirus* that can cause neurological infections and is now considered an important cause of aseptic acute meningitis, encephalitis in endemic areas (Ayhan and Charrel, 2020). Initially, the neurovirulence of the TOSV was illustrated with the initial case of possible encephalitis in a Swedish man who had visited Portugal a decade after its first isolation in 1971 (Ehrnst et al., 1985). However, like many arboviruses, TOSV infection is mostly asymptomatic or comprises acute fever and nausea, vomiting, and myalgia.

In summary, with arbovirus infections on the rise, they are of growing clinical importance. Therefore, understanding these viruses, their pathogenesis and the host response to infection, is important if we are to improve the care and design treatments for these patients.

Figure 1.2: Distribution of West Nile virus (WNV), Dengue virus (DENV), and Toscana virus (TOSV) in Europe and Mediterranean Region

Geographical distribution of Toscana virus (TOSV), West Nile virus (WNV), and Dengue virus (DENV) in Europe and the Mediterranean basin. Countries highlighted in red indicate the presence of both WNV and TOSV, those in orange indicate the presence of TOSV, pink represents regions where WNV is circulating, and green indicates areas where WNV, TOSV, and DENV are circulating in the population. Created with mapchart.net.

1.2.2 A Rising Threat of Medically Important Arthropod-borne Viruses

The burden of arboviral disease on human health is profound and recently the global incidence of arthropod-borne viruses has grown dramatically. Alarmingly, between 2000 and 2019, the WHO recorded a many-fold rise in reported cases of DENV globally, escalating from 500,000 to an estimated 96 million symptomatic cases (Bhatt et al., 2013; World Health Organization (WHO), 2023).

Over the last four decades, arboviruses have become a significant global health concern, experiencing a resurgence of viruses like WNV, DENV, and CHIKV. Additionally, unexpected emergence, as seen with ZIKV, has led to major outbreaks characterised by new clinical manifestations, such as congenital Zika syndrome and novel modes of transmission, like sexual transmission for ZIKV, previously unreported for arboviruses (Musso et al., 2022).

Not only human health, but importantly livestock are also adversely affected by arboviruses such as Rift Valley Fever virus (RVFV) and bluetongue virus (BTV).

RVFV, belonging to the *Hareavirales* order, is known to cause severe disease with high mortality and abortion in livestock such as sheep and cattle, occasionally spreading to affect other domestic animals and humans (Weaver and Reisen, 2010). BTV, a member of the *Orbivirus* genus, can lead to death and cause abortion or deformities in lambs or calves; outbreaks have been reported widely worldwide, reaching 3 billion US dollars in losses by BTV infectious globally (Ben Salem et al., 2024).

Despite this, no specific treatments are currently available for these viruses (Weaver and Reisen, 2010), and few have vaccines (Monath, 2005). The reasons for this are multi-factorial and include economics, the unpredictable nature of outbreaks, and their genetic diversity, all of which help conspire to make developing and stockpiling targeted antiviral drugs or vaccines difficult. Even for those viruses for which we have effective vaccines, stockpiling and timely distribution can hinder response to outbreaks, as e.g., evidence by the recent, poorly contained outbreaks of YFV in Africa (Lindsey et al., 2022).

1.2.3 Alphavirus Genus

Alphaviruses, which belong to the family *Togaviridae,* are transmitted by mosquitoes (e.g., *Aedes sp.* mosquitoes) and are widely distributed across continents, relying on the presence of competent vectors. There are noteworthy examples of emerging and re-emerging alphaviruses, due to the expanding distribution of mosquito vectors. Recent examples include the ongoing CHIKV epidemics in India, since 2005, and the Americas (since 2012), and outbreaks of Mayaro virus (MAYV) in tropical regions of South American countries (Azar et al., 2020). In addition, some alphaviruses also pose a threat as potential bioterrorism agents; Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV) and Western equine encephalomyelitis virus (WEEV) are important encephalitic pathogens that can be transmitted by aerosols (Roy et al., 2010).

Although most of these viruses are maintained involving zoonotic host species and mosquito vectors in sylvatic transmission cycles, due to a variety of determinants (e.g., urbanisation, climate change), they can spill over into humans (Kim and Diamond, 2023). CHIKV has evolved to enable efficient mosquito-borne transmission between humans in urban environments, which does not require other vertebrate hosts.

1.2.3.1 Taxonomy

Alphaviruses are classified based on their genetic similarity and clinical characteristics. Encephalitic alphaviruses, including EEEV, WEEV and VEEV responsible for meningitis and encephalitis, frequently leading to enduring neurological complications (e.g., paralysis) (Sah et al., 2023). CHIKV, Ross River virus (RRV), O'nyong'nyong virus (ONNV), Barmah Forest virus (BFV), MAYV, Sindbis virus (SINV), and Semliki Forest (SFV) induce musculoskeletal illness marked by symptoms including fever, rash, joint pain (arthralgia), muscle pain (myalgia), muscle inflammation (myositis), and both acute and chronic polyarthritis (Zaid et al., 2021).

When based on genetic similarity and serological cross-reactivity, Alphaviruses are also grouped into eight antigenic complexes. These are: Barmah Forest, Eastern Equine Encephalitis (EEEV), Venezuelan Encephalitis (VEEV), Western Equine Encephalitis (WEEV), Middelburg, Ndumu, Semliki Forest, and unclassified complex. Generally, viruses that belong to the Semliki Forest, Middelburg, and Ndumu complexes are only present in the "Old World," (a notable exemption being CHIKV which has spread to the Americas). This contrasts with the WEEV, EEEV, and VEEV complexes that are limited to the Americas, aka the "New World" (Zaid et al., 2021).

This next section will discuss CHIKV, a medical and socioeconomic importance, and SFV, the prototypical laboratory alphavirus.

1.2.3.2 Structure and Replication

The alphaviruses are small (around 65-70nm in diameter), spherical viral particles comprising a 12-kilobase (kb) positive-sense single-stranded RNA genome enclosed within an icosahedral protein nucleocapsid, which is then enveloped by a lipid membrane derived from the host (Figure 1.3) (Richardson and Vance, 1976; Strauss and Strauss, 1994).

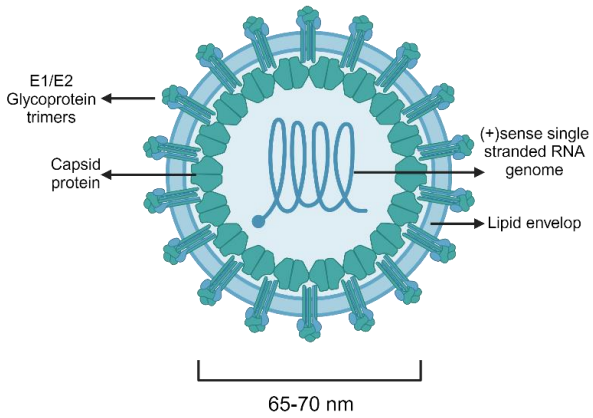


Figure 1.3: Alphavirus Virion Structure
Alphaviruses are small (65-70nm in diameter), enveloped viruses and possess a positive-sense single-stranded RNA genome. Their capsid exhibits icosahedral symmetry. The viral envelope, derived from the host cell's lipid membrane, is embedded with 80 glycoprotein spike structures, each consisting of a trimer of E1/E2 proteins. This figure was created on BioRender.com.

The genome of alphaviruses contains two open reading frames (ORFs) enclosed by untranslated regions (UTR) with a 5' cap and a 3' poly(A) tail. The 5' ORF encodes the non-structural polyproteins (nsP1, nsP2, nsP3, and nsP4), involved in virus replication and pathogenesis, translated from the genomic RNA. Meanwhile, the 3' ORF is translated from subgenomic 26S RNA and encodes structural polyproteins (capsid, E3, E2, 6k/TF, and E1) that compose the virion (Figure 1.4). Together, these proteins moderate viral transcription, replication, and host cell antagonism (Jose et al., 2009; Kim and Diamond, 2023).

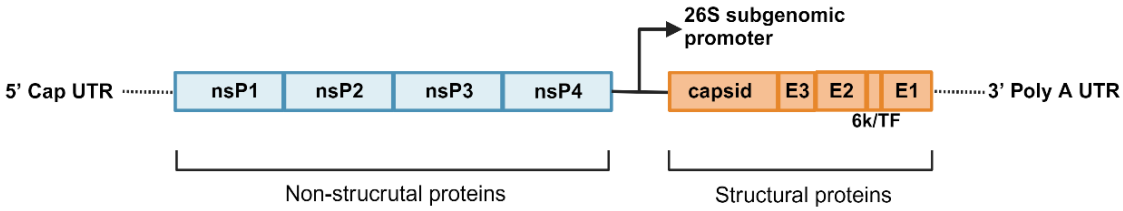


Figure 1.4: Alphavirus Genome Structure
The genome is ssRNA(+) that features two open reading frames (ORFs) and is capped at the 5′ end, with a 3′ poly(A) tail. The 5' ORF encodes the non-structural (ns) proteins, translating into nsP1, nsP2, nsP3, and nsP4. The 3' ORF encodes for the structural proteins and gets translated through the 26S subgenomic promoter into the capsid, 6k/TF, and the glycoproteins E1, E2, and E3. This figure was created on BioRender.com.

The virus attaches to a cellular receptor (possible receptors, e.g., heparan sulphate, CD209, L-SIGN) and is internalised by endocytosis. Inside the cell, the virus undergoes pH-dependent membrane fusion with the endosome, releasing the nucleocapsid into the endosome. Negative-strand RNA is firstly synthesised and serves as a template for both

genomic RNA and subgenomic RNA. The subgenomic RNA is translated to produce a structural polyprotein, which is then cleaved into individual structural proteins. Both structural and non-structural proteins assemble into immature virions in the endoplasmic reticulum (ER). Finally, the mature virus buds from the cell (Strauss and Strauss, 1994; Klimstra et al., 2004; Rupp et al., 2015).

1.2.3.3 Semliki Forest Virus – a well characterised model virus that infects mice.

In 1942, female *Aedes* mosquitoes were caught and pooled for a field investigation on the epidemiology of yellow fever. However, this study unexpectedly revealed an unknown alphavirus in the Semliki Forest region of Bwamba, Uganda (Smithburn and Haddow, 1944; Manwaring, 1945). This positive single-stranded RNA alphavirus was named after the forest where it was discovered, SFV. SFV circulates in sub-Saharan, central Africa and has been detected in various animal species, including rodents, horses, monkeys, and rabbits (Kemp et al., 1974; Hubálek et al., 2014). Small wild animals, birds, and nonhuman primates might the natural hosts of SFV (Ylösmäki et al., 2013). Its primary transmission vectors are *Aedes aegypti* and *Aedes Africanis* mosquitoes (Fazakerley, 2002).

In the 1980s, an outbreak of 22 symptomatic patients was associated with SFV infections in the Central African Republic (Mathiot et al., 1990). Although SFV infection causes self-limited febrile diseases in humans, only one reported fatal encephalitis is associated with SFV infection (Willems et al., 1979). However, SFV-infected mice can develop fatal central nervous system (CNS) infections (Fazakerley, 2002). Therefore, alphavirus infections, mainly SFV, in rodents has been investigated as a model for studying viral encephalitis.

Following the first isolation (Smithburn and Haddow, 1944), the original strain, along with three additional isolates from passaged in small mammals, including mice, were studied, and various SFV strains have been adapted for use in the laboratory (Bradish et al., 1971). SFV strains can be broadly categorised into two groups: virulent strains and avirulent strains, L10 (the original SFV isolate), V13, SFV4, and SFV6; and avirulent A8, A7, A7(74), MRS MP 192/7, respectively (Atkins et al., 1985; Fazakerley, 2002). However, these so-called virulent strains exhibit a strict host age dependency and become virulent in neonatal or young suckling mice (Bradish et al., 1971; Pusztai et al., 1971). All strains of SFV infect neurons and oligodendrocytes (Fragkoudis et al., 2009). As only SFV4 has been used for this thesis, it will be discussed further. The SFV4 strain, was generated by in vitro transcription and transfection of RNA from an

infectious cDNA clone (pSP6-SFV4) derived from the virulent prototype strain (Liljeström et al., 1991). SFV4 is also neurovirulent and fatal in adult mice following intracerebral inoculation. However, this strain rarely triggers clinical disease, especially CNS infection, when is inoculated intranasally, intraperitoneally, or subcutaneously by needle in the absence of mosquito saliva (Fragkoudis et al., 2007; McKimmie and Fazakerley, 2016).

SFV is advantageously classified as a biosafety level 2 pathogen, while other medically important alphaviruses commonly require BSL-3 facilities. SFV has been utilised as an experimental virus to understand the mechanism of viral encephalitis, and several studies have focused on pathogenesis and dissemination in neural tissue after initiating the SFV infection (Atkins et al., 1985; Atkins et al., 1999; Fazakerley, 2002; Fragkoudis et al., 2009). Furthermore, (Shi et al., 2023) developed a recombinant SFV-eGPB reporter virus, a safe surrogate for highly pathogenic alphaviruses, as a tool for antiviral study. It also showed that SFV replicates and spreads effectively in both immune-competent mice and Aedes mosquitoes (Rodriguez-Andres et al., 2012; Ferguson et al., 2015). Therefore, an in vivo model of SFV has been developed to investigate the pathogenesis of virus infection following the subcutaneous inoculation of the virus in the skin in the presence of mosquito bite (Pingen et al., 2016). The study showed that following subcutaneous injection of SFV4, the virus disseminates to the draining popliteal lymph node (dLN) within up to 6 hours post-infection, with peak viremia observed 24 hours after infection. Moreover, the virus infection can be detected more enhancement in the presence of mosquito bites with higher virus RNA than the control group.

With respect to this thesis, we assess whether sandfly saliva could modulate host susceptibility to SFV infection, a well-defined mouse model of arbovirus infection. This will be discussed in greater detail in section 3.5 of Chapter 3.

1.2.3.4 CHIKV – a medically important alphavirus.

In 1953, Chikungunya, which refers to contorted posture in the Kimakonde language, the virus was first isolated in Tanzania from the blood of a febrile patient. CHIKV infections can progress with severe fever, headache, vomiting, rash, myalgia and commonly polyarthralgia with crippling joint pains (Ross, 1955). Moreover, this debilitating rheumatologic disease can become chronic in some patients (Hawman et al., 2013). After the first isolation in Africa continent, CHIKV was defined in patients in Thailand, Asia in 1958 (Hammon et al., 1960). Later, substantive outbreaks of CHIKV

infection were reported in India, Africa, the Indian Ocean islands, Europe, Southeast Asia, the Caribbean islands, and the Americas (Schuffenecker et al., 2006; Dash et al., 2007; Rezza et al., 2007; Sergon et al., 2008; Hapuarachchi et al., 2010; Van Bortel et al., 2014; Weaver and Lecuit, 2015). Therefore, CHIKV infection has become a global health concern since it was first reported.

Aedes aegypti mosquitoes, is an important vector for CHIKV, are highly anthropophilic and geographically widespread. Importantly, *Aedes albopictus*, an invasive species, was also shown to be vector competent for CHIKV (Vega-Rúa et al., 2020). Originating in tropical Southeast Asia, *Aedes albopictus* has progressively played a larger role in the transmission of CHIKV in numerous regions where it has expanded its habitat. For example, over 200 autochthonous cases of CHIKV were reported in 2007 in two villages in Italy, and positive CHIKV sequences were detected in captured *Aedes albopictus* mosquitoes in the same region (Rezza et al., 2007). This is due to a mutation in the virus's E1 envelope protein gene, which allows efficient infection of *Aedes* albopictus mosquitoes (Tsetsarkin et al., 2007).

CHIKV has three genotypes based on the geographical location of its origin: West African, East/Central/South African (ECSA), and Asian strains (Volk et al., 2010). Phylogenetic analysis showed that genetic changes in the ECSA strain occurred when the virus spread in recent outbreaks. These CHIKV outbreaks had severe social and economic consequences, especially in the 2006 CHIKV epidemic on La Réunion island, and the 2014 US Virgin Islands outbreaks, which cost over £30 million for their economies (Soumahoro et al., 2011; Feldstein et al., 2019).

Animal models have been established to investigate CHIKV immunity and pathogenesis and serve as a nonclinical model for the development of anti-CHIKV drugs or vaccines. One of the first CHIKV in vivo infectious studies assessed pathogenesis on young immunocompetent mice model via intracerebral injection route (Ross, 1956). One of the challenges of using a wild type, e.g., C57BL/6, adult mice is that CHIKV does not disseminate efficiently. However, the high titre of CHIKV in young mice, make this a model for studying some aspects of CHIKV infection, such as viral persistence in joint tissues (Morrison et al., 2011; Haese et al., 2016). Other mouse and non-human primate CHIKV models, including IFN receptor-deficient mice have also been used to investigate antivirals and vaccines' efficacy (Constant et al., 2021).

1.2.4 Flavivirus Genus

The Flavivirus genus contains >70 members, including several that are known human and animal pathogens that are transmitted by arthropods. Flaviviruses infectious can cause asymptomatic or mild fever to haemorrhagic disease [e.g., DENV, yellow fever (YFV)] or neuropathological diseases [e.g., ZIKV, Japanese Encephalitis (JEV), WNV] (Gould and Solomon, 2008). Medically important flaviviruses circulate on all continents. These pathogens impose a substantial disease burden globally, with some estimates of dengue alone at up to 400 million infections annually of ~ which 30–50% are symptomatic million cases annually (Bhatt et al., 2013). The classification of flaviviruses is continually evolving to incorporate newly discovered viruses (Moureau et al., 2015). In this section, I will provide a brief overview of flavivirus structure and replication mechanisms, with a particular focus on ZIKV infection.

1.2.4.1 Structure and Replication

Flaviviruses are small (~50 nm) spherical, enveloped virus particles. The flavivirus positive sense single-stranded RNA genome (~11kb size) encodes three structural proteins, E (envelope) protein, C (capsid) protein and pr/M (premembrane/membrane) protein, as well as seven non-structural proteins. The nucleocapsid is surrounded by a lipid bilayer derived from the host cell (Figure 1.5) (van Leur et al., 2021).

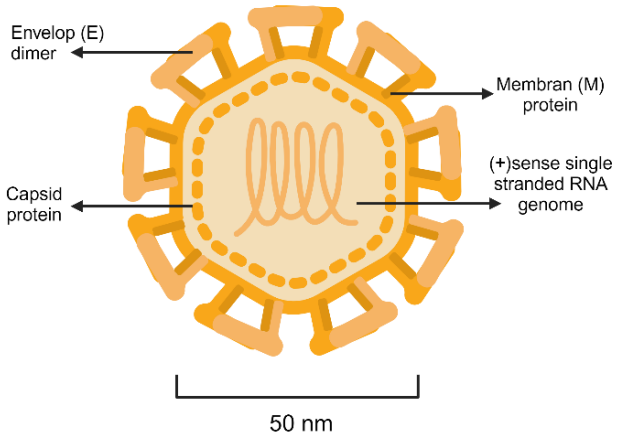


Figure 1.5: Flavivirus Virion Structure

Flaviviruses are small (50nm in diameter), enveloped, icosahedral viruses containing an unsegmented, positive-strand RNA genome. The C protein coats the viral nucleic acid, and the M and E proteins form symmetric structures. This figure was created on BioRender.com.

The genome possesses a single open reading frame (ORF) which codes for a single polyprotein is initially produced, which is then cleaved to generate both structural and non-structural proteins and is framed by 5’ and 3’ untranslated regions (UTRs).

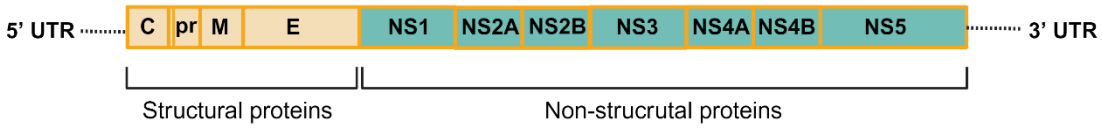


Figure 1.6: Flavivirus Genome Structure
The genome contains a single open reading frame (ORF) flanked by 5’ and 3’ untranslated regions (UTRs). This ORF encodes a polyprotein, which is cleaved by host and viral proteases during the replication cycle into three structural proteins—capsid (C), premembrane/membrane (pr/M), and envelope (E)—as well as seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. This figure was created on BioRender.com.

The viral glycoprotein E attaches to a cellular receptor to initiate internalization through endocytosis. Although the specific entry receptor of individual flaviviruses remains unidentified, the host receptors: DC-SIGN, Tyro3 and tyrosine kinase receptor AXL are linked to ZIKV entry (Hamel et al., 2015). The acidic pH environment inside host cell endosomes induces a conformational change in the E protein of the virus, facilitating fusion between the viral envelope and the endosomal membrane. This fusion event enables the release of the viral genome into the cytosol (Fritz et al., 2008). Within the cytoplasm, the viral RNA (vRNA) is translated by the host's translational machinery, producing a polyprotein. This polyprotein is then inserted into the membrane of the endoplasmic reticulum (ER). The polyprotein is then cleaved by both host and viral proteases, resulting in the generation of three structural proteins and at least seven non-structural proteins. Following, the production of new viral particles initiates within vesicle packets (VP) formed by the endoplasmic reticulum (ER). Subsequently, the VP can bud into the ER, forming an immature virion. Then, viral maturation occurs in Golgi apparatus. The infectious viral particles are released from the host cell by exocytosis (van Leur et al., 2021).

1.2.4.2 ZIKV – a medically important member of *Flavivirus.*

ZIKV was initially isolated in 1947 from a rhesus monkey in the Zika forest of Uganda, marking its first discovery (Dick et al., 1952). ZIKV is one of the important but neglected mosquito borne Flaviviruses. Although *Aedes sp.* mosquitoes principally transmit the virus, there are also reports of human sexual transmission and vertical transmission from infected pregnant woman to their foetus. (Miner, 2017; Pletnev et al.,

2021). ZIKV causes a self-limiting febrile illness with rash, headache, conjunctivitis, and muscle pain. ZIKV can result in congenital abnormalities in foetuses and newborns, including conditions like cortical atrophy with microcephaly, along with functional challenges such as dysphagia and epilepsy. These malformations may manifest following either symptomatic or asymptomatic infection of pregnant women (Miner and Diamond, 2017; Smoots et al., 2020; Paixao et al., 2022). In addition to this, Guillain-Barré syndrome (GBS) is a neurological sequela in adults who were diagnosed with ZIKV infection (Acosta-Ampudia et al., 2018; Sánchez et al., 2021).

Prior to 2007, there were less than 20 reported ZIKV human cases after its identification in 1954 (MacNamara, 1954; Musso and Gubler, 2016). In 2007, the first documented ZIKV outbreak was reported on the Pacific Island of Yap (Duffy et al., 2009). In 2013-2014, a large outbreak started in French Polynesia, that reached Brazil in 2015 and was subsequently detected in much of the Americas. As of July 2019, autochthonous ZIKV infection has been described in over 80 countries worldwide (Gatherer and Kohl, 2016; Petersen et al., 2016). In the last few years, the number of reported ZIKV cases have declined, likely due to underreporting and lockdowns due to the COVID-19 pandemic. However, epidemics and outbreaks continue in some regions, including India and Southeast Asia and it is expected to initiate new outbreaks as e.g., host immunity declines (Giraldo et al., 2023).

ZIKV does not replicate in immunocompetent mice e.g., wild-type (WT) C57BL/6, BALB/c (Rossi et al., 2016; Lazear et al., 2016). Arboviruses have evolved mechanisms to antagonise host interferon (IFN) responses, including ZIKV. Type I interferon signalling is inhibited by the NS5 viral protein of ZIKV, which cause the degradation of STAT2 (JAK- STAT pathways in humans), an IFN-regulated transcriptional activator (Grant et al., 2016a). However, ZIKV N5 protein is not able to bind, degrade or antagonise mouse STAT2, therefore this effect is species specific (Wu et al., 2017; Shu et al., 2021). As a result, ZIKV mouse models have been developed, which depend on the absence of interferon or mouse STAT2 proteins for understanding disease and assessing vaccines and antivirals (Morrison and Diamond, 2017). More recently, a mouse model of an immunocompetent transgenic knock-in mouse expressing human STAT2 instead of mouse STAT2 has been established. This mouse is susceptible to ZIKV replication and pathogenesis (Gorman et al., 2018a). Apart from mouse models, there are limited non-human models for ZIKV infection (Morrison and Diamond, 2017).

Although ZIKV is not the focus of this thesis, its recent emergence serves as an important example by which arboviruses can go from complete obscurity to the causative agent of a WHO-declared pandemic.

1.2.5 Phlebovirus Genus

Phleboviruses are a genetically diverse group of viruses from the genus *Phlebovirus*, family *Phenuiviridae*, order *Hareavirales*. In the 2022 report of the International Committee on Taxonomy of Viruses (ICTV), the *Phlebovirus* genus contains 67 known virus species (Walker et al., 2021; Sasaya et al., 2023), of which more than 40, including 11 species linked to human illness, have been identified in sand flies (Diptera: Psychodidae, Phlebotominae) through isolation or detection (Kuhn et al., 2020). However, some of *Phlebovirus* members can also be transmitted by mosquito (e.g., RVFV) and ticks (e.g., Mukawa Virus) (Oker-Blom et al., 1964; Nanyingi et al., 2015). *Phleboviruses* are globally distributed, with notable presence across the Mediterranean, Middle East, sub-Saharan Africa, and Africa. Like most arboviruses, these infectious in humans are often asymptomatic or cause flu-like symptoms but can also lead to life-threatening diseases such as acute viral haemorrhagic fever (Elliott and Brennan, 2014).

The impact of *Phleboviruses* on public health and the agroeconomic are noteworthy. For example, RVFV causes abortion and 95–100% mortality among newborns of domestic ruminants due to severe hepatic damage, and e.g., the 2007 RVFV outbreak in Kenya and Tanzania cost ~$66 million USD (Pepin et al., 2010; Peyre et al., 2015). In this section, I will discuss the *Phlebovirus* genus structure, replication, and sandfly-borne viruses, particularly TOSV, which is studied in this thesis.

1.2.5.1 Structure and Replication

Phleboviruses are spherical, enveloped RNA viruses approximately 100nm in diameter. They possess a tri-segmented negative-sense RNA genome named after their size: L, large, M, medium, S, and small. Virions consist of only four proteins: two glycoproteins known as Gn and Gc, which are integrated into the Golgi-derived viral membrane, and two internal proteins; the nucleocapsid (N) protein, responsible for encapsulating each of the three genome segments in the form of ribonucleoprotein complexes; and the L protein, functioning as an RNA-dependent RNA polymerase (Elliott and Brennan, 2014; Amroun et al., 2017).

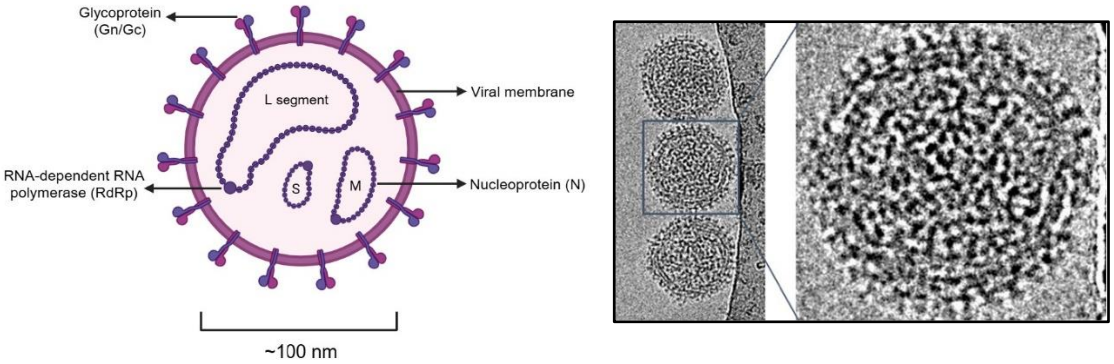


Figure 1.7: Diagrammatic Representation of *Phlebovirus* Virion In-cross Section (left) and Electron Microscope Image of TOSV (right)

The surface spikes consist of Gn and Gc glycoproteins. The three helical nucleocapsids, which are circular, each contain one of the unique single-stranded RNA segments (L, large; M, medium; S, small) encapsulated by the N protein and associated with the L protein. Electron micrograph of TOSV. Scale bar, 50 nm. Credit by (Koch et al., 2023) The left figure was created on BioRender.com.

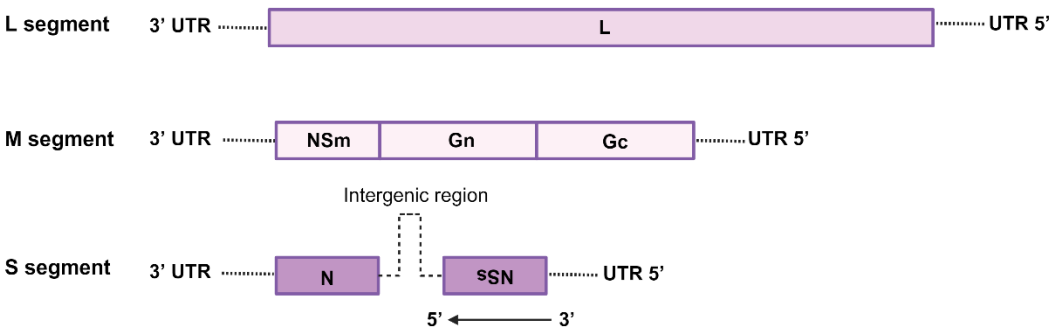


Figure 1.8: Phlebovirus Genome Structure

Phleboviruses contain tripartite single-stranded RNA genome. L segment (negative-sense) encodes the viral component of the RNA-dependent RNA polymerase (RdRp). M segment (negative-sense) encodes Gn/Gc and a non-structural protein (NSm). S segment encodes (ambisense) the nucleocapsid (N) protein and a non-structural protein (NSs). This figure was created on BioRender.com.

The S segment encodes the nucleocapsid (N) protein responsible for encapsulating the viral RNA-replication products to form the ribonucleoprotein (RNP) complex. The M segment encodes a polyprotein precursor, which then is cleaved into Gn and Gc components via host-cell proteases in the ER. The Gn-Gc heterodimer is involved in virus assembly and attachment to new target cells. The L segment encodes the viral component of the RNA-dependent RNA polymerase (RdRp) (Walter and Barr, 2011). The S and M segments also encode NSs and NSm which are non-structural proteins,

respectively (Piper et al., 2011; Walter and Barr, 2011). Although, NSs is not required for efficient *Phlebovirus* replication in cultured mammalian cells (e.g. RVFV) (Gerrard et al., 2007; Piper et al., 2011; Walter and Barr, 2011), they are required for efficient suppression of the interferon (IFN) response (Gori-Savellini et al., 2013; Kalveram and Ikegami, 2013; Savellini et al., 2019; Woelfl et al., 2020) and therefore likely have an important role in supressing anti-viral immunity during infection *in vivo*.

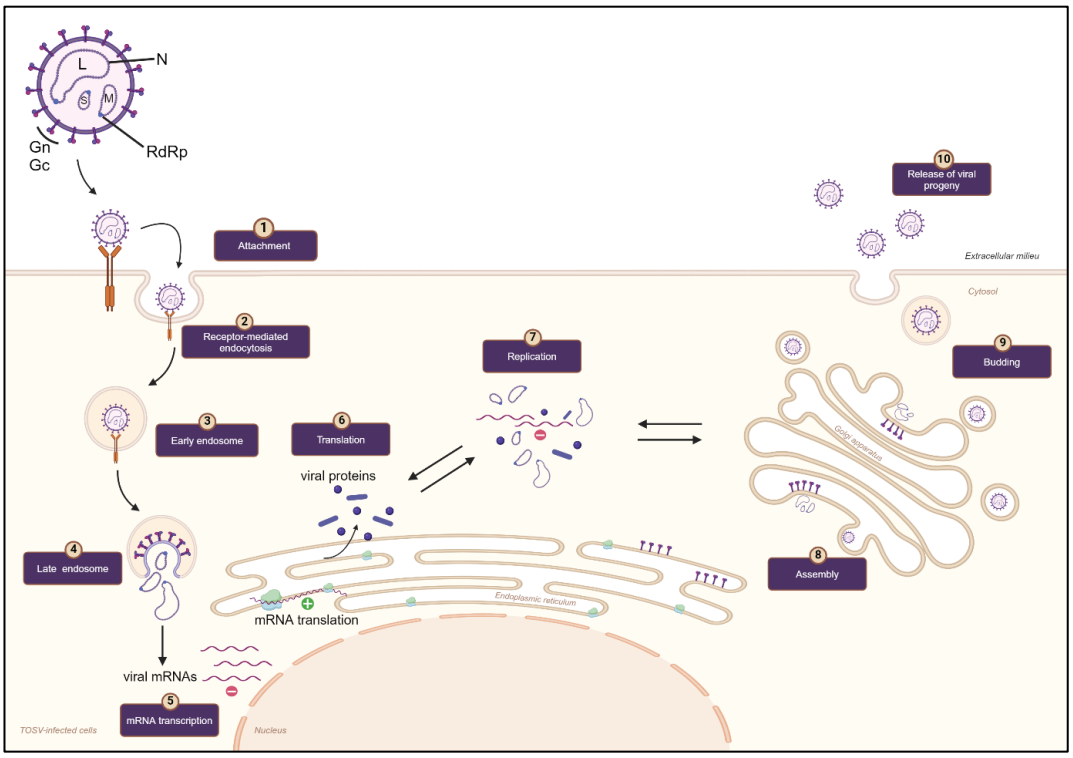


Figure 1.9: Replication Cycle of Phleboviruses

1. Phleboviruses, including TOSV, use virus encoded glycoproteins, Gn and Gc, to bind cell surface molecules DC-SIGN, L-SIGN and heparan sulphate (glycosaminoglycan) (Lozach et al., 2011; Spiegel et al., 2016). This is similar for the related phenuiviruses that also target DC-SIGN. Receptor use is likely cell-specific, e.g. with DC-SIGN mediated entry occurring in dendritic cells (DC). **2.** Virus enters the cell via receptor-mediated endocytosis (Koch et al., 2021). **3.** Once internalised, the viral particles move through early and late endosomes. **4.** In late endosomes, acidification induces the membrane fusion activity of the Gc protein with the endosomal membrane. This fusion triggers encapsidation of the viral genome and virus RdRp are released into the cytoplasm, where primary transcription and replication occur (Lozach et al., 2010). **5.** N interactions with RdRp allow access to the ribonucleoproteins (RNP), which serves as a template for the transcription of new mRNA (Lozach et al., 2010). Following translation of the viral mRNAs and genome replication **(6-7)**, viral Gn/Gc are cleaved by host-cell proteases in the endoplasmic reticulum, allowing Gn–Gc glycoprotein heterodimers to reach the Golgi apparatus (Spiegel et al., 2016), **(8.)** where viral assembly occurs (Hornak et al., 2016). **9.** Newly formed virions decorated with Gn and Gc in the Golgi bud via vesicles to the plasma membrane and **10.** are released from the host cell by exocytosis (Wuerth and Weber, 2016; Spiegel et al., 2016). This figure was created on BioRender.com.

1.2.5.2 Sandfly-borne Viruses

There are now more than 40 species of *Phleboviruses* that have been identified through either isolation from, or detection in, phlebotomine sandflies. Following infection in humans, they typically have an asymptomatic course. Some of these viruses can also cause mild, self-resolving, flu-like illness, but others have been associated with more severe illness, such as with TOSV infections, which can lead to meningitis and encephalitis. Interestingly, the genus name is derived from *Phlebotominae*, the taxonomic group of vectors for member species such as *Phlebovirus napoliense*, originating from the Greek word "phlebos," which means "vein" (Calisher and Calzolari, 2021). Sandfly-borne *Phleboviruses* are widely distributed and prevalent, facilitated by the presence of vectors from the genera *Lutzomyia* in Central and South America, and *Phlebotomus* and *Sergentomyia* in Africa, Europe, and Central Asia (Depaquit et al., 2010; Alkan et al., 2017). The interactions between vectors and viruses help determine the geographical spread and contribute to the inter-epidemic persistence and evolution of *Phleboviruses* (Horne and Vanlandingham, 2014).

1.2.5.2.1 *Phlebovirus napoliense* and *Phlebovirus siciliaense*

Sandfly fevers, known also as pappataci fever, were to first isolated from febrile United States (US) soldiers in Italy in 1943 and 1944 (Sabin, 1955). These viruses became important during the Second World War, as although infection with them typically caused flu-like in residents in the endemic region, immunologically naïve soldiers suffered long illnesses and were incapacitated in large numbers (Sabin, 1944). The symptoms of infections caused by *Phlebovirus napoliense* (sandfly fever Naples phlebovirus) and *Phlebovirus siciliaense* (sandfly fever Sicilian phlebovirus) are clinically comparable, often presenting as a 3-day fever marked by a sudden onset of fever, headache, muscle pain, sensitivity to light, and nausea (Ayhan and Charrel, 2017). Although the geographic distributions, their main vector (*Phlebotomus papatasi*) and pathogenesis of these viruses show similarities, according to the International Classification for Taxonomy of Viruses (ICTV), they are classified into two complexes, which are; 1.) the sandfly fever Naples serocomplex, also including TOSV; and 2.) the sandfly fever Sicilian serocomplex (Marklewitz et al., 2020). These virus serocomplexes have significantly different antigenic properties. Therefore, there is no immune cross-protection among them (Alkan et al., 2013).

They are extensively distributed across the Mediterranean region, the African continent, the Indian subcontinent, the Middle East, and Central Asia (Ergunay et al., 2017).

Sandfly fever virus serotypes were studied epidemiologically in a wide range, including Europe, Africa, and Mediterranean regions. In some regions around the Mediterranean, antibody prevalence rates for *Phlebovirus napoliense* and *Phlebovirus siciliaense* have been shown to be greater than 50% (R. Eitrem et al., 1991; Alkan et al., 2013; Ergunay et al., 2017). It is important to note that cross-reactions might have influenced the estimation of the seroprevalence rate. Serological cross-reactions have been documented within the sandfly fever Naples serocomplex, of which TOSV is a member (Depaquit et al., 2010).

Phlebotomus papatasi is the most well-defined vector of *Phlebovirus napoliense* and *Phlebovirus siciliaense*. In addition, *Phlebovirus napoliense* was also isolated from *Ph. perniciosus* in Italy (Verani et al., 1980) and from *Ph. perfiliewi* in Serbia (Gligić et al., 1982). Field and experimental studies have shown that transovarial transmission (female to offspring) and/or venereal transmission play important roles in maintaining viruses. Generally, while various sandfly-borne *phleboviruses* have been isolated from vertebrates (e.g., humans, bats), there is limited conclusive evidence indicating that vertebrates play a significant role in the natural life cycle of these viruses, suggesting they may simply serve as dead-end hosts (Ayhan and N. Charrel, 2019).

To develop *in vivo* models of these infections, patients' serum (containing *Phlebovirus napoliense* and *Phlebovirus siciliaense*) were injected via various routes into monkeys, hamsters, mice, rats, rabbits, and guinea pigs. However, they did not cause clinical symptoms. Subsequently, *Phlebovirus napoliense* and *Phlebovirus siciliaense* were effectively adapted to suckling mice through a series of consecutive passages (Sabin, 1955).

1.2.5.2.2 TOSV – an encephalitic *Phlebovirus.*

TOSV (*Phlebovirus toscanaense*) is an arbovirus within the genus Phlebovirus. (King et al., 2018; Kuhn et al., 2023). TOSV is one of the medically important arboviruses transmitted by *Phlebotomus sp.* sandflies. TOSV was initially isolated from *Phlebotomus (Ph.) perniciosus* and *Ph. perfiliewi*, that act as competent vectors of TOSV, collected in the Tuscany region of central Italy in 1971 (Verani et al., 1982). TOSV is the only sandfly-borne phlebovirus that can cause neurological infections and is now considered an important cause of aseptic acute meningitis, encephalitis, especially during the warm season in endemic regions (Ayhan and Charrel, 2020). Initially, the neurovirulence of the TOSV was illustrated with the initial case of possible encephalitis in a Swedish man who visited Portugal a decade after its first isolation in

1971 (Ehrnst et al., 1985). In 1983, it was isolated for the first time from patients with meningitis (Nicoletti et al., 1991).

Phylogenetic analyses have revealed three distinct lineages of TOSV, denoted as A, B, and C (Echevarría et al., 2003). Presently, there are no differences in virulence or clinical symptoms among these genetic lineages. In regions like France, Türkiye, and Croatia, at least two lineages are known to coexist (Ayhan et al., 2020). Recently, the complete sequence of the lineage A of TOSV, was made available, leading to the establishment of the first reverse genetic system capable of recovering infectious recombinant TOSV (rTOSV) from cDNA. This advancement allows for the creation of genetically modified versions of the virus. By generating an NSs-deficient rTOSV capable of expressing reporter genes, it enables visualization and tracking of intracellular replication, essential for further research and vaccine development efforts (Alexander et al., 2020). A developed reverse genetic system is also available for a lineage B strain of TOSV (Woelfl et al., 2020).

1.2.5.2.2.1 Epidemiology of TOSV: a historical context.

The burden of arboviral diseases on human health is profound and recently its incidence has grown, although increased diagnosis may also account for some of this. Approximately 250 million individuals in the Mediterranean region are at risk of TOSV infection. This heightened number is mainly attributed to factors including climate change and globalisation. Consequently, cases of TOSV and other arboviruses are on the rise (Ayhan, Baklouti, et al., 2017).

After TOSV's first identification in sandflies in Italy in 1971, early seroepidemiological studies frequently did not assess TOSV distribution and often was referred to as “Phlebotomus fever” or “Pappataci fever” (Sabin et al., 1944; R. Eitrem et al., 1991). It was not until ten years after its identification, that TOSV was registered in the International Catalogue of Arthropod-Borne Viruses (Karabatsos, 1978). The ability of TOSV to be neurovirulent was first shown following investigation of cerebrospinal fluid (CSF) from a Swedish patient with encephalitis, who had recently visited Portugal (Ehrnst et al., 1985). TOSV infection has also been reported in Swedish United Nations soldiers following stays in Cyprus (Niklasson and Eitrem, 1985; Eitrem et al., 1990). Perhaps not surprisingly, there is a high antibody prevalence rate of 20% to TOSV among Cyprus's local population (R. Eitrem et al., 1991). Travellers returning from TOSV endemic regions also regularly present with evidence of TOSV infection

(Calisher et al., 1987; Schwarz et al., 1993; T. F. Schwarz, Jäger, Gilch and Pauli, 1995; Schwarz et al., 1996; Dobler et al., 1997).

The clinical importance of these infections is underlined by a study assessing the viral aetiology of CNS infection among children in the Siena province of Italy. Importantly, this indicated that TOSV is responsible for at least 80% of children's acute viral CNS infections during the summer (A. Braito et al., 1998). This peak of CNS infection was coincident with a high frequency of adult insect vectors (*Ph. perniciosus* and *Ph. perfiliewi*), that typically peaks in August (Valassina et al., 1998). A similar study in Tuscany, Italy showed again that TOSV is the primary etiological agent (81%) of acute aseptic meningitis in the summer (Valassina et al., 2000). With increasing globalisation and frequency of tourists visiting these regions, the virus is regularly infecting tourists (Assunta Braito et al., 1998). In addition, seroprevalence studies suggest a large proportion of infection is common and occurs without obvious clinical CNS involvement. For example, in nine different regions of Spain, seroprevalence of TOSV in a random cohort of individuals was 26% (n= 1268 individuals) that had not presented with meningitis or febrile illness (Mendoza-Montero et al., 1998). Here, although antibodies to TOSV were found in younger age groups, they were detected at a higher frequency in older age groups (Cusi et al., 2010). This has also been shown in an Italian cohort, with an age-dependent seroprevalence of TOSV, with 19.8% in adults and 5.8% in children (Terrosi et al., 2009). Interestingly, individuals with increased exposure to sandfly-rich environments, such as forestry workers, showed seroprevalence rates as high as 77.2% (Valassina et al., 2003). A more recent assessment of sandfly transmitted virus in Italy over a ten-year period, described TOSV seropositivity at between 22.95% and 26.75% (Marchi et al., 2017).

Further evidence for increased geographical dissemination, a recent prevalence study in Bulgaria found seropositivity at 24.4%, despite no previous history of TOSV infection (Christova et al., 2020). While in Southwest Germany, 4% of individuals with probable viral meningoencephalitis in a retrospective cohort analysis, exhibited neuroinvasive TOSV, despite having no prior history of visiting an endemic region (Dersch et al., 2021). To assess exposure to sandfly-borne infections in general, one study from Spain assessed seropositivity to sandfly salivary proteins. Here, *Ph. perniciosus* sand fly salivary gland homogenate (SGH) and recombinant protein rSP03B were investigated to detect sand fly exposure in blood donors and SGH and rSP03B seroprevalences were estimated to be 69% and 88%, respectively. In addition, the same study showed 26% of

TOSV seropositivity in the blood samples (Ortuño et al., 2022). For a full list of these and other epidemiological studies, see Table 1.2.

In summary, TOSV infection is now widespread across multiple countries, not only in those that border the Mediterranean, but also further north in Central and Southern Europe. It is likely that further spread to more temperate countries will occur as the climate warms and international travel continues apace.

Location	**Year**	**Cases/Findings**	**References**
United States	1985, 2009, 2015	Three imported cases of TOSV meningitis or meningoencephalitis from Italy were reported	(Calisher et al., 1987; Kay et al., 2010; Howell et al., 2015)
United Kingdom	2019	One imported case with TOSV encephalitis was determined	(Farrugia et al., 2020)
Sweden	1980s	An imported TOSV case was reported from Swedish tourists visiting Spain	(Rickard Eitrem et al., 1991)
Southern Europe			
Southern Italy	2000s	Meningitis and encephalitis cases were reported during the summer	(Di Nicuolo et al., 2005; Venturi et al., 2007; Venturi et al., 2009)
Southern France	2001, 2004	Two TOSV cases manifested as aseptic meningitis and influenza-like illnesses, while one case was diagnosed as acute meningitis	(Hemmersbach-Miller et al., 2004; Peyrefitte et al., 2005)
Catalonia, Spain	Early 2000s	A 6% seroprevalence of anti-TOSV IgG was found, with 2 acute clinical cases with viral meningitis or meningoencephalitis	(Cardeñosa et al., 2013)
Spanish Mediterranean & Madrid	Early 2000s	The overall seroprevalence was 24.9%, with higher rates observed in rural populations compared to urban areas	(Echevarría et al., 2003; Navarro et al., 2004; de Ory-Manchón et al., 2007; Sanbonmatsu-Gámez et al., 2009)
Emilia-Romagna and Umbria, Italy	2002, 2003	The first reports of CNS infections caused by TOSV were documented	(Portolani et al., 2002; Francisci et al., 2002)
France	2003, 2006	Imported acute meningitis and meningoencephalitis cases due to TOSV infection were reported	(Defuentes et al., 2005; Epelboin et al., 2008)
Portugal	2002-2005	TOSV meningitis was confirmed in 6 (5.6%) cases out of 106 samples tested	(Santos et al., 2007)
Portugal	2004-2008	The prevalence was 4.2% in those with neurological symptoms and 1.3% in those without neurological symptoms	(AMARO et al., 2012)
Kosovo	2005	The presence of TOSV in the population was suggested	(Venturi et al., 2011)
Bosnia and Herzegovina	2006-2008	Anti-TOSV IgG and IgM were analysed in 68 human serum samples, revealing recent infection in seven patients (10.29%)	(Hukić and Salimović-Bešić, 2009)
Croatia	2007-2009	TOSV seropositivity was 37.5% among healthy residents	(Punda-Polić et al., 2012)

Ionian Islands, Greece	2010	TOSV IgG antibodies prevalence was 51.7% in Corfu and 39% in Cephalonia	(Papa et al., 2010)
Northern Italy	2010	Acute meningitis cases due to TOSV infection were reported	(Vocale et al., 2012)
Greece	2010-2014	TOSV was responsible for 10% of CNS infections	(Papa, Kontana, et al., 2015)
Greece	2010-2014	Three different TOSV cases were reported, two of which showed neurological symptoms	(Papa, Mallias, et al., 2014; Papa, Paraforou, et al., 2014; Papa, Kesisidou, et al., 2015)
Tuscany, Italy	2012	A total seropositivity of 10% was recorded for TOSV	(Remoli et al., 2018)
Sicily, Italy	2012	TOSV-specific IgG prevalence was 25% in those with neurological symptoms and 10.8% in those without neurological symptoms	(Colomba et al., 2012; Calamusa et al., 2012)
Emilia-Romagna, Italy	2012	Among 120 suspected neuroinvasive infection cases, TOSV was detected in 28.3%. Of these, 79.4% were in the acute phase of infection	(Pierro et al., 2014)
Aegean Sea islands, Greece	2013	TOSV seroprevalence was 21%	(Vassiliki Anagnostou and Papa, 2013)
Northern Greece	2013	TOSV seroprevalence was 11.26%	(V. Anagnostou and Papa, 2013)
Corsica, France	2014	TOSV RNA was detected in Phlebotomus species sandflies	(Bichaud et al., 2014)
Portugal	2009-2018	Six TOSV cases were identified from patients who had CNS infections	(Amaro et al., 2021)
Madrid, Spain	2007, 2018-2019	The seroprevalence was 34.5% overall, with anti-TOSV IgG at 41.5% in 2007 and 21.3% in 2018-2019	(Martinez et al., 2022)
Elba Island, Italy	2018	Twelve cases of TOSV meningoencephalitis with symptoms were reported	(Quattrone et al., 2020)
Southern Tuscany, Italy	2011-2019	TOSV positivity was 4.6% in CSF samples, and TOSV-specific IgM was 27.1% in sera	(Gori Savellini et al., 2020)
Southwest Portugal	2019	Neutralising antibodies to TOSV were found in 5.3% of healthy blood donors	(Maia et al., 2022)
Corsica, France	2019	TOSV antibodies were found in 22.5% of cases using virus microneutralization assay	(Masse et al., 2019)
Central Europe			
Germany	1993-1994	Thirteen acute TOSV infections were reported in German citizens returning from Southern France, Greece, Italy, and Portugal	(Schwarz et al., 1993; T. F. Schwarz, Jäger, Gilch and Pauli, 1995; Dobler et al., 1997)
Germany	1993-1995	Out of 317 patients, 13 (4.1%) tested positive for TOSV antibodies. These cases were imported from Italy (11 cases, 84.6%), Portugal (1 case, 7.7%), and Türkiye (1 case, 7.7%)	(Schwarz et al., 1996)
Netherlands and Germany	2000s	Imported TOSV infectious-caused CNS diseases were reported	(Beersma et al., 2004; Imirzalioglu et al., 2006)

Switzerland	2008, 2009, 2012	Imported aseptic meningitis cases due to TOSV infection were reported from Swiss tourist who visited Italy	(Sonderegger et al., 2009; Gabriel et al., 2010; Cordey et al., 2015)
Africa			
Tunisia	2003-2009	IgM (10%) and IgG (7%) antibodies for TOSV were identified in patients with neurological diseases	(Bahri et al., 2010)
Djibouti, Africa	2010-2011	The circulation of Toscana-related viruses was 3.7%	(Andayi et al., 2014)
Tunisia	2013	Anti-TOSV IgG positivity was 9.5% in healthy individuals	(Fezaa et al., 2013)
Tunisia	2013	TOSV neutralising antibodies were present in 41% of human sera, with confirmed co-circulation with Punique virus	(Zhioua et al., 2010; Sakhria et al., 2013)
Northern Algeria	2013	The presence of TOSV was confirmed in sandflies and the population with almost 50% seropositivity	(Alkan et al., 2015)
Tunisia	2014	Anti-TOSV IgM was 12.16% in serum samples, TOSV positivity was 12.86% in CSF samples, and TOSV RNA was found in pooled sandfly samples	(Fezaa et al., 2014)
Algeria	2016-2018	TOSV infection was found in 3.8% of patients with neurological symptoms	(Benbetka et al., 2023)
Libya	2013-2014	TOSV seroprevalence was 25% in the population	(Saadawi et al., 2022)
Middle East			
Türkiye	2010	Fourteen TOSV infection cases were reported in patients initially diagnosed with aseptic meningoencephalitis	(Koray Ergunay et al., 2012)
Central, North, and Southeast Anatolia, Türkiye	2011-2012	TOSV seroprevalence was 17.8% in asymptomatic blood donors and 15.7% in patients with CNS infections of unknown cause	(K. Ergünay et al., 2011; K. Ergunay et al., 2012)
Eastern Thrace, Türkiye	2012	TOSV seroprevalence in blood donors was 14.4% and the first co-infections of WNV and TOSV were reported	(Erdem et al., 2014)
Central Anatolia, Türkiye	2012	Among 94 patients investigated, TOSV seroreactivity was found in 37.2% (35 patients)	(Ocal et al., 2014)
Mediterranean Region, Türkiye	2011-2012	Neutralising antibodies to TOSV were detected in 13.9% of healthy blood donors	(TEZCAN et al., 2015)
Western Border of Iran	2013	Among military personnel, 1% TOSV was revealed in serum samples	(Shiraly et al., 2017)
Türkiye	2014	A patient who was HIV positive was also found to have an acute TOSV infection	(Kuşçu et al., 2014)
Western Saudi Arabia	2012-2016, 2019	The circulation of TOSV showed an overall seroprevalence of 0.8% in residents	(Al-numaani et al., 2023)

Table 1.2: Epidemiological Studies of TOSV.

The time frame indicates the years during which the samples were collected or when TOSV diagnoses were made in patients.

1.2.5.2.2.2 Potential Vertebrate Reservoirs of TOSV

Numerous species of vertebrates have been proposed as TOSV reservoirs, although firm proof is still lacking. Given the high frequency of either TOSV RNA detection or neutralising antibodies found in canine blood samples taken during the sandfly season in Mediterranean Anatolia, Türkiye (Dincer et al., 2016), Portugal (Alho et al., 2016), Corsica (Dahmani et al., 2016) and Algeria (Tahir et al., 2016; Sellali et al., 2022), dogs have been proposed as a reservoir host. Indeed, seroprevalence of TOSV was 6.8% in dogs and 3.7% in cats in the Portuguese study (Alwassouf, Maia, et al., 2016). Another seroprevalence study showed that guard dogs seroprevalence rate of 7.5% in two different regions of Tunisia (Sakhria et al., 2014), while there was 8.4% seropositivity in dogs from Greece (Alwassouf, Christodoulou, et al., 2016). Antibodies in cats to *Ph. perniciosus* saliva (47.7%, 350/167) and neutralising antibodies against TOSV (4.9%, 18/365) show that cats are bitten by sandflies, and can be infected with TOSV (Pereira et al., 2019). However, it is not clear if either species supports the TOSV transmission cycle, due to the low level of viremia that results from infection and inability to excrete virus (Muñoz et al., 2020). However, experimental infection of dogs by *Ph. perniciosus* feeding has been recently documented in a natural setting (Dachraoui et al., 2023). It has also been suggested that *L. infantum*-infected canines demonstrate enhanced vectorial capacity for TOSV (Dincer et al., 2015; Maia et al., 2017), compared to healthy dogs that do not have *L. infantum*.

Livestock may also act as a reservoir, with e.g., serum samples exhibiting seropositivity in 4.7% (Kosovo; n= 1,086) (Ayhan, Sherifi, et al., 2017), and up to 7.8% (Saudi Arabia) (Al-numaani et al., 2023) of samples. In some studies, issues relating to cross-reactivity have made it difficult to assess true seropositivity for TOSV specifically. TOSV RNA is typically not detected in most cases, complicating host range definition (Navarro-Marí et al., 2011), although infection of sheep and goat have also been suggested. Cattle are nonetheless clearly bitten by sandflies, as shown by presence of their blood in engorged females, particularly *Ph. perniciosus* (Messahel et al., 2022).

For several neurotropic arboviruses, such as WNV, birds have a role as viral amplification hosts, vector dispersion vehicles, and sources of new strains by interspecies transmission. Birds passing through known migratory routes in the Hatay province of Türkiye, have been shown to be positive for TOSV RNA in samples taken from the brain and kidney(Hacioglu et al., 2017). In addition, the migratory common quail (*Coturnix coturnix*) exhibit a high seroprevalence rate of 42.45% in Spain (Ayhan

et al., 2023). Furthermore, a recent study conducted in the northern wetlands of Türkiye revealed the presence of both TOSV RNA and infectious virus in bird populations (Hacioglu and Ozkul, 2023). These findings indicate that birds may be a reservoir or act as amplifying host for TOSV.

Bats have been recognised as important reservoirs of many zoonotic viruses worldwide (Moratelli and Calisher, 2015). However, there is little information about the role of bats in the ecology of phleboviruses, including TOSV. While TOSV was once isolated from a bat's brain (*Pipistrellus kuhlii*) (Verani et al., 1988), TOSV exposure rate can be considered low in bats as long-lived animals, with an antibody seroprevalence at 10%. Therefore, bat colonies are not likely to play a reservoir role for TOSV (Ayhan et al., 2021).

1.2.5.2.2.3 Public Health Importance

TOSV is among the top three aetiological agents of aseptic meningitis after enteroviruses and herpesviruses, especially in the summertime, because its vector is most active in the warmer months (Ayhan and Charrel, 2020). This seasonality can lead to outbreaks during specific times of the year, placing a periodic burden on healthcare systems. Therefore, possible outbreaks of TOSV can strain healthcare resources and lead to economic loss, particularly in tourism-dependent regions where the fear of infection may deter visitors. There are no specific antiviral treatments or vaccines available for TOSV. In addition, TOSV infection often presents with nonspecific symptoms such as fever, headache, and fatigue, which can be mistaken for other illnesses. This can lead to underreporting and misdiagnosis, complicating public health responses.

Overall, the public health importance of TOSV infection lies in its potential to cause significant neurological disease, its prevalence in endemic regions, the challenges in diagnosis and treatment, and its economic impact on affected communities.

1.2.5.2.2.4 Clinical Manifestations of TOSV Infection

Similar to many arboviruses, the majority of TOSV infections are either asymptomatic, mild or undiagnosed (Scott C Weaver et al., 2018); however, an increasing number develop severe disease that can be life threatening or leave disabling sequelae, including those that involve deafness (Martínez-García et al., 2008). In the Mediterranean region, the majority of TOSV infection occur in the warmer months between May and October, peaking in August. This timeframe corresponds to the peak activity of the sandfly

vector (Nicoletti et al., 1991). Importantly, TOSV is the only sandfly-borne phlebovirus that can cause neurological disease (Ayhan and Charrel, 2020). Following a bite by an infected sandfly, the virus is assumed to replicate in the skin, during the so-called incubation period that original estimates suggest range from 3 to 7 days before the onset of more severe clinical signs.

Typically, the infection manifests as a mild febrile illness, commonly marked by elevated body temperature, headaches, skin rashes (exanthema) with haemorrhagic features, feelings of sickness, muscle pain, headache, joint pain, arthritis and occasionally nausea and vomiting (AMARO et al., 2012; Papa, Kesisidou, et al., 2015). However, the incubation period of this infection can be prolonged. In one case report, the incubation period extended to 17 days in a patient who developed extreme lethargy, malaise, anhedonia and decreased hearing (Howell et al., 2015), while another study assessing infection in a travel-acquired cohort, estimated the incubation period at an average of 12 days (Laroche et al., 2021). During the early-stage phase of infection that coincides with viraemia, TOSV RNA can also be detected in urine, a characteristic that is sometimes observed in virus infections that involve the nervous system (Mathur et al., 1995; Barzon et al., 2013; Ergunay et al., 2015; Popescu et al., 2021).

1.2.5.2.2.5 Complications of TOSV Infection

Signs of meningitis, sudden hearing loss and other neurological involvement can develop in some cases 2 weeks post fever. These are diverse and can manifest differently and can encompass Kernig's sign (resistance to knee extension with hip flexed), stiffness in the neck, light sensitivity, tremors, nystagmus (involuntary eye movements), muscle weakness, specific neurological impairments, double vision, sleep disturbances, prolonged fatigue, altered mental alertness, and changes in consciousness. Typically, these symptoms can endure for several weeks, while persistent alterations in personality linked to TOSV encephalitis have also been reported (Calisher et al., 1987; Tino F. Schwarz et al., 1995; Pauli et al., 2008; Serata et al., 2011; Papa, Mallias, et al., 2014; Ludlow et al., 2016; García San Miguel et al., 2021), which can also occur without concurrent meningitis (Dionisio et al., 2001; Papa, Paraforou, et al., 2014). TOSV has the potential to cause fatal encephalitis in humans, although this is rare. In one fatal case, diagnosis was based on positive serological results and the patient's travel history to Tuscany prior to the development of symptoms, which indicated progressive encephalitis linked to TOSV infection (Bartels et al., 2012). Moreover, in Romania, severe encephalitis and meningoencephalitis caused by TOSV was identified

through RT-PCR testing, following 5 deaths out of 8 patients (Popescu et al., 2021). More frequent is the presentation of TOSV-linked hydrocephalus, a complication of viral meningoencephalitis that is otherwise highly uncommon. Patients with CNS TOSV infection have also reported testicular pain including epididymal-orchitis, epididymitis, and genital vasculitis (Baldelli et al., 2004; Zanelli et al., 2013; Gonen and Sacagiu, 2013; Tschumi et al., 2019; Mascitti et al., 2020), although no evidence yet suggests direct infection of testes (Zanelli et al., 2013). Interestingly, TOSV RNA was detected in seminal fluid samples from a patient with TOSV meningitis (Matusali et al., 2022), suggesting transmission through sexual intercourse may be possible.

Persistent sequalae of infection have also been noted. In one case, a patient presented with severe neurological features after returning from Umbria, Italy. Here, serum and CSF were positive for antibodies against TOSV, and recovery was associated with persistent headaches (Oechtering and Petzold, 2012). There have also been reports of siblings who contracted severe life-threatening meningoencephalitis following TOSV infection, and have since experienced long-lasting neurological complications, including hydrocephalus (Baldelli et al., 2004).

TOSV infection associated with peripheral neuropathy has been reported to mimic a Guillain-Barré like syndrome. Although, one case report was insufficient to demonstrate a definitive association (Rota et al., 2017), in a further case-control study, TOSV was recognised as a contributing factor to the development of GBS (Okar et al., 2021). Due to scarcity of cases, it is not clear whether these TOSV neurological manifestations of the peripheral nervous system can be considered an established feature of this disease. TOSV infection also has the capacity to rarely cause persistent neurological infections accompanied by ischemic complications (Sanbonmatsu-Gámez et al., 2009), sensory polymyeloradiculopathy (Gonen and Sacagiu, 2013), and brachial plexus involvement (Vilibic-Cavlek et al., 2020).

Complications arising from TOSV infections do not always involve CNS disease (Braito et al., 1997); lymphadenopathy (Ranaldi et al., 2011), pancytopenia during acute infection (Ocal et al., 2014), benign myositis and fasciitis (Mosnier et al., 2013) have been described. While the connection between myositis and viral infections is well-established (Crum-Cianflone, 2008). The mechanisms behind these viral-induced pathologies myositis remain unclear and warrant further research. In summary, TOSV infections are of growing clinical importance. Understanding the molecular and cellular

basis for these diverse pathologies, and the host response to infection, is important if we are to improve the care and treatment of these patients.

1.2.5.2.2.6 Diagnosis, Treatment and Prevention

The diagnosis of TOSV infection typically involves a combination of clinical evaluation and laboratory testing. Clinical evaluation involves assessment of symptoms, medical history and travel history to TOSV-endemic areas. With the rise in travel to TOSV-endemic regions, it has become increasingly important to diagnose imported cases of TOSV in countries where the virus is not endemic (Charrel, 2012). In particular, medical professionals are advised to include TOSV as a potential consideration when differentiating diagnoses in cases of acute meningitis and encephalitis (AME) in individuals who have recently travelled to the Mediterranean region (Howell et al., 2015).

For patients with CNS involvement, differential diagnosis can be aided through virological tests to confirm TOSV infection and differentiate it from, for example, WNV, enterovirus, and herpesvirus infections. These assays are typically PCR based and can detect low concentrations of viral RNA. Sensitivity is an important consideration for cases with neurological involvement, as the prior acute viraemic phase is rapid and virus can be cleared from the blood before clinical presentation (Valassina et al., 2002). To date, several PCR assays has been established to detect TOSV, including nested RT-PCR of serum and CSF samples, which are additionally employed for those with the acute meningitis (T. F. Schwarz, Jäger, Gilch and Nitschko, 1995; Valassina et al., 1996; Sánchez-Seco et al., 2003). Typically, assay primers amplify the TOSV S fragment (Valassina et al., 2000). More recently, real-time PCR assays have been adopted due to their time-saving benefits and reduced risk of contamination (Pérez-Ruiz et al., 2007). Although, CSF sampling is most informative, recent developments have optimised detection of TOSV RNA using real-time RT-PCR in more accessible blood samples from patients with CNS disease (Weidmann et al., 2008; K. Ergünay et al., 2011; Brisbarre et al., 2015). Lately, a TOSV real-time RT-PCR assay has been established to target three specific genomic regions within the nucleoprotein gene. This assay offers a robust and sensitive method for detecting TOSV by targeting multiple genomic regions, enhancing specificity and reducing the risk of false negatives (Thirion et al., 2021). Additionally, diluted urine samples have been shown to be suitable for TOSV RNA detection using this Trio TOSV RT-qPCR system (Mori et al., 2024). Finally, there now exists a multiplex PCR where several sets of

primers are employed in a single reaction to identify infections caused by either TOSV or enterovirus (Valassina et al., 2002).

Serological assays, such as Enzyme-Linked Immunosorbent Assay (ELISA) and Immunofluorescence Assay (IFA) can be used to detect current and previous infections. As such, they are less informative for diagnosis but may offer the only sign of TOSV infection if the acute viraemic is over and TOSV RNA can no longer be detected. These assays detect specific antibodies (IgM and IgG) against TOSV in the patient's blood serum. There is limited cross-reactivity among viruses belonging to the *Phlebovirus* genus, particularly between the TOSV and *Phlebovirus napoliense,* mainly because of the significant similarity in the N protein of these viruses (Amodio et al., 2012). As such, these tests are of most use when screening many specimens rapidly e.g., for seroprevalence studies. Notably, the neutralization assay has a lower likelihood of cross-reactions compared to indirect immunofluorescence (IIFA) or ELISA (Mendoza-Montero et al., 1998; Zhioua et al., 2010). Several commercial assays are available for the detection of TOSV antibodies, providing diagnostic options for healthcare professionals when assessing potential TOSV infections (Koray Ergünay et al., 2011).

Alternatively, diagnosis can be based on isolating the virus. Here, clinical samples, especially CSF, are employed to infect cells (e.g., Vero or BHK-21) and detect cytopathic effects (CPE) on cells, or additionally in animals, preferably combined with sequencing to define the aetiological agent. However, in practice this is rarely undertaken due to its complexity and the availability of other diagnostic techniques. In addition, concerns that these cell-culture based approaches are not sufficiently sensitive have been raised, as only 14% of the CSF samples that tested positive for TOSV by PCR successfully resulted in viral isolation when introduced to Vero cells (Charrel et al., 2005).

In a recent study, metagenomic next-generation sequencing (mNGS) has been used as a differential diagnostic tool in undiagnosed meningitis cases and revealed eight cases (8/23) caused by TOSV (Gámbaro et al., 2023).

In summary, prompt diagnosis allows for appropriate medical care and management of patients with TOSV infections (Cusi and Savellini, 2011). In addition, differential diagnosis of TOSV is important to find its accurate seroprevalence in the endemic areas since many of the symptoms of TOSV infections overlap with those of other viral infections or neurological conditions. It helps to track the distribution of TOSV, which

is essential for implementing control measures and understanding the virus's behaviour. Sandflies transmit TOSV, and understanding the geographical distribution of TOSV infections is vital for vector control efforts. Diagnosing cases helps identify areas at risk and implement preventative measures.

Since no specific antivirals and vaccines exist for TOSV, therapeutic approaches mostly include the use of anti-inflammatory drugs, antipyretics, and analgesics. The prevention of getting a sandfly bite is also essential for avoiding infection. These topics have been discussed in detail in the 1.3.4 and 1.7 sections.

1.2.6 *Leishmania* Parasites – medically significant parasites transmitted by sandflies.

Leishmania (Kinetoplastida: Trypanosomatidae) is an obligate protozoan parasite that causes Leishmaniasis and is transmitted by sandfly bites. Almost one hundred sandfly species have been associated with Leishmaniasis transmission. There are 31 species of *Leishmania* known to parasitise mammals, with over 20 species capable of causing disease in humans (Maroli et al., 2013). Natural transmission occurs through either zoonotic or anthroponotic routes. In humans, the disease presents in three primary clinical forms: Visceral Leishmaniasis (VL), also termed kala-azar, which is the most severe and potentially fatal without treatment; Cutaneous Leishmaniasis (CL), the most prevalent form with 0.7-1.2 million cases annually; and Muco-Cutaneous Leishmaniasis (MCL) (Maroli et al., 2013; Pal et al., 2022). VL is characterised by fever, weight loss, enlargement of the spleen and liver, and anaemia, while CL causes skin lesions, mainly ulcers around the bite site, leaving permanent scars. MCL leads to partial or total mucous membrane damage to the nose, mouth, and throat (Desjeux, 2004; Bañuls et al., 2011).

Leishmaniasis, endemic across many regions encompassing the tropics, subtropics, and the Mediterranean basin, affects approximately 12 million individuals, with an additional 350 million at risk of infection across 98 countries (Alvar et al., 2012). Additionally, an estimated 700,000 to 1 million new cases occur each year. However, given that the disease is more prevalent in endemic geographies inhabited by impoverished communities with inadequate healthcare systems and hygiene practices, the actual number of cases may be higher due to underreporting. For instance, in one region in India, the underreporting of VL was as high as eight times the actual prevalence of the disease (Singh et al., 2006; Okwor and Uzonna, 2016). Although this sandfly-borne parasite disease has affected many individuals worldwide, it is still on the

neglected tropical diseases (NTDs) list (Pal et al., 2022). It continues to re-emerge due to urban immigration, tourism to endemic regions, deforestation, conflict, and seasonal workers (Oryan and Akbari, 2016; Chastonay and Chastonay, 2022).

Notably, the prevalence of Leishmaniasis is also show parallelism of its vector distribution. For example, *L. major*, cause CL, tends to infect *Ph. papatasi* because, as a result of the coevolution process, it has a specific midgut receptor for this parasite species (Kamhawi et al., 2004). As climates change, the rising temperature has provided more favourable conditions for sandflies (Figure 1.10). Leishmaniasis is endemic in all southern European countries, including Greece, Italy, Portugal, Spain, and Türkiye, and the increasing distribution of its vectors may create new public health risks in more northern regions (Chalghaf et al., 2018; Hosseini et al., 2021; Tunalı and Özbilgin, 2023).

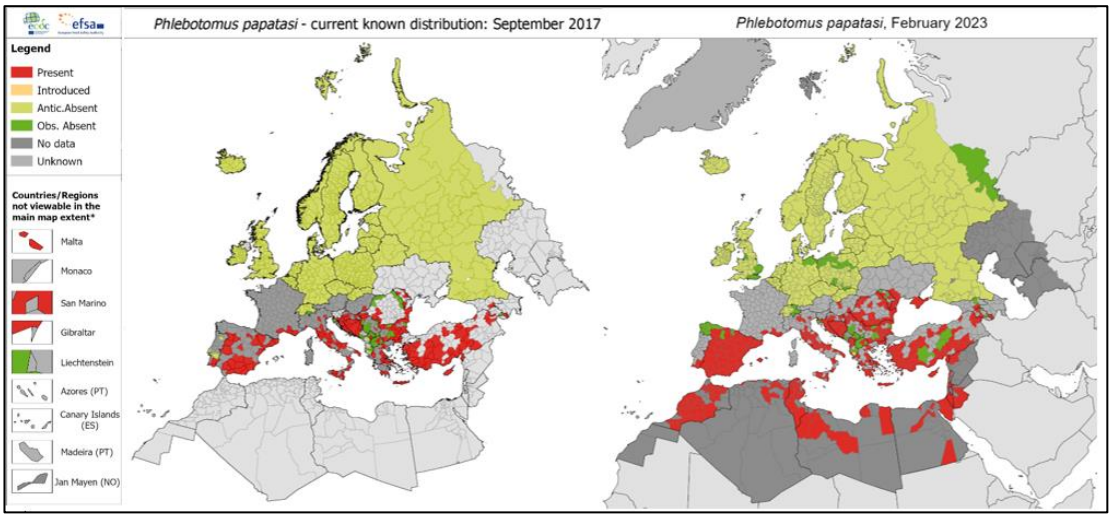


Figure 1.10: Distribution of Phlebotomus papatasi in 2017 (left) and in 2023 (right) in Europe and Neighbouring Regions
Note how the distribution of the vector increases northward over time. (European Centre for Disease Prevention and Control, 2023)

The type of treatment depends on the disease form, prognosis, parasite species and geographic location and patients need to get supportive treatment, such as rehydration. Generally, there are antileishmanial drugs such as (FDA)-approved oral miltefosine for CL, can be used for treatments but most of them have life threating side effects (Santos et al., 2008; Aronson et al., 2017). In addition, *Leishmania* drug-resistance was observed (Ponte-Sucre et al., 2017). At present, there is no commercially available effective vaccine for humans, although effective vaccines have been successfully developed for dogs (Moafi et al., 2019; Karmakar et al., 2021; King, 2023). Vector control strategies including insecticide-treated nets (ITNs), topical and spatial repellents

can decrease disease prevalence by reducing the number of sandflies (Balaska et al., 2021).

Leishmania and TOSV can co-circulate and/or cause co-infection in sandfly and vertebrate hosts due to the same vector species being shared, and similar geographical distribution e.g., as shown in dogs, which may act as a reservoir for TOSV (Dincer et al., 2015). Moreover, co-infection of TOSV and *Leishmania infantum* was determined in captured *Ph. tobbi* sandflies in eastern Thrace and Northern Cyprus, Turkiye (Ergunay et al., 2014), while infected dogs could transmit both these pathogens to sandfly vectors (*Ph. pernicious*) following a bite. Regarding human co-infections, (Bichaud et al., 2011) identified a noteworthy correlation between *L. infantum* seropositivity and TOSV during a retrospective serological screening conducted in southern France. This study provided the initial compelling evidence suggesting an epidemiological connection between *Leishmania* parasites and TOSV infections.

1.3 ARTHROPODS: VECTORS OF INFECTIOUS DISEASE

1.3.1 Mosquitoes

Mosquitoes (Diptera: Culicidae) are flying, relatively small insects. Over 3,700 types of mosquitoes have been identified worldwide. Some mosquito species impact human and animal health as vectors of infectious pathogens (Benelli et al., 2016). Mosquitoes belonging to the *Anopheles* genus transmit the *Plasmodium* parasite that causes Malaria, a life-threatening disease with over 200 million cases annually (Ashley et al., 2018). Viral infections can be transmitted by multiple species of mosquitoes. ZIKV, DENV, and CHIKV are transmitted by the *Aedes aegypti* and *Aedes albopictus* species (Kraemer et al., 2019).

Mature female mosquitoes lay their eggs in or just above water shortly after feeding on blood. These eggs typically hatch within 1-2 days of being submerged. The larvae then reside in the water, feeding on aquatic microorganisms and / or debris until they develop into pupae. Adult mosquitoes emerge from the pupae after 1-3 days. By 3-5 days post-emergence, adult mosquitoes become sexually mature. While most mosquitoes primarily feed on plant nectar and rotting fruit, females of certain species require blood meals to complete their reproductive cycle, which involves egg maturation and oviposition (Paaijmans and Thomas, 2011; Eckhoff, 2011). During a subsequent blood meal, the mosquito injects the virus into a new host along with its saliva (Mellor, 2000; Leitner et al., 2015). Apart from the salivary glands, mosquito ovaries can be infected

by related arboviruses, which provide additional vertical transmission (Borucki et al., 1999).

1.3.2 Ticks

Ticks are blood-feeding arthropods, of which there are approximately 900 species, classified as the *Argasidae* (soft bodied ticks) and the *Ixodidae* (hard bodied ticks) (Guglielmone et al., 2010). *Ixodidae* members transmit most pathogens, including bacteria, protozoa, and arboviruses such as tick-borne encephalitis virus (TBEV), Crimean-Congo haemorrhagic fever virus (CCHFV), African swine fever virus (ASFV) (Bell-Sakyi and Attoui, 2013). Medically important ticks typically take a blood meal from small mammals, birds, and reptiles during their immature stages, while adults feed on larger herbivores and carnivores. This diverse feeding behaviour allows ticks to acquire pathogens from various vertebrate hosts and subsequently transmit them to humans. Human exposure to ticks, often characterised by generalist host behaviour, increases the risk of pathogen transmission (Anderson, 2002; Salim Abadi et al., 2010).

Ixodid ticks progress through three active stages in their life cycle: larva, nymph, and adult. Most ticks of the *Hyalomma sp*. possess a three-host cycle that involves each of the three life stages seeking a new host to obtain a blood meal, but some exhibit two-host behaviour, with larvae and nymphs feeding on the same host (Bonnet et al., 2023). Larvae hatch, feed, moult to nymphs while attached to the host, and then drop to the ground for further development. Adults, resulting from this process, find a host, feed, mate, and lay eggs in protected sites with high humidity. Each blood meal can allow ticks to become infected with virus (e.g., CCHFV), while transmission can also occur through transstadial or transovarial routes. Transmission occurs between ticks and mammals or through co-feeding ticks. For CCHFV, human infection can result from the bite of an infected tick or exposure to the body fluids of a viraemic animal or CCHF patient (Bente et al., 2013).

As an example of the impact of tick-borne disease, CCHFV will be discussed briefly. In 1944, the haemorrhagic fever disease was first defined in Crimea (Crimean haemorrhagic fever) (Watts et al., 2019). However, the initial isolation of the virus dates back to the 1960s in Congo (Simpson et al., 1967). Subsequent investigations unveiled that the virus responsible for these cases shared antigenic similarities with the pathogen that causes diseases in the Congo (Casals, 1969). CCHFV can induce fatal haemorrhagic fever in humans with a mortality rate that can reach 40% (Bente et al.,

2013). CCHFV is prevalent across Africa, the Middle East, Southeast Asia, and southern and eastern Europe, closely mirroring the distribution of its vector, the *Hyalomma* tick (Messina et al., 2015). Furthermore, the range of *Hyalomma* ticks, specifically *Hy. marginatum* and *Hy. rufipes*, has extended as far north as Sweden (Grandi et al., 2020). Currently, there is no specific vaccine and therapeutics for CCHFV (Ahata and Akçapınar, 2023).

1.3.3 Phlebotomine sandflies

Geographically, sandflies are present between 50 ° N and 40 ° S latitudes but absent in New Zealand and the Pacific islands (Cecílio et al., 2022). In addition to *phleboviruses*, phlebotomine sandflies are also responsible for the spread of *Leishmania* discussed in Section 1.2.6 and *Bartonella bacilliformis* (Ready, 2013; Ayhan and Charrel, 2017). Of the 900 sand fly species, less than one hundred can transmit *Leishmania* parasites, while just nine species of sandflies transmit *phleboviruses*, including TOSV (Charrel et al., 2005).

1.3.3.1 Taxonomy

Sandflies belong in the order Diptera, suborder Nematocera, family *Psychodidae*, and subfamily *Phlebotominae*. Six primary sandfly genera are recognised, three of which are found in the Old World (*Phlebotomus*, 13 subgenera; *Sergentomyia*, ten subgenera; and *Chinius*, four species), and three of which are found in the New World (*Lutzomyia* 26 subgenera and groups; *Brumptomyia*, 24 species; and *Warileya*, six species) (Akhoundi et al., 2016). Notably, the genera *Lutzomyia* and *Phlebotomu*s and some *Sergentomyia* (Remadi et al., 2023) are those that are anthropophilic and exhibit competence to transmit pathogens (Cecílio et al., 2022).

1.3.3.1.1 TOSV and *Phlebotomus* sp.

Phlebotomus perniciosus and *Ph. perfiliewi* have been identified as vectors of TOSV (Verani et al., 1988; Nicoletti et al., 1996). Although not yet documented, it is likely that other related species such as *Ph. sergenti, Ph. longicuspis, Ph. neglectus, Ph. tobbi, Sergentomyia minuta* etc. could also participate in TOSV transmission (Ergunay et al., 2014; Es-Sette et al., 2015; Ayhan and Charrel, 2018).

The contributing factors for TOSV maintenance in nature are not well-known. Interestingly, both male and female sandflies have been identified with TOSV infection. As in other hematophagous insects, only females acquire blood meals; therefore, infection in male sandflies suggests vertical and/or transovarial transmission (Coluzzi et

al., 1988). Indeed, experimentally infected sandfly species, including *Ph. perniciosus*, can transmit TOSV transovarially (Tesh and Modi, 1987; Ciufolini et al., 1989; Maroli et al., 1993). In addition, transovarially infected female sandflies can transmit TOSV by biting a susceptible vertebrate (Ciufolini et al., 1989). Experimental evidence further suggests venereal infection in female sandflies, which might serve as an infection amplifier in the absence of other reservoirs (Tesh et al., 1992). However, the presence of vertebrate reservoirs is likely to be required for TOSV maintenance, as viral infection rates in sandfly colonies not exposed to viremic vertebrates, steadily drop with each succeeding generation of the colony (Tesh and Modi, 1987; Ciufolini et al., 1989; Maroli et al., 1993; Bergren and Kading, 2018).

The capacity of TOSV to circulate horizontally among members of the same generation has also been suggested based on work on the related phlebovirus Massilia virus (MASV) (Charrel et al., 2009; Jancarova et al., 2019). Interestingly, infected sandflies could deposit virus particles on plants, which uninfected sandflies could then acquire when feeding on nectar. This suggests that the virus deposited as they seek sugar meal may be an effective route for virus spread (Laroche et al., 2023).

1.3.3.1.2 TOSV and *Sergentomyia* sp.

Sandflies belonging to the genus *Sergentomyia* feed on a wide range of animals including reptiles, birds and a diverse array of mammals, occasionally including humans (Charrel et al., 2006; Moncaz et al., 2014; González et al., 2020; Pombi et al., 2020). Some of these flies, including *S. minuta,* have been found positive for both human and Leishmania DNA (Maia et al., 2015), and can transmit *Leishmania* to reptiles (Akhoundi et al., 2016), and possibly to humans (Berdjane-Brouk et al., 2012; Campino et al., 2013; Maia and Depaquit, 2016; Latrofa et al., 2018). Importantly, this species may also transmit TOSV to vertebrates as they have been found harbouring TOSV RNA (Charrel et al., 2006). Furthermore, the Phlebotomus vectored Chandipura virus (CHPV) has also been detected in *Sergentomyia* sandflies from India. CHPV is a member of *Rhabdoviridae* that can, like TOSV, cause encephalitis (Geevarghese et al., 2005). Together, this suggests *S. minuta* may also be a competent for transmitting phleboviruses, including TOSV (Ticha et al., 2023).

1.3.3.1.3 TOSV and *Lutzomyia* sp.

A pressing concern is the possibility that *Lutzomyia sp.* sandflies could transmit TOSV. This New World sandfly genus is found across South, Central and North America and

consists of over 400 species, including *Lu. longipalpis* (Diptera: *Psychodidae: Phlebotominae*). This species is an important vector of several medically importance pathogens, including *Leishmania* and potentially also arboviruses (Akhoundi et al., 2016). Indeed, it is the main vector of *L. infantum* in the Americas that e.g., causes VL. *Lu. longipalpis* has broad-range feeding habits and is to different habitats, including rural and urban areas feeding on humans, pets, livestock, rodents, bats and opossums (Dias et al., 2003; Afonso et al., 2012; Soares et al., 2014).

While laboratory-based infection of *Lu. longipalpis* with Phlebotomus-transmitted *Phlebovirus siciliaense* and *Phlebovirus napoliense* is inefficient (Jennings and Boorman, 1980), there is evidence to suggest competence as an arboviral vector. For example, Punta Toro virus (PTV, also a phlebovirus) is transmitted by *Lutzomyia* species in Panama (Tesh, R. B., Chaniotis, B. N., Peralta, P. H., & Johnson, 1974), while the Candiru complex viruses (family *Phenuiviridae*) have been isolated from *Lutzomyia* species, some of which cause febrile illness in humans (Palacios et al., 2011). *Viola phlebovirus*, a putative new viral species and a novel Phlebotomus fever serogroup member, was identified in *Lu. longipalpis* species in Brazil. The ability of this new sandfly-derived virus to replicate within mammalian cell lines and express NSs and NSm proteins suggests that the virus may be a novel arbovirus (De Carvalho et al., 2018).

A *Lu. Longipalpis* cell line can be infected and replicate a wide range of arboviruses, although PTV infection was in efficient (Tesh and Modi, 1983; Ferreira et al., 2018). This is surprising as PTV has been isolated from both humans and sandflies and may suggest the cell line may be derived from a cell type resistant to infection. Interestingly replication of *Phleboviruses* in *Lu. longipalpis* is nonetheless possible as e.g., RVFV can replicate following intrathoracic inoculation (albeit not via blood feeding) and be transmitted to RVFV-susceptible mammalian hosts (Hoch et al., 1984). This is intriguing considering the geographic separation of vector (Americas) with RVFV (Old World) and their lack of co-evolution to date (Hoch et al., 1984).

To date, with the geographic range of *Lu. longipalpis* and TOSV not overlapping, it is not surprising that there is no field evidence that this sandfly can be infected or transmit TOSV to vertebrate hosts. There may also be sequence adaptions required by TOSV for infection of *Lutzomyia* species for transmission to be sufficiently efficient. However, as with the ZIKV outbreaks in the Americas, the increasing pace of globalisation and

international travel will increase the risk of TOSV viremic individuals becoming exposed to biting sandflies of the Americas (Peterson et al., 2017).

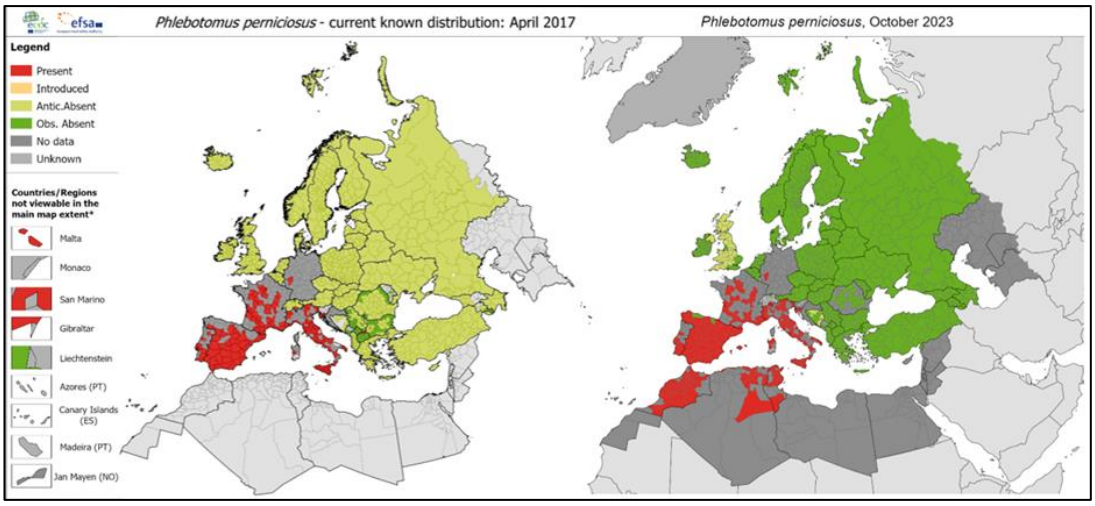
For this thesis, note that salivary factors from *Lu. Longipalpis* are used in chapter 4.

1.3.3.2 Effect of The Climate Emergency on Sandfly Distribution

Climate has multiple impacts on the dynamics and prevalence of arthropod-borne infections. Invertebrate vectors, including sandflies, are ectothermic and changes to the environment due to climate change can impact life cycle, movement, feeding activity and survival (Caminade et al., 2019). Increasing temperatures can result in expansion of geographic range of vectors and their ability to accomplish a higher number of life cycles in a given summer. Not all changes that accompany the climate emergency are beneficial and e.g., extreme weather events can be devastating on population numbers. In addition, if the current endemic areas become overtly hot or dry for optimal survival, their abundance can decrease consequently, as the presence of water is necessary for larval stages (Volf and Volfova, 2011).

Historically, the increasing incidence of arthropod-borne diseases has been overlooked (Rocklöv and Dubrow, 2020). ZIKV, DENV and CHIKV are good examples of arboviruses transmitted by mosquitoes whose distribution has spread to over 130 countries (Kraemer et al., 2019). As climate change becomes more pronounced, it is anticipated that, in addition to mosquitoes, other hematophagous insects like sandflies, will also undergo geographical expansion. Many sandfly species have already been established across the Mediterranean region. Models predicted that just a 1°C increase could create optimal environmental conditions for certain sandfly species (*P. mascittii, P. neglectus*) in Austria (Aspöck et al., 2008). Subsequently, *Ph. (Transphlebotomus) mascittii*, was newly documented in Austria and across the Western Europe (Naucke et al., 2011), while *Ph. mascittii* has now been confirmed in central Europe, north of the Alps, France, Switzerland, Belgium, and Germany (Poeppl et al., 2013). As such, the TOSV vectors *Ph. mascittii* and *Ph. perniciosus* are now not only confined to Southern Europe but also identified in Germany (Oerther, 2020), where autochthonous cases of TOSV meningoencephalitis have been reported (Naucke et al., 2008; Dersch et al., 2021). In addition, changes in *Ph. ariasi, Ph. mascittii, Ph. neglectus, Ph. perfiliewi, and Ph. perniciosus* (Figure 1.11), distribution are being observed, with these species spotted in northern regions and higher altitudes, a shift attributed to climate change. The occurrence of imported TOSV infections in these locations poses a risk for potential local outbreaks if the competent vector species become established, as observed with

other diseases transmitted by vectors. In summary, TOSV could extend its activity to new temperate regions where suitable vector species exist (Knechtli and Jenni, 1989; Maroli et al., 2013; Liang et al., 2015; Ayhan et al., 2020).



Note how the distribution of the vector increases northward and African continent over time. (European Centre for Disease Prevention and Control, 2023)

1.3.3.3 Sandfly Life Cycle

Sandflies are delicate, tiny insects (1.5-3.5 mm in length) with a hairy appearance and large black eyes, but the rest of the parts are beige. Sandflies have the characteristic form of hairy, vertical V-shaped wings when they are at rest or blood-feeding (Figure 1.12) (Lane and Crosskey, 2012).

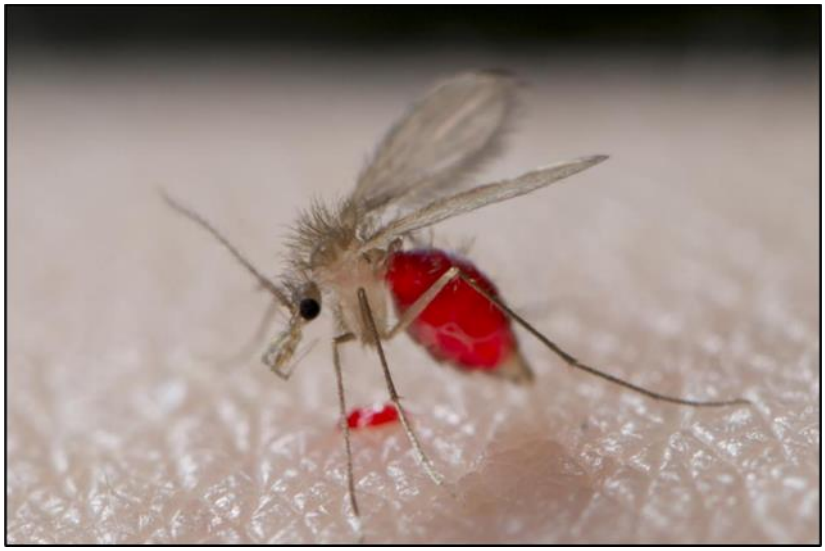


Figure 1.11: Blood Fed Female Sandfly
(Photo © IRD – N. Rahola)

Sandflies' life cycle begins with the egg (~300-400µm). Female sandflies lay their eggs in terrestrial environments characterised by abundant organic material, which serves as

a nutrient for the larvae. Eggs take up to 20 days to hatch, followed by four larval instars, which take up to 30 days, depending on species, temperature, and nutrient availability, then pupae and finally mature form emerge, firstly males then females (Figure 1.13) (Ready and Croset, 1980; Alkan et al., 2013). Sandflies exhibit egg and larval dormancy as well as diapause, which can be influenced by environmental factors such as temperature and photoperiod (Ready, 2013). Male pheromones lure females to mating locations, with the timing of this mating varying among species and occurring either before or after the blood meal (Hamilton, 2022).

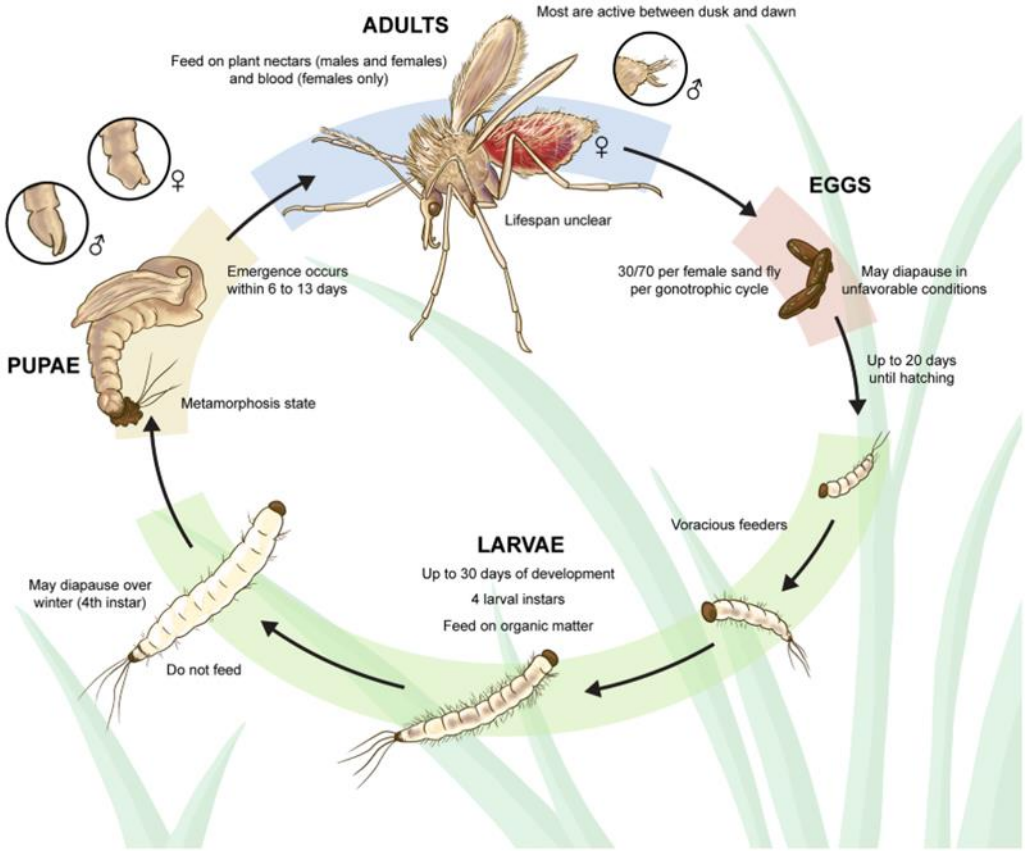


Figure 1.12: Sandfly's Life Cycle Schematic Representation

The sand fly life cycle consists of four main stages: eggs (pink background), larvae with four instars (green background), pupae (yellow background), and adults (blue background). The last two stages include distinct morphological features (highlighted within the circles) that help differentiate between genders. Key characteristics of each stage (or sub-stage) are noted alongside the images and the average development duration for each stage (Cecílio et al., 2022).

While male and female sand flies both consume nectar, only the females require a blood meal to reproduce. Female sandflies are not picky hematophagous insects, and their feeding choices cover a wide variety of vertebrate hosts, including humans, livestock, dogs, amphibians, and birds. (Charrel et al., 2005; Anaguano et al., 2015; Dincer et al., 2015; Costa et al., 2021) Although some studies have suggested preferential feeding on

certain species (Pérez-Cutillas et al., 2020), the consensus of the literature is that sandflies prefer blood meals from which ever species is the most abundant, thus, minimizing the need for substantial movement that is otherwise limited by their short flight range. When taking a blood meal, pathogens are transmitted to a host (Kamhawi, 2000). Temperature, humidity, and airflow all have an impact on feeding activity. Though biting during the day might happen indoors in darkened areas or among shaded vegetation/trees, most species feed around sunset and night when the temperature drops, and the humidity rises (Killick-Kendrick, 1999). Adult sandflies can be found in caves and rock crevices, tree trunks or tree hollows, domestic animal enclosures, masonry crevices, and other dark, humid locations such as basements and wells (Durden and Mullen, 2018). Sandflies typically have a silent and short-range with a characteristic short hopping flight, though their maximum dispersion rarely extends beyond one kilometre (Killick-Kendrick et al., 1986). In specific regions, sandflies were observed to have a natural lifespan of 2-3 weeks, while in nature or captivity were documented to survive for up to 2 months (Lewis, 1971; Ready and Croset, 1980).

1.3.4 Vector Control Measures

In the absence of widely used drugs and vaccines for vector-borne diseases, vector control is the sole available method of protecting against outbreaks of disease. For some insects, various strategies have been employed to reduce vector populations, encompassing insecticides and more innovative methods, like genetic modifications. Understanding vector ecology and epidemiology and implementing environmental management tailored to the ecology and behaviour of local vector species are crucial to achieving these goals (Wilson et al., 2020).

1.3.4.1 Conventional Methods – insecticides.

Insecticides used for disease prevention started with the discovery of 1,1,1-Trichloro-2,2′bis(p-chlorophenyl) ethane, commonly known as DDT, in the early 1940s. DDT's initial large-scale application was post-World War II when it was deployed to combat a typhus outbreak transmitted by body lice in war-affected European populations (Wheeler, 1946). DDT were then used widely in the eradication programs against mosquito-borne malaria in Europe (Bruce-Chwatt and De Zulueta, 1980) and the US (Andrews et al., 1950). Despite the successes achieved using DDT, it is highly persistent in the human body and its environment and causes harmful effects on human and animal health (Beard, 2006). Studies suggested that DDT had adverse impacts on the reproductive tract e.g., in wild birds (Ratcliffe, 1970). In addition, a high level of

DDT in the postpartum was related to the reduced lactation period (Gladen and Rogan, 1995). Together these led to a ban of the use of DDT in the US in the 1970s (Kutz et al., 1977; Benelli et al., 2016). It should also be noted that malaria has since resurged in incidence throughout much of the range in which DDT were used.

Insecticide remains the cornerstone of control efforts. These applications (e.g., larvicide and aerial ULV) were also performed to control WNV spread into the US population (Ruktanonchai et al., 2014; Nasci and Mutebi, 2019) and CHIKV in southern Europe (Venturi et al., 2017). Organophosphates like malathion, temephos, and pyrethroids such as deltamethrin, permethrin and carbamates have been employed since the 1990s. Pyrethroids have also been utilised for long-lasting insecticidal nets (LLINs), thermal fogging, and indoor residual spraying (IRS) for two decades (Tangena et al., 2018). However, the use of these chemicals has bioaccumulation risks, and insecticide resistance is now today's reality. Their broad activity against multiple species also causes death of non-target insects. In phlebotomine sandflies *(Ph. argentipes*, the vector of VL in India), resistance mutations were detected to DDT and pyrethroids (Gomes et al., 2017).

Briefly, larvicides (e.g., temephos), pyrethroid-treated ITNs, insecticide-treated materials for personal protection, spraying of residual insecticides (typically either pyrethroids, carbamates, or organophosphates), insecticidal treatment of habitat or animals (e.g., cattle), insecticide-treated traps and target, topical repellent [e.g., N,N-diethyl-meta-toluamide (DEET)], and spatial repellent have been using as chemical vector control (Wilson et al., 2020), and WHO encourage the rotation of insecticide use, to limit emergence of resistant variants (Knapp et al., 2015).

Despite insecticides' proven benefits in combating vector-borne diseases, their widespread use has raised concerns, underscoring the necessity for alternative approaches to address this global challenge.

1.3.4.2 Innovative Approaches

Recently, environmentally friendly, gene-driven alternatives of vector control have been developed. Biocontrol strategies, particularly for mosquito, include mosquitocides, the plant-based ovicides and larvicides (Tu, 2011), Bti and entomopathogenic fungi (Scholte et al., 2004; Lacey, 2007), and mosquito predators (e.g., larvivorous fish) (Griffin and Knight, 2012).

Additionally, the release of male mosquitoes infected with endosymbiotic bacteria *Wolbachia* has become popular, novel strategy of vector control. *Wolbachia* naturally infects more than 50% of the insect species worldwide (Hilgenboecker et al., 2008), transmitted transovarially and reduce the fertility of the infected insect such as causing unviable offspring (Shaw and Catteruccia, 2018). These features led to consider candidate for the biological control of vector-borne viruses. Studies have illustrated that *Wolbachia*-infected mosquitoes are no longer susceptible to DENV and CHIKV based on disrupting arboviral replication and transmission (Walker et al., 2011; van den Hurk et al., 2012). Trials with *Wolbachia* positive mosquitoes demonstrated promise in establishing naturally occurring populations of *Ae. aegypti* mosquitoes infected with the bacteria (Hoffmann et al., 2011).

Genetic modifications of mosquitoes are also being studied. These includes the release of sterile mosquitoes that generating lethal mutations in their progeny. This approach decreases the ratio of fertile wild-type males to released infertile males, which ultimately diminishes or eradicates new offspring as the sterile males mate with wild females (Benelli et al., 2016). A similar approach has been used with *Glossina austeni* tsetse flies from Unguja Island, Zanzibar (Vreysen et al., 2014). Mosquitoes have also been genetically modified to transmit a dominant lethal transgene to their offspring, resulting in their death during the larval stage. This approach, known as RIDL (Release of Insects carrying Dominant Lethal transgenes), is presently deployed in the field to combat *Ae. aegypti* mosquitoes (Harris et al., 2012).

1.4 IMMUNOLOGY

Immunology was established as a scientific field in the 1800s, but our comprehension of immunity traces back much further. As early as the 5th century BC, the Athenian historian Thucydides noted individuals who were resistant to a second infection of the plague. He used the Latin words “immunitas” and “immunis,” which means “exempt” in English (Holladay and Poole, 1979; Silverstein and Bialasiewicz, 1980). By the 1700s, in the Ottoman Empire, active applications known as “variolation” to confer immunity to smallpox were undertaken widely (Boylston, 2012). This practice involves transferring superficial skin wounds of material from smallpox pustules. Later, Edward Jenner observed that cowpox or vaccinia exposure provided immunity to smallpox. He demonstrated that individuals who were already exposed to these less pathogenic disease materials two months prior to exposure, were protected from smallpox inoculation, and used the term "vaccination" to describe the procedure (Jenner, 1802).

In the late 19th century, Robert Koch defined that specific microorganisms are the causative agents of infectious diseases (Kaufmann and Winau, 2005), while Louis Pasteur also developed vaccines for cholera in chickens and rabies (Smith, 2012). In addition to these groundbreaking practices, the fundamentals of immunology were defined with the discovery of cellular immune responses (e.g., macrophages) and the concept of “humoral” immunity by Metchnikoff, Von Behring, and Kitasato (Kantha, 1991; Tauber, 1992).

Together, these have paved the way for advancing our understanding of immunology.

1.4.1 The Immune System Tissues

The immune system safeguards the host against various threats, including pathogens (e.g., viruses), harmful substances (e.g., toxins), and tissue damage. For this purpose, it is specialised as a biological system consisting of organs and tissues interconnected by a large web of lymphatic vessels (Schultz and Grieder, 1987). These organs can broadly be classified into three categories: the primary, secondary, and tertiary lymphoid organs. During embryonic development, primary lymphoid organs such as the bone marrow and thymus form, play a crucial role in generating lymphoid cells. The bone marrow is where both innate immune cells and adaptive immune cells (T and B lymphocytes) are generated, and the thymus is where T-cells mature from thymocytes produced in the bone marrow (Boehm et al., 2012).

Secondary lymphoid organs constitute lymph nodes, spleen, Peyer's patches in the small intestine, and mucosal membranes (e.g., in the bowel), and tonsils (Ruddle and Akirav, 2009). All have special zones for naïve T and B lymphocytes. Secondary lymphoid organs, such as lymph nodes, serve as the site for initiating primary immune responses to antigen derived from peripheral tissues e.g., the skin. Lymph nodes are located along lymphatic vessels and act as filters, gathering antigen and antigen-presenting cells from non-lymphoid organs. In contrast, mucosal lymphoid organs like Peyer's patches and tonsils acquire antigens directly through the mucosal epithelium, as they lack afferent lymphatics (Randall et al., 2008). The spleen, which also lacks afferent lymphatic vessels, directly surveys the bloodstream for pathogens such as virus. Here, the spleen stores various immune system cells such as splenic dendritic (DC) subsets, macrophages and monocytes, which can migrate into blood stream or clear pathogens directly from the circulation (Lewis et al., 2019). Tonsils are barriers that stop potentially harmful pathogens from entering the body via the mouth and nose (Perry and

Whyte, 1998). The colon, an example of a mucous membrane associated with lymphoid tissue, defends the body with its mucous wall, which hosts cells that e.g., express protective IgA antibodies (Mowat and Agace, 2014).

Lastly, tertiary lymphoid organs are ectopic accumulations of immune cells, akin to secondary lymphoid organs, which emerge in peripheral tissues outside of the lymphatic system, triggered by persistent local inflammation, such as in patients with rheumatoid arthritis (Shipman et al., 2017).

1.4.2 Innate Immunity: primary defence against pathogens.

Innate immunity encompasses the initial defence mechanisms deployed against pathogens and constitutes the first line of protection (Medzhitov and Janeway, 2000). From microscopic unicellular eukaryotes to all multicellular animals and plants, they all possess an innate immune system (Buchmann, 2014; Ji et al., 2022). Hence, it is not surprising that innate immune features such as antimicrobial peptides, phagocytosis, complement, and Toll-like receptors, emerged approximately 700 million years ago. In comparison, adaptive immunity, which is present only in more complex multi-cellular animals, arose in jawed vertebrates (fish) around 450 million years ago (Buchmann, 2014). In addition to swiftly identifying and eradicating harmful foreign invaders, innate immunity plays a crucial role in preserving the equilibrium of tissue function. This includes detecting and facilitating the repair of damaged tissues, as well as disposing of ageing or dying cells to uphold tissue integrity. Importantly, the innate immune cells called antigen-presenting cells (APCs), such as DCs, process antigens to be recognised by lymphocytes and thereby initiate adaptive immunity (Nussenzweig, 2011). However, innate immune responses have potential to cause tissue damage and on their own have no capacity to generate immune memory to infection. (Yatim and Lakkis, 2015). Dysfunctional activation of innate immune responses can also contribute towards development of autoimmune diseases (Lang et al., 2007).

The first initial defence mechanism consists of anatomic (e.g., skin, oral mucosa, respiratory epithelium, intestine) and chemical (e.g., antimicrobial proteins) barriers. Anatomic barriers have epithelial tight junctions that establish cell-cell contact, which provide mechanical protection against pathogen invasion. Epithelial surfaces also secrete antimicrobial proteins (e.g., defensins, cathelicidins) and antimicrobial enzymes (e.g., lysozyme, secretory phospholipase A_2 in tears and saliva) (Medzhitov and Janeway, 2000; Schleimer et al., 2007). If these defence mechanisms are insufficient to

prevent breach of anatomical barriers, plasma components, including complement can also limit infection. The complement system consists of over 50 plasma proteins which are mainly generated by the liver and can be activated by three distinct pathways: the classical, alternative, and lectin pathways. Binding of complement to pathogens can lead to their lysis, opsonisation or phagocytosis, and the initiation of inflammatory responses (Kemper et al., 2023). Specifically, complement activation can limit arbovirus infections, as mice that lack complement receptors are more susceptible to WNV infection, resulting in earlier viral dissemination to the CNS (Mehlhop and Diamond, 2006). In addition, NS1 protein in flaviviruses, including DENV, binds complement proteins (C4, C1s) and attenuates classical and lectin pathway activation (Avirutnan et al., 2010), underlying their importance in host immunity.

1.4.2.1 Pattern Recognition Receptors

In 1989, Charles Janeway Jr. proposed the presence of receptors expressed by innate immune cells responsible for recognising pathogen encoded molecules, which are known as pattern recognition receptors (PRRs) (Janeway, 1989). PRRs are present in various subcellular compartments, including cellular and endosomal membranes, the cytosol, and extracellularly in secreted forms circulating in the bloodstream and interstitial fluid. Upon binding their ligands, PPRs activate inflammatory responses required to eliminate infectious pathogens, clear out damaged cells and tissues, and initiate the healing process. Additionally, PRR signalling triggers co-stimulatory signals in DCs that permit them to activate adaptive immune cells, especially T lymphocytes. These PRR ligands are referred to as Pathogen-Associated Molecular Patterns—PAMPs, and Damage-Associated Molecular Patterns—DAMPs. In this section, we will explore the primary PRRs essential for the host to detect viral encoded molecules and initiate antiviral responses (Medzhitov and Janeway, 1997; Amarante-Mendes et al., 2018).

Toll like receptors

The anti-microbial function of the Toll protein was first found in *Drosophila melanogaster*, where it is required for the fruit fly's antifungal response. Homologs of Toll, called Toll-like receptors (TLRs), are also expressed in vertebrate cells, and possess either anti-viral, bacterial, and fungal activity (Takeuchi and Akira, 2010). Humans have 10 distinct TLRs which are located on the cell surface membrane (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) and in intracellularly (TLR3, TLR7, TLR8,

TLR9, TLR11, TLR12 and TLR13) (Kawai and Akira, 2010). TLRs possess a transmembrane protein with leucine-rich repeats (LRRs) responsible for their horseshoe shape, facilitating the recognition of PAMPs. Additionally, they share a cytoplasmic tail containing a Toll–IL-1 receptor (TIR) domain crucial for intracellular signalling (Kumar et al., 2011). Although they have common structures, they have evolved to recognise motifs expressed by diverse pathogens including bacterial lipopolysaccharide (TLR4) (Qureshi et al., 1999), viral single stranded RNA (TLR7-TLR8), DNA with unmethylated CpG (TLR9), and double-stranded RNA (either as genomes or replication intermediaries in viruses) (TLR3) (Nazmi et al., 2014).

Once these diverse motifs bind their TLRs, they lead to the expression of inflammatory cytokines, chemotactic molecules, antimicrobial peptides, and type I IFN, such as IFN-α and IFN-β, which are potently anti-viral. Following ligand binding, TLRs recruit TIR domain-containing adaptor proteins such as MyD88, TIRAP, TRIF and TRAM. Most TLRs use only MyD88, or other adaptor proteins paired with MyD88, whilst TLR3 interacts only with TRIF. TLR signalling with MyD88 activates the transcription factor NFκB and mitogen-activated protein kinases (MAPKs), which induces the expression of pro-inflammatory cytokines. In addition, TLR-3 and TLR-7, TLR-8, and TLR-9 activate members of the IRF family, which are localised in the cytoplasm via TRIF and MyD88, respectively. IRF family members IRF3 and IRF7 are key for activating antiviral type I IFN expression.

RIG-I like receptor family

The RIG-I-like receptor family (RLRs) senses viral RNAs produced within a cell, found in the cytoplasm, and includes RIG-I (retinoic acid-inducible gene I), MDA-5 (melanoma differentiation-associated 5) and laboratory of genetics and physiology-2 (LGP-2). RIG-I and MDA-5 contain an RNA helicase-like domain, a C-terminal regulatory domain (CTD) and two N-terminal caspase recruitment domains (CARDs). LGP-2 also has similar helicase and CTD domains, but it lacks CARDs (Reikine et al., 2014). RIG-I detects the unaltered 5′-triphosphate end of single-stranded RNA (ssRNA) viral genomes. The RNA transcripts of Flaviviruses, along with those of numerous other ssRNA viruses, possess this unmodified 5′-triphosphate end, making them recognizable by RIG-I (Chow et al., 2018). In contrast, MDA-5 recognises double-stranded RNAs (dsRNA). Although CARDs are absent in LPG2, LPG2 can detect dsRNA and may

regulate the activities of RIG-I and MDA-5 during viral RNA recognition (Kell and Gale, 2015).

Upon binding, virus RNA alters the conformation of these receptors, allowing RIG-I and MDA-5 to interact with the mitochondrial antiviral signalling protein (MAVS). MAVS propagates signals by recruiting various TRAF family members, like TRAF3, which leads to the activation of TBK1 and IRF3/IRF7, the expression of type I IFN, and the activation of NFκB (Zhang et al., 2010).

Nucleotide oligomerization domain (NOD) like receptors

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are innate immune receptors found within the cytoplasm of epithelial and immune cells, capable of recognizing various bacterial peptidoglycans such as γ-glutamyl diaminopimelic acid (iE-DAP) and lipopolysaccharides (LPS). Some NLRs stimulate NFκB to initiate inflammatory responses like TLRs, whereas others initiate a separate pathway leading to cell death and the generation of pro-inflammatory cytokines (Liu et al., 2017; Kelley et al., 2019).

Cytosolic DNA sensors

Lastly, there are over ten innate sensors of cytoplasmic DNA, called Cytosolic DNA sensors, that can lead to the production of type I interferon. Their discovery stemmed from observations showing that cells lacking TLR9 could still generate type I IFN in response to double-stranded DNA (dsDNA). A cytosolic sensor called cGAS binds to dsDNA, initiating the production of cyclic GMP-AMP (cGAMP). cGAMP then binds to and activates the stimulator of interferon genes (STING) dimer located on the endoplasmic reticulum (ER) membrane. This STING homodimer recruits and activates TBK1, which subsequently phosphorylates and activates IRF3, resulting in the expression of type I IFN (Goubau et al., 2013; Paludan and Bowie, 2013).

1.4.2.2 Cells of Innate Immunity

Innate immune cells play a pivotal role in detecting PAMPs, executing effector functions like phagocytosis, and aiding in activation of adaptive immune responses. The leukocytes which form the innate immune system arise from pluripotent hematopoietic stem cells in the bone marrow (Collin and Milne, 2016). However, some tissue-resident macrophages and Langerhans cells derive from the embryonic yolk sac and the foetal liver (Collin and Milne, 2016). Pluripotent cells divide to generate two types of stem

cells. One, the common lymphoid progenitor, gives rise to the lymphoid lineage of leukocytes including innate lymphoid cells (ILCs), natural killer (NK) cells, and T and B lymphocytes. The other, the common myeloid progenitor, gives rise to the myeloid lineage, which encompasses the remaining leukocytes, red blood cells (erythrocytes), and megakaryocytes responsible for platelet production (Bernareggi et al., 2019). Briefly, the key innate immune cells and their importance in arbovirus infection will be discussed here.

Granulocytes (Polymorphonuclear leukocytes)

Granulocytes originate from granulocyte/macrophage progenitor cells and undergo maturation within the bone marrow. Their numbers increase in response to immune activation, prompting their migration to sites of infection or inflammation, but are short-lived. Eosinophils, basophils, and neutrophils are characterised by the presence of dense granules containing a variety of enzymes and toxic proteins within their cytoplasm, and nuclei which have distinctive shapes. Eosinophil granule proteins (e.g., α defensin), along with reactive oxygen species (ROS) and pro-inflammatory cytokines, are deployed to target various pathogens, including helminths and bacteria respectively. Eosinophils are also involved in allergic reactions (Gigon et al., 2023). Basophils, the only histamine-containing circulating leukocyte, play a crucial role in hypersensitivity reactions and primarily contribute to the defence against parasites (Schwartz et al., 2016).

Neutrophils, the most abundant leukocyte, are pivotal cells in innate immune responses that express cytokines and can engulf a diverse array of microorganisms via phagocytosis, leading to their neutralisation within intracellular vesicles. This destruction is facilitated by utilising degradative enzymes and other antimicrobial substances stored within their cytoplasmic granules (Borregaard, 2010). Neutrophils are generated in large numbers (1 to 2 x 10^{11} each day in humans) and are often stored in the bone marrow before their release into the circulation following inflammatory cues. Here, they are released quickly in significant numbers, facilitated by upregulation of the chemotactic cytokine receptor CXCR2. These cells circulate in the bloodstream in a dormant state. Following infection, mature neutrophils transmigrate from blood into infected tissues (Eash et al., 2010; Borregaard, 2010), where they can carry out various actions to combat pathogens, including phagocytosis, generation of neutrophil extracellular traps (NETs), and the expression of chemotactic and inflammatory

cytokines, which recruit additional neutrophils and other immune cells, such as monocytes, dendritic cells and T cells, to the site of infection (Rosales, 2018).

Mast cells

Mast cells are hematopoietic myeloid cells that originate from the bone marrow. They are long-lived tissue-resident cells. Mast cells typically do not circulate in their mature form. Instead, they complete their differentiation and maturation locally, following the migration of their precursors into specific vascularised tissues including the skin or cavities (e.g., airways and gastrointestinal tract) where they will ultimately settle. Their granules hold numerous inflammatory mediators, including histamine and various proteases, which contribute to shielding internal surfaces from pathogens, including parasitic worms and allergic reactions (Kawakami and Galli, 2002; Galli et al., 2005). When multivalent antigen crosslinks with IgE bound to FcεRI, it triggers the activation of mast cells. Therefore, degranulation, pro-inflammatory lipid mediators, and the synthesis and secretion of cytokines and chemokines result from FcεRI-dependent cell activation (Krystel-Whittemore et al., 2016).

Monocytes

Monocytes are mononuclear myeloid cells that develop from haematopoietic stem and progenitor cells in the bone marrow under the control of the transcription factor PU.1 and circulate in the bloodstream, where they can be stimulated by multiple cytokines, including the macrophage colony-stimulating factor (M-CSF) (Ito et al., 2009). The controlled release of inflammatory monocytes into the blood is regulated by expression of the chemokine receptor CCR2 (Mitchell et al., 2014). Here, circulating TLR ligands active bone marrow cells to express CCL2, which in turn binds to CCR2 on monocyte, triggering their release into the bloodstream (Shi et al., 2011). In addition, a reservoir of monocytes in the adult spleen serves as a quick response to injury or inflammation (Swirski et al., 2009).

Two primary types of monocytes have been extensively studied in mice and humans: classical ($Ly6C^{hi}$ CD43− monocytes in mice, equivalent to $CD14^{hi}$CD16-monocytes in humans) and non-classical ($Ly6C^{lo}$ CD43+ monocytes in mice, corresponding to CD14-$CD16^{hi}$ monocytes in humans) (Coillard and Segura, 2019; Wolf et al., 2019). Intermediate monocytes ($Ly6C^{int}$ CD43+ monocytes in mice and CD14+ CD16+ monocytes in humans) have also been identified (Zhu et al., 2016; Villani et al., 2017;

Hamers et al., 2019). Among these three subsets, classical monocytes constitute approximately 85% of monocytes, the percentage of intermediate and non-classical monocytes are ~5-10% (Coillard and Segura, 2019). Functional differences, such as migration to tissues, between these subsets exist (Geissmann et al., 2003); classical monocytes are rapidly be recruited to the inflammation site and give rise to macrophages or monocyte-derived dendritic cells (moDCs) or remain as monocytes with antigen-presenting abilities. These cells have diverse roles in controlling infection, limiting inflammatory damage, and initiating tissue repair. In contrast, non-classical monocytes are recruited to non-inflamed tissues through a process reliant on the chemokine receptor CX3CR1. They are distinguished by their capacity to patrol the resting vasculature, clear cellular debris, and facilitate endothelial repair under homeostatic conditions. Intermediate monocytes also share characteristics similar to classical and non-classical monocytes (Auffray et al., 2007; Yona et al., 2013; Wolf et al., 2019).

Macrophages

Macrophages are larger phagocytic cells that are either recruited to tissues from the blood precursors or can be already tissue resident. Recruited macrophages tend to be derived from monocyte precursors that differentiate in situ. These cells execute several immune and inflammatory process and tend to be short lived. In contrast, tissue-resident macrophages reside in most tissues from birth, originating from the embryonic yolk sac and foetal liver during development. Macrophages continue to replenish throughout life through the differentiation of classical monocytes.

The diverse appearance and function of macrophages across different tissues have been defined, exemplified by microglial cells, osteoclasts, Kupffer cells, and bronchoalveolar macrophages under resting conditions (Ginhoux and Guilliams, 2016; Link et al., 2018). Macrophages, proficient in phagocytosis, play a pivotal role in eliminating cellular debris, apoptotic cells, and pathogens by degrading them within phagolysosomes. Moreover, they interact collaboratively with other cells of the innate immune system, such as neutrophils, innate lymphoid cells, and natural killer (NK) cells (Molgora et al., 2018).

Macrophage activation can be triggered by PAMPs or DAMPs by pattern recognition receptors (PRRs). Activated macrophages can be categorised into two groups: M1-like macrophages and M2-like macrophages. M1 macrophages are typically activated

following exposure to bacterial components, like lipopolysaccharide (LPS) and the Th1 cell cytokine interferon-gamma (IFN-gamma). M2 macrophages are typically induced by Th2 T cell derived cytokines such as IL-4 and IL-13 (Egawa et al., 2013). Despite increasing evidence suggesting mixed and context-dependent macrophage phenotypes, the fundamental classification of M1-like and M2-like macrophages persists (Edwards et al., 2006; Martinez and Gordon, 2014). As this thesis does not directly address this issue, further elaboration on macrophage differentiation will not be provided [reviewed in detail in ref (Mosser and Edwards, 2008)].

Dendritic cells

Dendritic cells (DCs) are a heterogeneous population and constitute 'professional' antigen-presenting cells. They detect and capture antigens and present them on their cell surface in the context of major histocompatibility complex (MHC) molecules. DC, being heterogeneous in nature, can be categorised into various groups according to their anatomical localization, developmental origin, and primary functions. They arise either from embryonic progenitor cells, which produce resident dendritic cells including Langerhans cells, or from specialised myeloid-derived hematopoietic stem cells termed macrophage/DC progenitors, which generate conventional DC and plasmacytoid DC. Alternatively, DC can arise from $Ly6C^{hi}$ monocytes, which, upon migration into tissues, differentiate into inflammatory DC also called monocyte-derived DCs (moDCs) (Hespel and Moser, 2012). Both inflammatory and conventional DC exhibit common characteristics including morphology, migratory properties, surface markers such as CD11c, MHC II, and costimulatory molecules like CD80/86 and CD40, which facilitate their ability to activate naïve T cells (Hespel and Moser, 2012).

Conventional DC (cDC) display high mobility, participating in diverse tasks such as patrolling barrier tissues for pathogens. Following acquisition of antigen, these cells relocate from peripheral tissues to lymph nodes to support either immune tolerance (in absence of PRR signalling) or activation of T cells (in presence of PRR signalling). So called 'immature' cDCs that have yet to encounter a PAMP, demonstrate adept phagocytic capabilities, which diminish upon encountering pathogens and subsequent maturation. Once matured, cDCs present processed antigens from pathogens on MHC II molecules, migrate to the T-cell zones in lymphoid organs, and secrete cytokines such as IL-12. This enables the exposure of naïve T lymphocytes to antigens carried by cDCs

and DC-derived cytokines, which can result in the activation of T cells (Sichien et al., 2017).

Plasmacytoid DC (pDC) are not efficient antigen presentation to T cells. Instead, they can rapidly express abundant quantities of type I IFN upon detecting viral RNA via TLR7. Initially situated in the bone marrow, pDCs migrate to peripheral tissues in response to chemotactic signals that activate CCR2 (Cella et al., 1999; Coccia et al., 2004).

Inflammatory DCs have been described during pathogenic inflammations, including cutaneous *Leishmania major* infection, fungal infections, pulmonary influenza infection, and rheumatoid arthritis and have modulatory functions by producing inflammatory cytokines and presenting antigens to CD4+ T cells (Segura and Amigorena, 2013; Liu et al., 2021).

Innate lymphoid cells

Innate lymphoid cells (ILCs) are a type of lymphocyte that lacks antigen-specific receptors found on T cells and B cells. They develop in the bone marrow from the same common lymphocyte progenitor (CLP) that gives rise to B and T cells. The innate lymphoid subgroup consists of NK cells, ILC1, ILC2, ILC3 and LTi cells (Eberl et al., 2015). ILC1s, ILC2s, and ILC3s exhibit functional similarities to CD4+ T helper (Th)1, Th2, and Th17 cells, respectively, while natural killer (NK) cells perform functions akin to CD8+ cytotoxic T cells. ILC1s and Th1 cells respond to intracellular pathogens like viruses and tumors; ILC2s and Th2 cells react to large extracellular parasites and allergens; and ILC3s and Th17 cells combat extracellular microbes such as bacteria and fungi (Vivier et al., 2018). ILCs will not be discussed in further detail due to their absence of relevance in this thesis.

1.4.2.3 Innate Immune Response to Arthropod-borne Infection

Immune responses to arbovirus infections are quickly activated and have evolved to robustly control levels of virus in tissues and blood. Consequently, most infections result in a short course of illnesses (e.g., acute fever) and are self-limiting e.g., as compared to immune responses to *Herpes* viruses that are lifelong. Innate immune responses, such as the early generation of neutralising antibodies and the type I interferon (IFN) responses have an important role in controlling these infections in the first few days of infection (Elong Ngono and Shresta, 2018).

The study found that blood cells from TOSV-infected patients released significantly more IFN-α in response to TLR3 stimulation with the poly(A) agonist compared to healthy controls. In addition, the CSF of the patients who suffer from meningitis or meningoencephalitis associated with TOSV included a high IFN-α level. Together these suggest an involvement of TLR3 in the antiviral response to TOSV (Varani et al., 2015).

Many arboviruses have evolved viral mechanisms to antagonise IFN responses, including ZIKV and TOSV (Gori-Savellini et al., 2013; Grant et al., 2016a). Type I interferon signalling is inhibited by the NS5 viral protein of ZIKV, which cause the degradation of STAT2 (JAK- STAT pathways in humans), an IFN-regulated transcriptional activator (Grant et al., 2016a). Moreover, TOSV can antagonise the IFN system by its non-structural protein (NSs) (Savellini et al., 2011; Gori-Savellini et al., 2013).

Neutrophils can play important roles in response to vector-borne infections by arboviruses or parasites. For instance, once a mosquito bites and exerts its saliva into the skin inoculation site, the recruitment of neutrophils rapidly occurs. Following this phenomenon, the influx of other immune cells to the site of infection is triggered, and these newly recruited immune cells become targets of virus infections, including for SFV, DENV, and BUNV. Experimental depletion of neutrophils reduced host inflammatory response to bites and led to a better clinical outcome in virus infections (Pingen et al., 2016; Schmid et al., 2016). Additionally, it has been shown that neutrophils are susceptible to WNV infection and become infected. It has been suggested that WNV-infected neutrophils may transport the virus into the central nervous system (CNS), potentially contributing to neurological diseases like encephalitis (Paul et al., 2017). In *Leishmania* infection, once a sandfly deposits its saliva into the skin, it triggers a rapid influx of neutrophils. These neutrophils can be infected by *Leishmania* parasites and exacerbate the infection in a mammalian host, serving as a “Trojan horse” reservoir for parasite infection (Peters et al., 2008).

The role of monocytes in arbovirus infections can vary with each virus infection. For instance, studies suggested that monocytes/macrophages are susceptible to CHIKV and cause persistent infection, particularly in joints (arthralgia), which is related to continuously releasing their inflammatory cytokines from infected these cells (Dupuis-Maguiraga et al., 2012). Likewise, monocytes have been shown to be susceptible to infection by both DENV and ZIKV (Durbin et al., 2008; Michlmayr et al., 2017). This

was further demonstrated by infecting mice that lack the chemokine receptor CCR2. These mice lack skin and circulatory monocytes and were protected from mosquito bite enhancement (detail discussed in section 1.6.1.1) of SFV (Pingen et al., 2016). However, monocytes have been suggested to protect against worse infection outcomes, like CHIKV and Ross River virus (RRV); in mice defective in the circulating monocytes model, the blood and tissues had higher viral loads of these viruses (Haist et al., 2017). The same controversial study results have been shown for WNV infection (Terry et al., 2015). These conflicting results underscore additional research's need to elucidate whether monocytes predominantly exert a protective or detrimental effect during arbovirus infections.

Resident macrophages in the skin are strategically positioned near the initial site of viral entry, enabling rapid detection of invading pathogens through a variety of PRRs and initiation of antiviral responses. However, research indicates that macrophages are primary targets for infection by most mosquito-borne viruses and may serve to replicate arbovirus in the skin (Pingen et al., 2016). Contribution of macrophages to the pathogenesis of DENV has been suggested. Macrophages are one of the main targets for DENV infection, with C-type lectin domain family 5, member A (CLEC5A), serving as the main receptor on macrophage surfaces for DENV. Following dissemination from the skin, macrophages within both lymphoid and nonlymphoid tissues act as primary reservoirs for DENV (Khanam et al., 2022). In addition, macrophage-secreted TNF-α has been shown to cause endothelial cell damage in DENV infection (Züst et al., 2014). Macrophages responses may also enhance JEV pathogenesis in the CNS. Here, the CLEC5A receptor on macrophages binds JEV, causing excessive cytokine expression (e.g., TNF-α, IL-1, IL-6), CXCL10, and other macrophage-derived chemokines. This phenomenon helps permeabilise the blood brain barrier and allow JEV to pass more easily into the brain and infect neurons (Yadav et al., 2022).

Skin DC express IFN following infection at mosquito bites (Bryden et al., 2020). These cells have important roles in immune responses to arboviruses infection, because they are some of the first cells to encounter virus and activate adaptive immune responses (Albornoz et al., 2016). Interestingly, many viruses have evolved to infect DC, such as TOSV, which infects DCs using the DC-SIGN receptor (Lozach et al., 2011). Furthermore, cDCs have demonstrated high susceptibility to infection by various arboviruses, including RRV, EEEV, VEE, CHIKV, and DENV (Marovich et al., 2001; Schilte et al., 2010). It has been suggested that infected cDCs may migrate to lymphoid

organs, and in doing so help with virus dissemination from the skin bite site. However, it should be noted that cell free virus can also disseminate efficiently from skin to lymph node via the lymphatics without requirement for cell migration (Iannacone et al., 2010). Interestingly, DCs infected with DENV cannot trigger efficient antiviral responses and are compromised in their ability to activate T-cells. This implies that some arboviruses have evolved mechanisms to suppress DCs' immune functions (Salazar et al., 2014; Santos Souza et al., 2016).

1.4.3 Adaptive Immunity

The innate immune system offers essential mechanisms for promptly detecting and eradicating pathogens, while adaptive immunity response is slow but has evolved to provide a more comprehensive recognition of antigens. Adaptive immunity contains a carefully regulated interaction among antigen-presenting cells and T and B lymphocytes, enabling pathogen-specific immune responses, developing immunologic memory which responds more effectively to restimulation, and maintaining host immune homeostasis. This section will discuss the fundamental mechanisms of the adaptive immune response, its primary cell types, and the significance of the adaptive immune response in arbovirus infections.

1.4.3.1 Cells of Adaptive Immunity

T-cells and B-cells derived from the common lymphoid precursor in the bone marrow (Kondo, 2010). The cells of the adaptive immune system encompass T lymphocytes, which undergo maturation in the thymus, and B lymphocytes, which develop in the bone marrow and play a vital role in humoral immunity by producing antibodies. Lymphocytes exhibit high mobility; following development in the primary lymphoid organs (thymus and bone marrow), they migrate to secondary lymphoid organs including the lymph nodes and the spleen, which capture circulating antigens from lymph and blood, respectively (Bonilla and Oettgen, 2010).

T-cells

T cell progenitors migrate from the bone marrow to the thymus. Within the thymus, T cells undergo a series of developmental processes including commitment to the T cell lineage under control of Notch signalling, rearrangement of the classical α/β T-cell receptor (TCR), positive and negative selection, and differentiation into specific lineages. After TCR recombination, T cells undergo positive selection, whereby those with receptors capable of recognizing self-MHC molecules survive, while T cells

lacking this ability undergo apoptosis. Around 95% of developing T cells are eliminated via this selection. In the subsequent phase of T-cell development, termed negative selection, the thymocytes possessing a strong affinity for MHC/self-peptide complexes are eliminated through apoptosis. Next, developing T cells differentiate into two lineages based on their TCR: CD4+ and CD8+ cells. CD4+ T cells are selected on MHC class II molecules, whereas CD8+ cells are selected with MHC class I. Following maturation as antigen-naïve cells, both lineages migrate into the circulation (Zúñiga-Pflücker, 2004).

1.4.3.1.1 CD4+ T Cells

Most T cells in the human body are CD4+ T cells. They play a crucial role in defending against infectious diseases but can also contribute to the development of allergies and autoimmune conditions. CD4+ T cells are commonly known as "helper" T cells (T_H) because their main function is to deliver cytokine signals to other effector cells and to assist in promoting high-affinity antibody production by plasma B cells (see section below) (Chopp et al., 2023). CD4+ T cells are activated when their TCR recognises a particular peptide displayed on MHC-II expressed by antigen-presenting cells (APCs). After recognition, naïve CD4+ T cells undergo differentiation, influenced by factors such as the intensity of the TCR signal, the cytokine microenvironment, and transcription factor expression. T cell subsets include Th1, Th2, Th9, Th17, Th22, T follicular helper (Tf_h), and several types of Tregs (Li et al., 2014; Hirahara and Nakayama, 2016). Naïve CD4+ T cells become T_H1cells under the instructive influence of interleukin-12 (IL-12) and IFNγ, and secrete pro-inflammatory cytokines (e.g., IFNγ, IL-2 and TNFα) which are responsible for cell-mediated immunity. Interestingly, Th1 T cells are the primary producers of IFNγ, e.g., accounting for roughly 50% of all detected IFNγ during acute CHIKV infections in mice (Teo et al., 2013). Th2 cells develop via activation with IL-4 and IL-10, and then express IL-4, IL-5, IL-6, IL-10, and IL-13. They play a role in response to extracellular immunity (e.g., anti-parasitic) and mediate mast and eosinophil cell-related allergy. Regulatory T-cells (Tregs), an anti-inflammatory CD4+ population, secrete inhibitory cytokines IL-10, IL-35, and TGF-β. These cytokines regulate inflammatory CD4+ T cells and thereby maintain immune balance and facilitate tissue repair. There is evidence of Tregs' involvement in CHIKV and ZIKV pathogenesis (Guerra-Gomes et al., 2021; Gois et al., 2022). Finally, Th17 cells develop in the presence of IL-6, TGF-β, IL-23 and, IL-1β and secrete the IL-17 pro-inflammatory cytokine. Th17 cells protect the host from bacterial and fungal

infections on mucosal surfaces but is also associated several inflammatory diseases (e.g., psoriasis and rheumatoid arthritis), and CHIKV and RRV- induced arthritis (Mostafavi et al., 2022a; Liu et al., 2022; Gomez-Bris et al., 2023). Each subset stimulates an immune response against a distinct group of pathogens by activating cells such as CD8+ T cells, NK cells, B cells (Chopp et al., 2023).

1.4.3.1.2 CD8+ T Cells

Naïve CD8+ T cells activate upon recognizing specific peptides displayed by MHC class I on APCs in peripheral lymphatic organs. Co-stimulatory signals and cytokines from APCs or CD4+ T cells can also shape CD8+ T cell differentiation into cytotoxic T-cells (CTL). CTLs contain perforin granules and granzymes, which when released into target (e.g., infected) cells triggers infected cells' death via lysosomes and apoptosis. Moreover, they produce cytokines (e.g., IFNγ and TNFα) that increase cytotoxicity and stimulate macrophages (Mittrücker et al., 2014; Hay and Slansky, 2022). CD8+ T cells are vital for efficiently controlling CCHFV infection, by likely producing antiviral cytokines and killing the infected cells. (Rao et al., 2023) showed that mice lacking IFNγ (Ifnγ−/−) and mice depleted of CD8+ T cells had higher viral loads and worsened pathogenesis. Conversely, the cytotoxic effect of CD8+ T cells can be harmful in the later stages of arboviral pathogenesis. For example, CD8+ T cell activity is responsible for killing SFV-infected oligodendrocytes in the CNS, which results in demyelination (Amor et al., 1996).

1.4.3.1.3 γδ T cells

Lastly, γδ T cells constitute a minor subset of T cells, comprising approximately 5-15% of T cells in the peripheral blood and less than 5% in the tissues (e.g., dermis, spleen) but important. They recognise antigens independently of MHC. The potential role of γδ T cells has been suggested in several arbovirus infections. For instance, these cells can act as an initial source of IFN-γ during dengue virus infection and enhance host immune responses by eliminating virus-infected cells (Chen et al., 2022).

B-cells

B cells derive from hematopoietic precursor cells (HSC) in the bone marrow (BM). Due to the rearrangement of the H-chain (V, D, J) and L-chain (V, J) gene segments encoding the B-cell receptor (BCR), broadly three developmental stages (pro-B, pre-B, and immature B cells) are undertaken in the BM. The goals of B cell development are; generating a diverse antigen receptor which allow them to recognise over 5×10^{13}

different antigens; altering or eliminating self-reactive B-cells/B-cell receptors (Pieper et al., 2013).

After expression of IgM in immature B cells, they migrate from the bone marrow to secondary lymphoid organs (e.g., spleen), where they progress through specific transitional stages known as T1 and T2, ultimately maturing into long-lasting mature follicular (FO) or marginal zone (MZ) B cells. Naïve B cells can encounter antigens either though their B cell receptor (BCR) or through, B cell–T_{FH} cell interaction occurs. BCR activation and T cell help induces naive B cells to proliferate and differentiate into two primary cell types: memory B cell or long living plasma cells (Akkaya et al., 2020). The cues supplied by activated CD4+ T cells also facilitate immunoglobulin class switching in B cells from IgM and IgD to other isotypes like IgG, IgA, and IgE, which can occur along with somatic hypermutation to diversify their antigen binding repertoire (Stavnezer et al., 2008; Steele, 2009). This leads to expression of high-affinity antigen-binding antibodies. Antibodies protects the host by neutralising pathogens, marking them for phagocytosis (opsonization), and providing a framework for activating the complement system (classical complement pathway).

B cells and their antibodies exhibit a dual role in the immune response to arbovirus infections, as they can either provide protection or exacerbate pathogenesis. For instance, research indicates that antibodies like IgM and IgG produced in reaction to SFV in mice conferred protective effects (Amor et al., 1996). On the other hand, antibody-dependent enhancement (ADE) can occur for some arboviruses, when non-neutralising antibodies enhance the entry of a virus into target cells via interactions with Fcγ receptors on immune cells. For example, during a secondary DENV infection, DENV-antibody complexes attach to Fcγ receptors on cells like macrophages, aiding viral entry and replication. Elevated viral levels of DENV due to ADE prompt the generation of inflammatory mediators and increase vascular permeability. This phenomenon raises concern in vaccine development, particularly for viruses like DENV and ZIKV, where ADE has been observed (Bardina et al., 2017; Elong Ngono and Shresta, 2018).

1.4.4 Cytokines and Chemokines in Innate and Adaptive Immunity

A “cytokine” refers to a protein secreted by immune cells that influences the activity of nearby cells with compatible receptors. Cytokines, generated by both immune and non-immune cells (e.g., fibroblasts and endothelial cells), exert their effects through

autocrine, paracrine, or endocrine signalling by binding to specific receptors on target cells, eliciting diverse responses. They are classified into various groups based on their structure and functions (Zhang and An, 2007).

Chemokines, a distinct subset of secreted cytokines, serve as chemoattractant, recruiting leukocytes expressing correct chemokine receptors into chemokine expressing tissues. Chemokines also play a role in organizing distribution of different cell types within lymphoid tissues into specific areas, where specialised immune responses occur e.g., T and B cell zones in lymph nodes. Here, key cytokines and chemokines involved in both innate and adaptive immune responses will be briefly discussed, with special attention to the type I interferon family, which pivotal in innate antiviral immunity.

1.4.4.1 IL-1 Family

Interleukin 1 (IL-1) family, consisting of proinflammatory cytokines primarily linked to innate immune responses, and includes IL-1α, IL-1β, IL-1 receptor antagonist (IL-1RA), IL-18, and IL-33. During infection or inflammation, epithelial cells, monocytes, neutrophils, macrophages, hepatocytes, and tissue resident macrophages primarily express IL-1α and IL-1β, which are pro-inflammatory and upregulate acute phase response proteins in the liver (Lopez-Castejon and Brough, 2011). They also trigger the secretion of additional cytokines such as IL-6 and TNF and can contribute to the induction of Th17 adaptive immune responses (Chung et al., 2009).

1.4.4.2 Common γ Chain (γc) Family

The cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 belong to a family that shares the common cytokine receptor γ chain (γc) (Spolski et al., 2017). The γc family cytokines collectively regulate various aspects of lymphocyte development, growth, differentiation, and survival, with clinical relevance extending to allergic and autoimmune diseases, cancer, and immunodeficiency. CD4+ and CD8+ T cells predominantly produce IL-2 when their T-cell receptors are stimulated (Liao et al., 2013). IL-2 promotes the activation and proliferation of T helper (Th)1, Th2, and Th9 cells (Lin and Leonard, 2018). IL-4 serves as a potent cytokine for Th2 responses, plays a role against extracellular parasite infections, and contributes to allergic reactions by signalling B cells to undergo class switching to produce IgE immunoglobulins (Paul, 2015). IL-7 plays a pivotal role in the development and maintenance of T-cells, while IL-9 supports mast cell proliferation. Furthermore, IL-15 is essential for the formation

and persistence of NK cells, and IL-21 facilitates the transformation of B-cells into plasma cells, impacting immunoglobulin production (Spolski et al., 2017).

1.4.4.3 Common β Chain family

Cytokines that engage with a receptor containing the cytokine-specific α chain and the common β chain are categorised as the common β chain family. This group includes IL-3, IL-5, and GM-CSF, which primarily regulate the differentiation and function of myeloid cells, particularly in allergic responses. Specifically, IL-3 and GM-CSF contribute to development of immune cells including macrophages, mast cells, and polymorphonuclear cells, while IL-5, originating from T-cells, stimulates B-cell growth and maturation (Shearer et al., 2003).

1.4.4.4 Tumor Necrosis Factor Superfamily

The tumor necrosis factor superfamily (TNFSF) encompasses more than 20 cytokines and nearly 30 cytokine receptors, which together orchestrate signalling pathways crucial for immune regulation, cell growth, differentiation, and apoptosis. Among these, TNFα, primarily produced by macrophages and T-cells, is the most extensively studied. TNFα exists in soluble or membrane-bound forms and interacts with TNF receptors TNFR1 and TNFR2. While TNFR1 is present on leukocytes and stromal cells, TNFR2 is found on B-cells and T-cells. Upon TNFα binding, these receptors initiate signalling cascades that activate NFκB or induce apoptosis. Excessive TNFα expression in the blood is the primary mediator of endothelial permeabilisation that leads to organ failure during sepsis (Šedý et al., 2015; Song and Buchwald, 2015).

1.4.4.5 Interferons

Interferons (IFNs) are cytokines best known for their antiviral properties, but also are potent cell growth regulators and immunomodulatory activity. IFNs are divided into three families: type I, II, and III. Type I IFNs contain multiple subtypes (Katze et al., 2002), but IFN-α and IFN-β are the primary interests of this thesis. Type II IFN only has IFN-γ (Alspach et al., 2019), and the Type III IFN family, also known as IFN-λ, has IFN-λ1, -λ2, -λ3, -λ4, and is mainly generated by infected cells located in mucosal tissues, such as respiratory tract epithelial cells (Ye et al., 2019). IFN-α and IFN-β are produced by various cell types, while IFN-γ is specifically synthesised by select immune cells such as natural killer (NK) cells, CD4+ T helper 1 (Th1) cells, and CD8+ cytotoxic T cells (Katze et al., 2002).

Type I IFN is primarily produced by cells that sense viral PAMPs, with additional significant expression from plasmacytoid dendritic cells (pDCs), which make abundant type I interferons (IFN-α and IFN-β). Interestingly, pDCs typically trigger IFN production without being infected following TLR7 activation (Swiecki and Colonna, 2015).

Type I Interferons

The type I IFN family includes IFNα, IFNβ, IFNδ, IFNε, IFNζ, IFNκ, IFNν, IFNτ and IFNω, and some of them show diversity in mammals, and are key players in the immune response against viruses (Pestka et al., 2004). IFN-α and IFN-β bind to common cell-surface receptors, IFN-αR1/ IFN-αR2 [also known as the IFN-α/β (IFNAR) receptor] expressed on most differentiated cells (Dalskov et al., 2023).

IFN induction in response to viral recognition involves three main pathways, each mediated by specific adaptor proteins (STING, MAVS, and either TRIF or MyD88) that connect pattern recognition receptors (PRR) to downstream signalling components. These pathways converge to activate the kinase TBK1, which phosphorylates transcription factors IRF3 and IRF7 (Dalskov et al., 2023). These pathways also activate NF-κB (Honda et al., 2006).

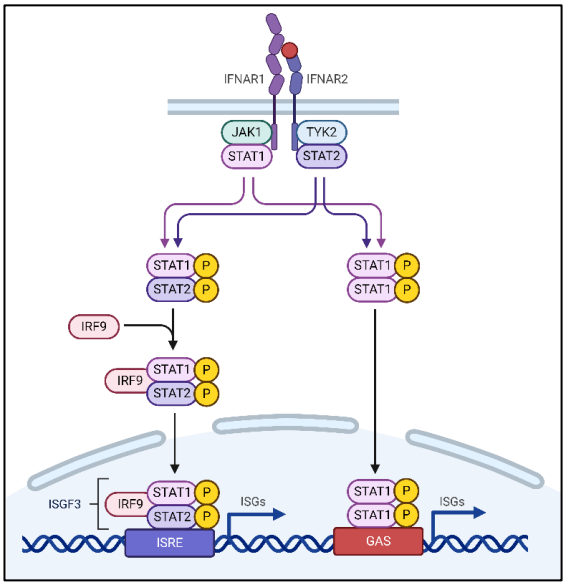


Figure 1.13: The Type I IFN Signalling Pathway
Upon binding type I IFNs to the IFNAR receptor, composed of two subunits, IFNAR1 and IFNAR2, JAK1 and TYK2 kinases are activated, which phosphorylate STAT1 and STAT2. These phosphorylated STAT proteins dimerize (STAT1-STAT2) and form a complex with IRF9, creating the ISGF3 complex. The ISGF3 complex translocates to the nucleus, where it binds to interferon-stimulated response elements (ISREs) in the promoter regions of interferon-stimulated genes (ISGs). Phosphorylated STAT1 can also dimerize with another phosphorylated STAT1 molecule, forming a STAT1-STAT1 homodimer. This homodimer translocates into the

nucleus, binding to Gamma-Activated Sequence (GAS) elements in the promoter regions of specific genes. Created with BioRender.com

Following their expression, type I IFNs initiate antiviral responses by binding to a shared IFNAR receptor. This binding activates the JAK1-STAT pathway, prompting the formation of the ISGF3 complex comprising STAT1-STAT2 dimers and IRF9. ISGF3 then binds to interferon-stimulated response elements (ISREs) in the nucleus, leading to the transcriptional activation of various interferon-stimulated genes (ISGs) (Figure 1.14) (Ivashkiv and Donlin, 2014; McNab et al., 2015).

Nearly all viruses, including arboviruses, have evolved diverse pathways to disrupt the IFN pathway (discussed in the 1.4.2.3 section). These include IFN induction/expression inhibition, intercepting receptor binding of IFNs through viral decoy IFN receptors, disrupting intracellular IFN signalling, and reducing ISG expression levels (Katze et al., 2002). It is known that IFNα/β receptor knockout mice, along with IFN-γ receptor knockouts, fail to generate an effective antiviral response (Kamijo et al., 1993). For example, SFV infection of IFNAR knockout mice did not survive long after 24 hours post-infection, unlike in wild type mice (Hwang et al., 1995).

Type II Interferons

IFN-γ has roles in innate and adaptive immune responses against viruses, bacteria, and tumors, its dysfunction is evident in promoting some immunopathology. IFN-γ expression primarily occurs in differentiated T lymphocytes and natural killer (NK) cells, ultimately resulting in the development of a polarised immune response consisting of T helper (T_H)1 CD4+ T cells and CD8+ cytolytic T cells. IFN-γ upregulates multiple IFN sensitive genes in many cell types and e.g., activates macrophages to become highly anti-microbial.

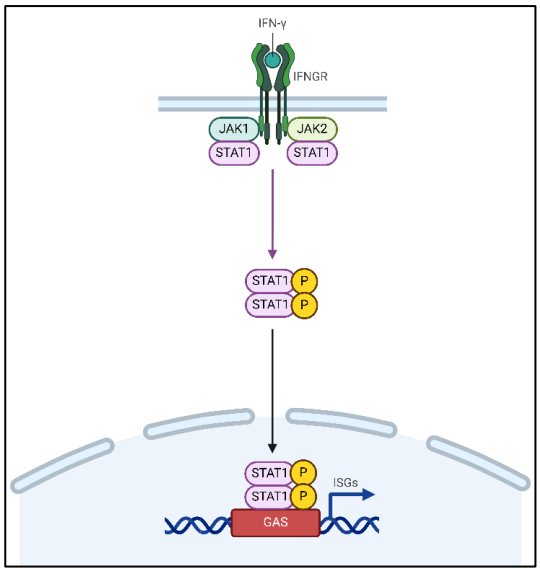


Figure 1.14: The Type II IFN Signalling Pathway

The sole type II IFN, known as IFN-γ, interacts with the cell-surface receptor called the IFNGR. Upon binding IFN-γ to the receptor, JAK1 and TYK2 kinases are activated and promote the formation of STAT1–STAT1 homodimers, which translocate to the nucleus and bind to Gamma-Activated Sequence (GAS) elements located in the promoters of specific interferon-stimulated genes (ISGs), thereby initiating their transcription. Created with BioRender.com

IFN-γ binds the receptor IFNGR contains chains IFN-γR1/ IFN-γR2 and activates JAK1, JAK2, STAT1, STAT3, and STAT5 (Figure 1.15). IFN-γ exhibits diverse functions such as promoting macrophage maturation and phagocytosis, boosting expression of antigen presentation via MHC I and MHC II molecules, inducing apoptosis in infected cells, activating NK cells, facilitating class switch B cell immunoglobulins from IgM to IgG and IgE, and promoting the differentiation of CD4+ T-cells into Th1 cells (Pestka et al., 2004; Alspach et al., 2019). IFN-γ or IFN-γ receptor knockout mice suffered from elevated viral loads in peripheral tissues early in WNV infection, resulting in rapid dissemination to the CNS and early mortality (Shrestha et al., 2006).

Type III Interferons

The type III interferon family includes IFNλ1, IFNλ2, IFNλ3, and IFNλ4 and share common heterodimeric receptor complex: IFN-λR1/ IL-10R2. When IFN-λ binds to its receptor, it triggers the upregulation of similar antiviral interferon-stimulated genes (ISGs) as Type I IFNs (Figure 1.16) while inhibiting the proliferation of epithelial and glioblastoma cells (Kotenko and Durbin, 2017). Importantly, the type III IFN system has evolved as the frontline defence at mucosal barriers, which are the initial targets of many invasive pathogens. For example, intestinal epithelial cells (IECs) depend on the IFN-λ-based autocrine system for antiviral protection. Intraepithelial T lymphocytes

(IELs), in constant contact with the epithelial layer, also secrete both type I and type III IFNs upon antigen stimulation (Swamy et al., 2015).

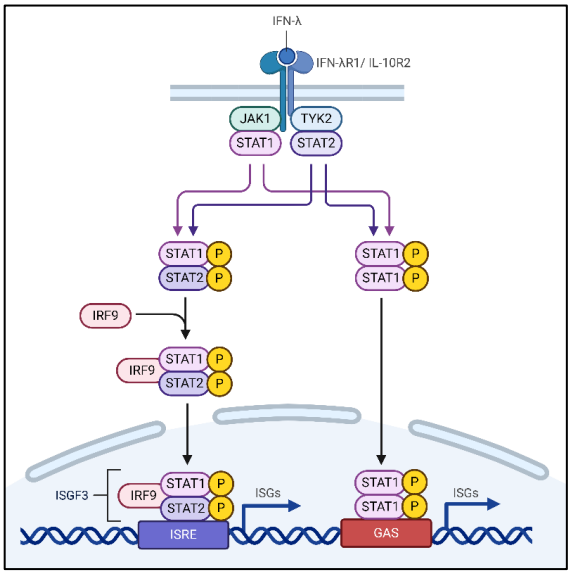


Figure 1.15: The Type III IFN Signalling Pathway

Type III IFNs bind to a receptor complex made up of the IFN-λR1 and the IL-10Rβ. The receptor binding activates the JAK1 and Tyk2 kinases, which phosphorylate STAT1 and STAT2—the phosphorylated STAT1 and STAT2 heterodimers complex with IRF9 to produce the ISGF3 transcription factor. The ISGF3 transcription factor binds to ISREs and promotes the expression of ISGs. Phosphorylated STAT1 can also dimerize with another phosphorylated STAT1 molecule, forming a STAT1-STAT1 homodimer. This homodimer translocates into the nucleus, binding to Gamma-Activated Sequence (GAS) elements in the promoter regions of specific genes. Created with BioRender.com

1.4.4.6 Interferon-Stimulated Genes

The IFN response to pathogens leads to the transcription of hundreds of IFN-stimulated genes (ISGs). IFN families trigger a distinct but partially overlapping group of ISGs. Furthermore, certain ISGs (e.g., viperin) can be directly activated by viral infections even without IFN production, being regulated directly by factors like IFN regulatory factors (IRF1, IRF3, IRF7), NFκB, or IL-1 signalling (Sarkar and Sen, 2004; Platanitis and Decker, 2018). After the initial discovery of the first ISGs two decades ago, a meta-analysis of ISG transcriptomes identified approximately 450 genes that were consistently activated by type I IFNs in various cell types from different mammalian species (Schoggins et al., 2011). It is now believed that the total number of ISGs may be even greater. In terms of effector functions, ISGs exert antiviral effects through several means: they directly disrupt viral replication (e.g., viperin), halt cellular metabolism that may otherwise aid viral replication (e.g., antiviral ISG IFITM3 inhibits viral entry), block protein synthesis, lower the threshold for apoptosis, increase the expression of

antigen presenting class I MHC molecules, and enhance leukocyte migration by upregulating chemokines; thereby boosting the likelihood of clearance by CD8+ T-cells (Schoggins, 2019). For example, IFITM proteins enhance the trafficking of these vesicles containing viruses to lysosomes, effectively blocking productive entry (Spence et al., 2019).

Several ISGs are studied in this thesis and the following text summarises their function.

RSAD2/viperin

Radical-SAM-domain-containing-2 (RSAD2) encodes viperin, an endoplasmic reticulum-associated, IFN-induced protein with broad antiviral activity across various mammalian species, as well as in fish, birds, and reptiles. RSAD2 can act at multiple stages of the virus life cycle including transcription, replication, assembly and release, and its function also includes a chain terminator during virus RNA synthesis (replication) in infected cells (Helbig and Beard, 2014; Rivera-Serrano et al., 2020). In terms of RSAD2's role in arbovirus infections, for instance, a longitudinal transcriptional study conducted on peripheral blood mononuclear cells (PBMCs) from individuals infected with CHIKV unveiled the role of RSAD2 in combating infection. Notably, RSAD2 showed significant induction in monocytes, the primary cellular target of CHIKV in the bloodstream. Additionally, *rsad2* knock-out mice exhibited elevated virus titres and more pronounced joint inflammation than those with normal *rsad2* expression (Teng et al., 2012). RSAD2's enzymatic function, along with protein kinase R (PKR), was also shown to be required for BUNV infection inhibition in a mouse model (Carlton-Smith and Elliott, 2012).

ISG15

ISG15, a 15-kDa ubiquitin-like protein (UBL), is known as a potent and swiftly upregulated interferon-stimulated gene (ISG) in response to pathogen invasion. ISG15 can conjugate with other proteins through an enzymatic cascade reaction, which is called ISGylation and has been discovered to be crucial in the innate immune responses triggered by type I IFN, playing a vital role in defending host cells against pathogens including RNA viruses (Skaug and Chen, 2010). Pathogen activated immune cells, including monocytes and lymphocytes express ISG15. Once inside the cell, ISG15 can interact with proteins involved in innate immune signalling pathways, activating e.g., IRF3, STAT1, JAK1; or inhibiting e.g., RIG-I; thus, modulating the expression of IFN.

Moreover, extracellular secreted ISG15 can bind to the LFA1 receptor on cell surfaces, stimulating the secretion of IFNγ from NK cells and T cells, as well as promoting NK cell proliferation and DC maturation (Zhang et al., 2021). Research into neonatal mice lacking ISG15 showed a rise in susceptibility to fatal CHIKV infection. However, mice deficient in the ISG15 E1 enzyme, which prevents conjugation, exhibited a viral load similar to wild-type mice. However, *isg15-/-* mice exhibited notably higher levels of pro-inflammatory cytokines and chemokines, leading to a reduced survival rate (Werneke et al., 2011). Counterintuitively, an *in vitro* study suggested that ISG15 actually promotes ZIKV replication in a ISGylation-dependent manner, and in its absence (*isg15*-/- cells) significantly reduced the ZIKV RNA level in A549 cells (Wang et al., 2020).

IFIT

Following pathogen invasion, host innate immune responses activate IFN-induced protein with tetratricopeptide repeats (IFIT) genes. The IFIT gene has counterparts in various mammalian species, such as birds, fish, and amphibians (Diamond and Farzan, 2013). The IFIT family, comprising IFIT1, IFIT2, IFIT3 in both humans and mice, and IFIT5 in humans, employs diverse antiviral mechanisms. Following binding to virus RNA, translation is inhibited. IFIT binding helps identifying viral RNAs without 2’-O methylation, and also with detecting viral RNAs that possess 5’-triphosphates (e.g., RVFV) (Pichlmair et al., 2011; Schoggins, 2014). IFIT proteins, found in the cytoplasm, mediate their function while lacking enzymatic activity. Notably, WNV has developed a 2’-O methylation of its RNA's 5’ cap to escape IFIT responses. When WNV is engineered to lack this methylation, it becomes more vulnerable to IFIT effects, hindering viral replication and mitigating CNS pathology; however, infectivity is restored in IFIT1-deficient macrophages (Daffis et al., 2010). In vitro studies conducted on A549 cells have demonstrated the significance of IFIT1 in limiting the translation of both ZIKV and DENV proteins (Frumence et al., 2016).

1.4.4.7 Chemokines

Chemokines constitute a group of compact, highly conserved proteins that play crucial roles in chemotaxis, haematopoiesis, and angiogenesis. They function by binding with cell surface G-Protein Coupled Receptors (GPCRs). Four classes of chemokines exist, distinguished by the positions of conserved cysteine residues: CC chemokines, CXC

chemokines, XC chemokines, and CX3C chemokines (Miller and Mayo, 2017). However, this thesis excludes discussion of the latter two chemotactic cytokines.

CC chemokines, comprising 28 members from CCL1 to CCL28, predominantly stimulate migration of leukocytes including monocytes, basophils, eosinophils, T-lymphocytes, and NK cells during inflammation and infection (Murphy et al., 2000). Generally, chemokines including CCL2, CCL3, CCL4, CCL5, CCL11, and CCL13 are categorised as inflammatory. For example, CCL2 is known for its chemotactic effect on monocytes, which it sends to the inflammation site via forming a chemokine/receptor complex (CCL2/CCR2) signalling (O'Connor et al., 2015). This thesis predominantly investigated at expressions of CCL2.

In contrast, CCL18, CCL19, CCL21, CCL25, and CCL27 are considered homeostatic. These homeostatic chemokines help orchestrate immune system function at rest e.g., enabling continuous recirculation of T cells between blood and lymph nodes. CCL1, CCL17, CCL20, and CCL22 have dual functions, serving both inflammatory and homeostatic roles (Le et al., 2004).

Currently, 17 members of CXC chemokines (CXCL1-CXCL17) have been defined. Similar to CC chemokines, CXC-class chemokines have also been classified into inflammatory (e.g., CXCL2), homeostatic (e.g., CXCL12), and dual-function chemokines (e.g., CXCL10) (Bendall, 2005). Importantly, cells such as macrophages express CXCL1 and CXCL2, which play a crucial role in recruiting neutrophils to the site of inflammation. Neutrophils are particularly responsive to these chemokines because they express receptors CXCR1 and CXCR2, which can bind to not only CXCL1 and CXCL2 but also other related chemokines like CXCL5, CXCL6, CXCL7, and CXCL8. As a result, recruited neutrophils support and amplify the immune response at the site of inflammation (Palomino and Marti, 2015). CXCL9 (monokine induced by gamma-IFN), CXCL10 (IFN-induced protein-10) are particularly involved in Th1 CD4 and CD8 T cell recruitment and can be expressed by both type I and type II IFN in numerous cell types, including fibroblasts. These chemokines function by binding to the CXCR3 receptor.

Chemokines can mediate influx of leukocytes into the CNS and cerebrospinal fluid (CSF) across the blood-brain barrier (BBB) in meningitis and meningoencephalitis. A study indicated that serum CXCL10 concentrations were significantly increased in the acute phase of Tick-borne encephalitis (TBE) compared to the control group patients

(Groom and Luster, 2011; Koper et al., 2018). In addition, CXCL10 is expressed by murine-cultured astrocytes and microglia and may have a role for both physiological and pathological processes in the CNS (Li et al., 2006). In a mouse model of SFV encephalitis, the influx of immune cells contributed to lethal encephalitis during the infection. Blocking CCR2 and CXCR3 receptors could reverse this pathogenic effect (Michlmayr et al., 2014). This thesis primarily examined the expressions of CXCL2 and CXCL10.

1.5 DERMAL BIOLOGY

1.5.1 Structure

The largest organ, the skin, provides a dual defence system, offering a physical barrier against external injuries and an immunological barrier against pathogens. Furthermore, it functions as a sensory organ capable of detecting stimuli such as temperature fluctuations and tactile sensations. Additionally, it plays a role in maintaining proper hydration levels and shielding against ultraviolet radiation and environmental toxins (Di Meglio et al., 2011).

The skin is comprised of three main layers: the epidermis, the dermis, and subcutaneous fat tissue (hypodermis) (Figure 1.17). The epidermis is composed of five layers: the stratum corneum (the outermost layer), responsible for providing the protective barrier between the inner and the external environment, stratum lucideum, stratum granulosum, stratum spinosum, and stratum basale (Bird et al., 2018). The epidermis contains keratinocytes (more than 90% of all epidermal cells) and resident dendritic cells, known as Langerhans cells (Nestle et al., 2009).

The dermis has collagen fibres that provide strength and elasticity structure of the skin and is categorised into upper papillary and lower reticular sub-layers. Although the reticular dermis includes hair follicles, sebaceous glands, and sweat glands, both dermis layers harbour fibroblasts, adipocytes, immune cells like dermal dendritic cells (DDCs), αβ T cells, γδ T cells, natural killer (NK) cells, B cells, mast cells, and macrophages, alongside nerve endings and blood vessels (Di Meglio et al., 2011; Nguyen and Soulika, 2019). In the context of arbovirus infection, the dermis, with its extensive network of superficial blood vessels, becomes a prime target for hematophagous arthropods seeking a blood meal, making it the primary site of virus deposition during infection (Briant et al., 2014).

Lastly, the hypodermis comprises fibroblasts and adipocytes. Adipose tissue in the skin stores energy through fatty acids and serves as an endocrine organ crucial for regulating glucose and lipid levels (Driskell et al., 2014). It releases various mediators, including growth factors, adipokines, and cytokines, and houses numerous immune cells (Cildir et al., 2013). Moreover, subcutaneous fat acts as insulation for the body, as fat is a poor conductor of heat (Nguyen and Soulika, 2019).

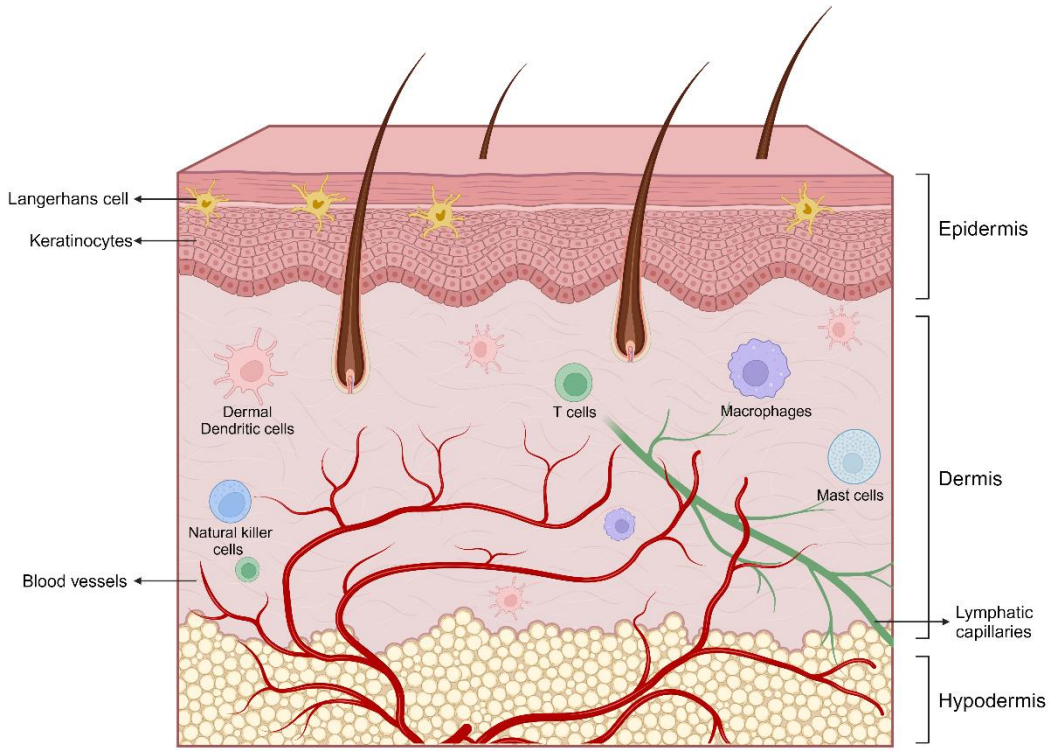


Figure 1.16: The Skin Structure with Its Three Layers and Cells

The skin comprises three distinct layers, each containing various cells and structures. The epidermis is the outermost layer, housing keratinocytes, melanocytes, and Langerhans cells. The dermis beneath the epidermis contains resident and migratory immune cells, connective tissue, hair follicles, blood vessels, lymphatic vessels, and sweat glands. The hypodermis is the deepest layer, consisting primarily of fat and connective tissue. Created with BioRender.com

1.5.1 Cells of the Dermal Compartment

One of the initial key stages in arbovirus infection involves the deposition of the virus by hematophagous insects (e.g., sandflies and mosquitoes) during feeding. The virus is mostly deposited in the dermis of the skin as the insects probe to locate a capillary or create a blood pool to initiate feeding. However, there is a lack of understanding regarding what occurs during the during the initial stages following the deposition of arboviruses (such as TOSV) in the skin. This includes the identification of the initial target cells of infection, as well as the involvement of local and migratory immune cells and fibroblasts in the cutaneous immune response. In this section, the main cellular

components of the epidermis and dermis and their influence on arbovirus infections will be elucidated.

1.5.1.1 Cells of the Epidermis

The epidermis protects the body from the outside world and senses pathogens through keratinocytes and Langerhans cells. As mentioned above, the most abundant cell type of the epidermis is the keratinocyte. Keratinocytes offer both structural integrity to the epidermis via keratin intermediate filaments and innate immune sensing, principally through TLR1-6 and TLR9. They are also involved in anti-bacterial and anti-viral defence via the secretion of β-defensins and type I IFN, chemokines, respectively (Miller, 2008). Studies with distinct arboviruses have shown cultured keratinocytes are susceptible to virus infection. For instance, WNV, DENV and CHIKV can infect these cells (Lim et al., 2011; Briant et al., 2014). The primary skin-resident immune cell is Langerhans cells (LCs) in the epidermis layer. Langerhans cells possess PRRs, including Toll-like receptors (TLRs) 1, 2, 3, 5, 6, 10, and C-type lectin receptors (CLRs), which enable them to detect and react to injury and infections effectively (Collin and Milne, 2016; Martin et al., 2022). LCs in humans express langerin (or CD207), a type II membrane-associated C-type lectin, at their cell surface and inside the Birbeck granules (BGs) in their cytoplasm. Langerin plays a role in the capture and degradation of viruses by LCs such as HIV-1 (Ribeiro et al., 2016). An *in vitro* study showed that USUV led to productive infection in human LCs as target cells for the virus by escaping langerin-induced restriction. USUV is an emerging arbovirus phylogenetically close to WNV (Martin et al., 2022). These cells are also susceptible to DENV infection (Wu et al., 2000; Cerny et al., 2014). Moreover, the epidermis harbours a distinct population of foetal-derived γδ T cells, express a conserved TCR, known as dendritic epidermal T cells (DETCs). Upon binding to their specific antigen, DETCs secrete IFN-γ and IL-2, thereby inducing local inflammatory reactions and facilitating leukocyte proliferation, differentiation, and activation (O'Brien and Born, 2015). Whether any of these data has *in vivo* relevance for arthropod transmitted virus is not clear.

1.5.1.2 Cells of the Dermis

The dermis comprises an extracellular matrix composing collagen and elastic fibres synthesised by fibroblasts. This matrix acts as a scaffold for the migration of immune cells and maintains the structural integrity of the dermis (Nguyen and Soulika, 2019). Fibroblasts also have immunomodulatory function by responding to inflammatory

signals via their PRRs and synthesising many pro-inflammatory cytokines and chemokines (Bautista-Hernández et al., 2017). CHIKV antigens were observed in fibroblasts of the deep dermis in the human and mouse skin, as well in fibroblasts located beneath the synovial wall of the joint connective tissue. Together, the study suggested that CHIKV primarily infects fibroblasts, which may also explain its preference for joints, and skin connective tissues (Couderc et al., 2008). CHIKV infection in fibroblasts is inhibited by IFNβ (Schilte et al., 2010). Moreover, the susceptibility of primary human dermal fibroblasts to DENV has suggested these skin cells as a significant site for primary replication (Bustos-Arriaga et al., 2011).

CD11b+ DCs (conventional), Ly6C^{hi} blood monocyte-derived dendritic cells (moDCs), and macrophages are also found in the dermis. CD11b+ DCs are involved in phagocytosis and antigen presentation via MHC class II molecules. Following antigen uptake, dendritic cells migrate to the lymph nodes (LNs), activating T cells and leading to the production of pro-inflammatory cytokines. moDCs exhibit poor migration function to LNs but can potentially activate T cells within the dermis. Dermal macrophage populations are crucial in recognizing pathogens through PRRs and mediate local immune responses by releasing proinflammatory cytokines and chemokines, which recruit neutrophils to the site of infection or inflammation (Tamoutounour et al., 2013). Dermal DCs and dermal macrophages are also primary cell targets for DENV.

Mast cells can also be found within the dermis (discussed in section 1.4.2.2), which are crucial for antimicrobial immunity and allergic responses. Lastly, the dermis also includes γδ T cells, which produce IL-17 type and IL-22 cytokines—dermal IL-17 production influences in promoting the development of inflammatory skin conditions such as dermatitis and psoriasis (O'Brien and Born, 2015).

1.6 VECTOR SALIVA AND INFECTION

Arboviruses infect susceptible arthropod vectors through a process that involves a cycle of transmission between vertebrate hosts and their vectors. Virus is transmitted to a new vertebrate host when the vector feeds for a blood meal. This transmission occurs when the vector inserts its mouthparts into the host's skin during which it deposits saliva. If infected, saliva may contain virus in addition to numerous vectors expressed factors that have potent biological effects on the skin (Lefteri et al., 2022).

When arthropods probe for blood vessels in the skin, they encounter a host response characterised by haemostasis, inflammation, and immunity (if bite experienced). Arthropods inject saliva composed of diverse molecules that overcome this response to facilitate successful blood feeding (Ribeiro and Francischetti, 2003). In general, hematophagous arthropods possess at least one factor that possesses anti-clotting, anti-platelet, and vasodilatory properties, which typically vary significantly at the sequence level among species. For instance, the saliva of *Aedes* species mosquitoes contains sialokinin, a vasodilatory tachykinin-like peptide, but the saliva of *Anopheles* species mosquitoes does not possess this exact molecule, but instead expresses different factors that have skin vasoconstricting function (Schneider and Higgs, 2008). Pathogens have also evolved alongside their vectors to enhance transmission efficiency. For example, *Leishmania* parasites (Titus and Ribeiro, 1988) effectively establish infections in experimental settings when transmitted with sandfly saliva, and mosquito saliva augments many genetically distinct arbovirus infections (e.g., SFV, BUNV, WNV) (Styer et al., 2011; Pingen et al., 2016). These results suggested that pathogen replication within the skin bite site marks a crucial phase of infection, before virus is able to disseminate to the blood and peripheral tissues distal from the bite.

This section will briefly focus on the effect of mosquito and tick saliva on arbovirus infections, as well as the composition of sandfly saliva and its effect on Leishmania parasite infection.

1.6.1 Mosquito Saliva

The injection of the virus in mosquito bitten skin, or virus co-injected with mosquito saliva, or salivary gland extract (SGE), compared to the inoculation of the virus alone (in an experimental setting), significantly influences the severity of virus infection (Pingen et al., 2016; Pingen et al., 2017). Dengue virus (DENV), West Nile virus (WNV), Semliki Forest virus (SFV) and Bunyamwera virus (BUNV) infections have been shown to be augmented by these mosquito-derived factors in a mammalian host (Styer et al., 2011; Pingen et al., 2016). The specific mechanisms underlying this pan-viral enhancement are still important research subjects.

Briefly, the vascular response against mosquito saliva can enhance viral infection and dissemination, via sialokinin (SK)-dependent vascular leakage. *Aedes* mosquito saliva contains SK, which rapidly increases the permeability of the blood vascular barrier. When virus is co-inoculated with SK alone in the absence of saliva, SK leads to a rapid

induction of oedema, and leukocyte influx. Infection of some of these leukocytes, primarily monocytic cells and their progeny leads to increased virus RNA at the skin inoculation site and enhanced dissemination to the blood, compared to the virus alone group (Lefteri et al., 2022). This is discussed in more detail below in section 1.6.1.1.

Another hypothesis regarding mosquito saliva-dependent enhancement suggests that mosquito saliva may assist virus infection by suppressing host immune responses at the site of infection. For example, cultured human keratinocytes showed significantly higher viral loads during early infection with DENV in the presence of *Aedes aegypti* saliva than those infected with the virus alone, suggesting that the enhanced infection was associated with expression levels of IFNs decreased by salivary gland extract (SGE) (Surasombatpattana et al., 2012). However, this mechanism is unlikely to account for virus enhancement by mosquito saliva, as studies have shown that mosquito saliva enhances DENV and ZIKV infection even when type I IFN is absent (Michael J. Conway et al., 2014a; Schmid et al., 2016; Lefteri et al., 2022). Furthermore, the presence of mosquito bites enhanced cutaneous type I IFN (e.g., IFN-β) and ISG gene expression, indicating that bites do not promote virus infection by suppressing the cutaneous induction of IFNs (Pingen et al., 2016).

1.6.1.1 The Host Inflammatory Response to Mosquito Biting Modulates Host Susceptibility to Arthropod-borne Infection

Mosquito bites and saliva induce inflammation, leading to the recruitment of immune cells to the site of inoculation. In addition, various immune cells are susceptible to arthropod-borne viruses discussed in 1.4.2.3 section. Previously, our group showed that mosquito bites caused an influx of inflammatory neutrophils, likely driven in part by neutrophil-attracting chemokines such as CXCL2 and promoted by IL-1β. Recruited neutrophils were themselves pro-inflammatory and resistant to virus infection. Recruited neutrophils expressed chemokines that in turn recruited monocytes, including CCL2. This resulted in an influx of virus-susceptible monocytic cells. The recruited monocytic cells become infected and replicated new virus. Moreover, when neutrophils are depleted, and IL-1β expression is reduced, the local skin response to bites is suppressed. This in turn reduced virus replication and spread within the host. Blocking the migration of virus-susceptible monocytes into the skin bite site, as seen in *ccr2* -/- mice, also prevents the enhancement of virus infection by bites (Pingen et al., 2016). Likewise, another independent study illustrated that mosquito SGE induces the

recruitment of inflammatory neutrophils and monocytes, potentially leading to increased DENV infection (Schmid et al., 2016).

1.6.2 Tick Saliva

Unlike mosquitoes, tick species feed on blood for extended periods ranging from hours to days or even weeks. As a result, tick saliva has evolved to contain numerous secreted proteins with functions that are not yet fully understood. Tick saliva comprises hundreds or thousands of proteins, a mixture of peptides, and non-peptide molecules, that aid in blood feeding by disrupting verterbrate hosts' haemostasis, inflammation, and immunity. The saliva also contains water and ions from the host blood meal, which regulates the tick's water balance and supports attachment (Nuttall, 2019a).

Importantly, ticks have developed a distinctive set of immunosuppressive factors, likely as an adaptation to their prolonged attachment to mammalian skin and avoid detection during blood taking (Déruaz et al., 2008; Nuttall, 2019b). For example, tick-derived cytokine binders, evasin proteins, directly inhibit the expression of pro-inflammatory cytokines (Hayward et al., 2017). These immunosuppressive effects seem to facilitate the transmission of tick-borne diseases indirectly. Tick saliva blocks the antiviral effects of IFN-β, leading to increased replication of tick-borne encephalitis virus (TBEV) in dendritic cells (Lieskovská et al., 2015). Tick saliva also exacerbates the infection in the host in terms of its role in the dissemination and pathogenesis of arboviruses, like mosquitoes. A study showed that tick saliva co-inoculated with Powassan virus (POWV), an encephalitic tick-borne flavivirus, also increases viral loads and alters the course of disease in the *in vivo* mouse model compared to infected mice with POWV alone (Hermance and Thangamani, 2015).

1.6.3 Sandfly Saliva

The salivary system of sandflies comprises two salivary glands, ducts, a pump, and a channel. These glands are paired, hollow organs surrounded by a single layer of epithelium (Figure 1.18). The glands may vary in size and shape depending on the sandfly species (Adler and Theodor, 1926).

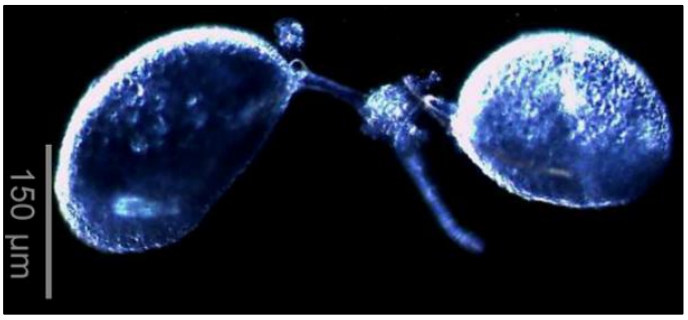


Figure 1.17: Dark-field Microscopy for *Phlebotomus duboscqi* Salivary Glands
From (Lestinova et al., 2017)

The saliva of sandflies consists of compounds with anti-haemostatic, vasoactive, immunomodulatory, and anti-inflammatory properties that counteract the host's natural defence mechanisms, and the makeup of the saliva may vary due to species, geographic locations, and gender. However, the protein concentrations typically range from about 0.18 to 0.8µg per gland. When comparing blood-feeding female sandflies to non-blood-feeding males, the levels of salivary proteins in *P. duboscqi* were nearly 30 times greater in females than in males (Volf et al., 2000), suggesting most of these factors are associated with facilitating blood feeding.

This includes immunomodulatory effects of sandfly saliva. For example, some *Lutzomyia* and *Phlebotomus* species' saliva has an inhibitory impact on the activation of T cells, while promoting the expression of Th2-type cytokines (Soares et al., 1998; Mbow et al., 1998). *Lutzomyia longipalpis* saliva is also inflammatory in mice, with a rise in macrophage numbers to skin, and increased expression of the monocytic chemokine CCL2, pro-inflammatory IL-6, CXCL8, and IL-12p40 (Costa et al., 2004). Saliva also has immune suppressant effects by stimulating the expression of IL-10 in DCs, while decreasing the expression of co-stimulatory molecules CD86 and also MHC-II on the surface of these cells. This effect hampers the dendritic cells' capacity to present antigens (Carregaro et al., 2008). *L. longipalpis* saliva itself *is* also chemotactic for macrophages (Teixeira et al., 2005)

1.6.3.1 Sandfly Saliva Composition

Protein groups found in certain *Phlebotomus* and *Lutzomyia* species include antigen 5-related proteins, apyrases, odorant-binding proteins (including D7-related and PpSP15-like proteins), yellow-related proteins (YRPs), silk-related proteins, and lufaxin-like protein. However, most salivary components remain unidentified; thus, their specific roles are still unknown.

Odorant binding protein-like families

The role of D7-related proteins, approximately 27 kDa in size, found in sandfly saliva is not fully understood. However, similar proteins in mosquitoes are known to bind biogenic amines or eicosanoids, acting as anticoagulants. PpSP15-like proteins, around 15 kDa, are specific to sandflies (Abdeladhim et al., 2012) and exhibit highly variable amino acid sequences, potentially leading to diverse functions among different sandfly species. In P. duboscqi, two SP15-like proteins (SP15a and SP15b) demonstrate a high affinity for the negatively charged surface of polymers like polyphosphate, heparin, and dextran sulphate. They compete with coagulation factor XII for binding sites and inhibit coagulation (Abdeladhim et al., 2014; Lestinova et al., 2017).

Antigen-5 protein family

The Antigen-5 family of proteins is part of the cysteine-rich secretory proteins, a protein family commonly found in the saliva of various blood-feeding insects. Our knowledge of these proteins is limited to other hematophagous insects, such as the kissing bug (*Dipetalogaster*, a genus of *Triatominae*) because the functions of Antigen-5 proteins in sand fly saliva has not yet been fully understood. Salivary Antigen-5 proteins from *Dipetalogaster maxima* hinder neutrophil oxidative burst and platelet aggregation (Assumpção et al., 2013; Abdeladhim et al., 2016).

Silk-related protein family

Silk-related proteins, also known as salivary proteins 32-like (SP32-like), were first identified in the salivary glands of *Phlebotomus papatasi* and share similarities with the silk protein of *Nephila clavipes*, a spider species (Valenzuela et al., 2001). This protein family is unique to sandflies. Importantly, PpSP32, a member of this protein family from *P. papatasi* saliva, stands out as the immunodominant antigen in people living where cutaneous leishmaniasis is prevalent. Therefore, it can potentially be an important tool for epidemiological studies and a biomarker for sandfly exposure, specifically for

P. papatasi (Marzouki et al., 2012; Marzouki et al., 2015). Recently, a study demonstrated that PpSP32 induces a strong immunomodulatory effect on monocytes and THP-1-derived macrophages derived from peripheral mononuclear cells of healthy individuals. In addition, this protein also has anti-inflammatory activity, such as reducing oedema *in the vivo* model (Souissi et al., 2023).

Yellow family of proteins (YRPs)

Yellow-related proteins are numerous in sandfly salivary glands. There are various recombinant YRPs depend on sandfly species such as LJM17, LJM17 and rSP03B and PpSP42 and PpSP44, from *Lutzomyia longipalpis, Phlebotomus perniciosus, Phlebotomus papatasi*, respectively. Yellow-related proteins were characterised as the binders of biogenic amines (e.g., serotonin, histamine) (Xu et al., 2011; Abdeladhim et al., 2016). Moreover, sand fly salivary yellow proteins display antigenic characteristics, being identified by serum antibodies from experimentally bitten mammals (e.g., mice) and naturally exposed animals such as dogs and humans (Rohoušová et al., 2012). Lastly, yellow proteins are being explored for potential use in anti-Leishmania vector-based vaccines. For instance, LJM17 from *L. longipalpis* induced leishmanicidal, kills leishmania parasites, Th1 cytokines in immunised dogs, while LJM11 provided protection to laboratory animals against both Le. major and Le. infantum infections (Rohoušová et al., 2012). Conversely, mice vaccinated with *P. papatasi* yellow-related proteins PpSP42 or PpSP44 triggered Th2 cytokine responses and worsened Le. major infection (Oliveira et al., 2008).

Apyrase

Apyrase is a universal enzyme that prevents blood coagulation by diverse hematophagous animals, such as mosquitoes and sandflies. This saliva component breaks down nucleotide triphosphates (ATP) and diphosphates (ADP) into a monophosphate (AMP) and an inorganic phosphate (Pi). Consequently, it eliminates a significant physiological trigger for platelet aggregation released from damaged tissues and blood cells (Rohoušová et al., 2012). Apyrases are categorised into three families: the 5´-nucleotidase family, initially identified in the salivary glands of *Aedes aegypti* (Champagne et al., 1995); the CD39 family nucleotidase; and the Cimex family, which is present in the salivary glands of sandfly species like *P. perniciosus* and *L. longipalpis* (Lestinova et al., 2017). In addition to their hydrolyzing function, apyrases in sand flies also exhibit antigenic properties. Antibodies from dogs exposed either experimentally or

naturally to *P. perniciosus* strongly reacted to recombinant PpeSP01 and PpeSP01B apyrases (Vlkova et al., 2011).

Lufaxin-like protein

An anticoagulant named Lufaxin was identified initially in *L. longipalpis* and then in all sandflies studied so far. Lufaxin is a potent inhibitor of the coagulation Factor Xa (FXa) that leads to thrombin production and fibrin clot formation (Collin et al., 2012).

Another compound in sandfly saliva is hyaluronidase, which helps take a blood meal. The hyaluronidase enzymes play a significant role in insects feeding on superficial haemorrhagic pools like sandflies.

The mentioned salivary components are incomplete, but a more detailed description is beyond the scope of this thesis and more comprehensive information can be found in (Abdeladhim et al., 2014).

1.6.3.2 Sandfly Saliva and Leishmania Interactions

Leishmania are obligate intracellular protozoan parasites that are responsible for causing leishmaniasis (discussed in 1.2.6 section). An infected sandfly probes the skin multiple times to find vessels, causing a blood pool from which it feeds. During this process, Leishmania parasites are deposited with saliva at the blood-feeding site. The impact of sandfly saliva on the host's haemostatic and immune systems alters the site, leading to rapid neutrophil infiltration, which facilitates the establishment of Leishmania infection, as the parasites can temporarily survive within these cells. The neutrophils then coordinate the recruitment of cutaneous monocyte-lineage immune cells, which are also targets for the parasites. (Peters et al., 2008).

The immunomodulatory capacity of *Lu. longipalpis* sandfly gland homogenate was demonstrated by enhancing the recruitment of leukocytes to the bite site when inoculated with *Leishmania chagasi*. This modulation could potentially facilitate disease worsening as both neutrophils and macrophages are susceptible to parasite infection (Teixeira et al., 2005). Furthermore, *in vitro* studies demonstrated that when exposed simultaneously to both *Leishmania chagasi* and sandfly salivary gland extract (SGE), neutrophils released increased higher levels of CCL2, a chemokine that attracts parasite-permissive macrophages (van Zandbergen et al., 2004; Prates et al., 2011).

Sandfly saliva substantially increase the host's susceptibility to infection by the *Leishmania* parasite. *Le. major*, the causative agent of cutaneous leishmaniasis, co-

inoculated with *Lu. longipalpis* SGE, caused a larger lesion size on the skin inoculation area and a higher parasite burden in a murine infection model (Titus and Ribeiro, 1988). Furthermore, another study employing *Ph. papatasi* SGE, a natural vector of *Le. major*, showed similar outcomes. Infections in the presence of SGE led to earlier, and more destructive dermal lesions (Figure 1.19) with higher parasite count, resulting in unrecovered disease overall (Samuelson et al., 1991; Lima and Titus, 1996; Belkaid et al., 1998; Rohoušová and Volf, 2006). Interestingly, the enhancement of Leishmania infection appears to be specific to sandfly saliva, as saliva from *Anopheles aegypti*, *Rhodnius prolixus*, or *Ixodes scapularis* did not show similar effects on *Le. major* infectivity in mice (Titus and Ribeiro, 1988).

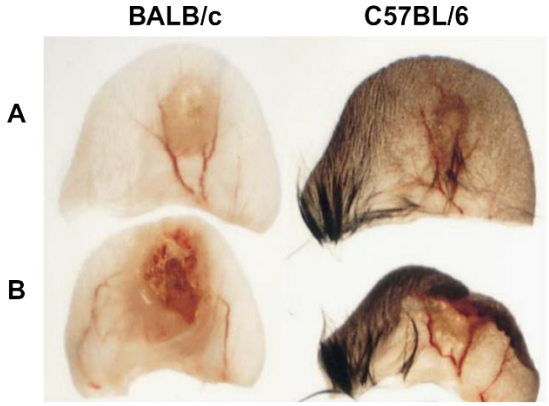


Figure 1.18: Photomicrograph of BALB/c and C57BL/6 Ears

10 weeks after intradermal injection of 1,000 *L. major* metacyclic promastigotes alone **(A)** or with salivary gland sonicate (SGS) **(B)**. From (Belkaid et al., 1998)

Interestingly, repeated exposure to bites from uninfected sandflies or immunization with specific salivary proteins, provides protection against *Leishmania* infection in hosts. As a result, sandfly salivary antigens are being utilised to develop a vaccine against leishmaniasis (Lestinova et al., 2017). However, this approach is not within the scope of the thesis, and no more detailed information will be provided.

1.7 ANTIVIRALS, VACCINES AND TREATMENTS FOR ARTHROPOD-BORNE INFECTIONS

Approximately one hundred antiviral drugs, whether as single agents or combinations, have received approval, with more currently in various stages of development. For example, broad-spectrum antivirals (e.g., ribavirin, favipiravir) has been using inhibit the replication of many viruses such as coronaviruses, influenza, and flaviviruses

(Ianevski et al., 2022). However, as of the time of writing this thesis, no specific antivirals have been approved for the treatment of any arbovirus infection. Moreover, there have been limited developments in vaccines for these infections. In practice, the standard clinical approach to treating numerous arboviral infections involves managing secondary symptoms with fluids, anti-inflammatory drugs, antipyretics, and analgesics. Additionally, in regions at risk, control measures targeting arthropod vectors are implemented, as discussed in 1.3.4 section detail.

We can consider several reasons that make developing antivirals and vaccines targeting arboviral disease difficult. These include the presence of an immense number of distinct arboviruses, the spontaneous and unpredictable nature of arboviral outbreaks, the high cost of developing new drugs and the time post infection when anti-virals might be administered.

Experimental antiviral agents come in various molecular forms, such as small molecules, peptides, neutralising antibodies, IFNs, CRISPR-Cas systems, si/shRNA, and other nucleic acid polymers (NAPs) (Ianevski et al., 2022). Indeed, anti-arboviral drugs exert their effects through various mechanisms in mammalian cells, primarily targeting viral proteins and/or host cell proteins to hinder viral propagation, RNA synthesis, entry, attachment, and secretion (Dong and Dimopoulos, 2021). This thesis will give a few examples of recent experimental antiviral studies on arbovirus infections. Sofosbuvir, a clinically approved nucleotide analogue, has demonstrated efficacy against DENV, ZIKV, YFV and CHIKV, with the latter shown both *in vitro* and *in vivo* systems, in which use reduced arthralgia and severe acute infection (Ferreira et al., 2019). Another antiviral is an approved drug in Japan against influenza T-705 (Favipiravir/Avigan) that also provides in vitro efficacy against a range of RNA arboviruses such as CHIKV, SFV, SINV, WEEV, WNV and ZIKV (Furuta et al., 2009; Franco et al., 2023). Ribavirin, a broad-spectrum antiviral that interferes with virus replication, is the only therapeutic agent for CCHF treatment, administered either orally or intravenously, recommended by the WHO (World Health Organization, 2022). Finally, polyphenols and plant extracts have undergone thorough investigation due to their potential as antiviral agents against arboviruses, showing encouraging outcomes in studies (Goh et al., 2020). As an example of these antiviral natural compounds, resveratrol, a polyphenol mainly present in red grapes, has potent anti-inflammatory effects. A recent study demonstrated that resveratrol disrupted the activation of MyD88-dependent NF-κB signalling in mouse skin mediated by AaNRP, a mosquito salivary

protein. Dietary supplementation of this phytochemical limited the enhancement of ZIKV infection by suppressing AaNRP-mediated skin neutrophil infiltration and the consequent recruitment of monocytic cells (Wang et al., 2024; Keskek Turk et al., 2024).

Developing safe and efficient vaccines for arthropod-borne viruses is a priority in public health. However, the licensed vaccine presently accessible for these viruses is limited. The live attenuated 17D vaccine, developed in the 1930s, remains the sole yellow fever (YF) vaccine in use. While a single dose of the 17D vaccine provides protection to over 95% of recipients within 30 days of vaccination, YFV continues to pose public health challenges in South America and sub-Saharan Africa. This is due to factors such as the cost and time required for vaccine production, alongside issues related to public awareness and education (Collins and Barrett, 2017). Moreover, four types of JEV vaccines are available: inactivated mouse brain-derived JEV, Vero cell-produced inactivated JEV, live-attenuated JEV, and live-attenuated chimeric YFV-17D/JEV vaccines (Srivastava et al., 2023).

DENV, which causes many millions of infections annually, is a prominent candidate for vaccine development. DENV presents four distinct serotypes in nature: DENV-1 to DENV-4. Initial infection with one serotype provides enduring immunity against that specific serotype and temporary immunity against the other three for approximately six months. The sole approved dengue vaccine, Dengvaxia (CYD-TDV), has restrictions on its use because of its limited effectiveness against DENV-1 and DENV-2 and the potential to cause severe dengue infection in individuals who have not previously been exposed to the virus (Lee et al., 2023).

Following the recent ZIKV outbreak, WHO announced a public health emergency and urged the global research and development sector to focus on preventive and treatment measures against the virus. Subsequently, numerous vaccine initiatives commenced, with several advancing to phase I trials and animal model kinetic studies, showing promising results (Abbink et al., 2018). Although many leading projects have been cancelled due to economic reasons and a decline in ZIKV cases, the results of two mRNA-based ZIKV vaccines (mRNA-1325 and mRNA-1893) phase 1 trials were published latterly. According to this, the first-generation mRNA-1325 vaccine demonstrated good tolerance levels but elicited weak ZIKV-specific neutralising antibody (nAb) responses. In contrast, the updated mRNA-1893 vaccine exhibited a satisfactory safety profile. It prompted robust and long-lasting Zika virus-specific nAb

and serum binding antibody (bAb) responses following two doses, irrespective of the individuals' baseline flavivirus serostatus (Essink et al., 2023).

1.8 THESIS AIMS

Although the arbovirus grouping of viruses, being not defined by phylogeny, consist of genetically highly diverse viruses, their transmission all occurs by hematophagous (e.g., mosquito, tick, sandfly) bites. This is a common aspect of the transmission route that includes the host inflammatory response to arthropod-derived factors that are deposited during virus inoculation. Including our group's previous research, several studies that investigated host responses to mosquito-derived factors have indicated that these factors have an important influence on infection and disease, aiding virus replication, and dissemination within the mammalian host. Mosquito bites and saliva led to enhanced virus replication in many infections, including SFV, BUNV, CHIKV, RVFV, DENV, WNV, and Cache valley virus (CVF) (Pingen et al., 2016; Pingen et al., 2017). The enhancement mechanism is associated with saliva-drive leukocyte influx, some of which become infected and replicate virus (Pingen et al., 2017). Besides mosquito-derived factors, tick saliva co-inoculated with an encephalitic tick-borne flavivirus (Powassan virus), also increases viral titre and worsens disease (Hermance and Thangamani, 2015). Taken together, these studies show that various arthropod-derived factors can enhance host susceptibility to infection with genetically diverse arboviruses.

Sandfly species and the capacity of their biting to influence host susceptibility to pathogens has been well studied in the context of *Leishmania* parasites. Here, sandfly bite/saliva/salivary gland extract elicits an immune response that enhances the *Leishmania* infection due to the inflammatory nature of various saliva components (Teixeira et al., 2005; Peters et al., 2008). Here, biting/saliva causes neutrophils and macrophages to infiltrate rapidly and localise to site where parasites are deposited, and become infected (Peters et al., 2008; Lestinova et al., 2017).

In summary, the saliva of sandflies plays a crucial role in aiding parasite establishment within the host. Importantly, there have been no published studies investigating the effects of sandfly saliva on virus infection, particularly at the skin inoculation site. Of viruses transmitted by sandflies, one of them stands out due to its potential to cause serious neurological disease, TOSV. Although TOSV was first isolated from sandflies in Italy, in 1970s and it is an important cause of meningitis, and encephalitis, it remains a neglected virus with limited published data. Importantly, no mouse models are

available for investigating TOSV pathogenesis. Furthermore, few *in vitro* and *in vivo* studies have examined the target cells of TOSV infection, especially in the cutaneous site. Indeed, the cell tropism of bunyavirales in skin are poorly defined in general. The influence of sandfly-derived factors on TOSV infection are also not known (Varani et al., 2015; Cusi et al., 2016). For this reason, the interactions between the virus, vector and the host are poorly understood.

In this thesis we **hypothesised** that inflammatory responses to sandfly bite and/or salivary factors will enhance infection with TOSV, supported by more frequent infection of inflammatory cells recruited to the skin and worsening clinical outcomes, including CNS infectiousness, of the virus.

Therefore, the aims of this thesis are:

1. **To establish a novel *in vivo* mouse model of TOSV infection that incorporates sandfly bite and salivary components.**

2. **To investigate whether sandfly bite and salivary compounds can modulate either TOSV infection, or SFV infection, a well-defined mouse model of arbovirus infection.**

3. **To define the cellular tropism of TOSV in the skin and whether sandfly-derived factors affect this tropism.**

Together, the findings obtained by this thesis will be crucial for our understanding of TOSV pathogenesis at the arthropod-vertebrate interface. In addition, a better understanding of the mechanisms responsible for arboviral enhancement gives the potential to identify new candidates for therapies that e.g., target arthropod saliva.

CHAPTER 2: MATERIALS AND METHODS

2.1 PLASTICS

Tissue culture plastics including plates, flasks, and 15ml, 50ml centrifuge tubes for this thesis, were purchased from Costar®/Corning®, USA. 1.5ml and 2ml microcentrifuge tubes were purchased from Eppendorf (Eppendorf, UK). 96 and 384 well PCR plates were purchased from Starlab (Starlab, UK). Filter pipette tips were purchased from Starlab (Starlab, UK).

2.2 VIRUSES

Virus stocks of Semliki Forest Virus (SFV4) were generated from plasmids containing the genomic sequence, kindly provided by Prof. Andres Merits, University of Tartu. Previously, plasmids had been electroporated into Baby Hamster Kidney (BHK)-21 cells to generate infectious virus. Toscana virus (strain 1812) from Italy, a strain known to infect mice, originally isolated from a patient in Italy, was kindly supplied Prof. Maria Grazia Cusi, University of Siena (Grazia Cusi et al., 2005). Virus stocks of BHK-21-derived TOSV and kidney epithelial cells originated from an African green monkey (Vero)-derived TOSV were cultured in the respective cell lines for 24 hours. Afterwards, the supernatant was collected and centrifuged at 1000xg to eliminate large cell debris, and virus titers were determined by applying plaque assays on BHK-21 cells. The genetically modified TOSV (strain 1500590, lineage A, obtained from an infected patient, which is an NSs-deletant rTOSV expressing mCherry, a reporter gene (ΔNSs:mCherry), was kindly provided by Prof. Alain Kohl at the University of Glasgow (Alexander et al., 2020).

2.3 CELL CULTURE

BHK-21 cells were used to grow-up Toscana Virus (TOSV) stocks. Furthermore, these cells were used for our plaque assays to titre the virus stock and to determine viral titres *in vivo* derived sampled. In addition, Vero cells were also used to obtain Vero-derived TOSV stock. The cells were kept at -195°C in the nitrogen tank for long-term storage and, the stock cell lines can be rapidly thawed in a 37°C water bath and transferred to a T75 (a surface area of 75 cm^2) (Corning®, USA) tissue culture flask or a T175 (a surface area of 175 cm^2) (Corning®, USA) flask containing the media when required. BHK-21 and Vero cells were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, USA), supplemented with 50 ml (10%) Foetal Bovine Serum (FBS) (Gibco™, USA), 100 units/ml Penicillin and 0.1% mg/ml

Streptomycin (Gibco™ USA), 1% GlutaMAX (Gibco™, USA) and 1% Tryptose Phosphate Broth (TPB) (Gibco™, USA).

2.3.1 Growing Primary Cell Cultures

For various experiments in this thesis, primary cells from C57BL/6 and *ifnar1-/-* mice were generated *in vitro.*

2.3.1.1 Fibroblastic-like Cells

Skin from the back of C57BL/6 or *ifnar1-/-* mice was dissected and immediately transferred into 500µl of complete DMEM (Gibco™, USA) supplemented with 10% FBS (Gibco™, USA), 1% TPB (Gibco™, USA), 5 ml Pen/Strep (Gibco™, USA) and 5ml GlutaMAX (Gibco™, USA). The dissected skins should be kept on ice until the digestion process begins. Tissues were transferred to a new sterile 1.5 ml microcentrifuge tube with 1 ml of digestion media containing 0.9ml of Hanks balanced saline solution (HBSS) (Sigma-Aldrich®, USA), 100µl Collagenase D (Roche®, USA) at a concentration of 1mg/ml (skin and spleen only), 50µl of Dispase II (Roche®, USA) at a concentration of 0.5mg/ml (skin), and 8µl of DNase I (Roche®, USA) at a concentration of 0.1mg/ml. Next, skin was chopped finely to aid the digestion process. Samples were incubated at 37°C for 50 minutes, shaking at 1100RPM on an Eppendorf thermomixer F1.5 (Eppendorf, Germany). After incubation, 500µl of complete DMEM is added to each sample to supplement the cells. Next, each sample is transferred to a new sterile 50ml centrifuge tube through a 70µM cell strainer (Greiner®, Austria), using 1000µl of complete DMEM to flush any remaining cells from the 1.5ml microcentrifuge tube and a further 1000µl of complete DMEM to flush the cell strainer. Cells were centrifuged at 300xg for 5 minutes at 4°C then re-suspended in DMEM twice to remove excess digestion media. Cells were then placed into a T25 (a surface area of 25 cm^2) (Corning®, USA) tissue culture flask (corning, USA) pre-coated with 0.2% gelatine (ScienCell™, USA) containing complete DMEM (Gibco™, USA), supplemented with 10% FBS (Gibco™, USA), 100 units/ml Penicillin and 0.1% mg/ml Streptomycin (Gibco™ USA), 1% GlutaMAX (Gibco™, USA) and 1% TPB (Gibco™, USA). Cells were maintained at 37°C 5% CO_2 for several passages until only fibroblast remained alive. Such cells were previously validated as fibroblastic, based on morphology and flow cytometry assessed expression (CD45- CD40+) (Bryden et al., 2020).

2.3.1.2 M-CSF Macrophages

Removed skin from the back of wild-type C57BL/6 or *ifnar1-/-* mice and made an incision on either side of the spinal cord to cut out the femur. The femur was kept on ice until processing. Bone marrow from the femur was extracted by flushing cells from the femur using 26-gauge needle and complete DMEM (Gibco™, USA), supplemented with 10% FBS (Gibco™, USA), 100 units/ml Penicillin and 0.1% mg/ml Streptomycin (Gibco™ USA), 1% GlutaMAX (Gibco™, USA) and 1% TPB (Gibco™, USA). Cells were dissociated by gently pipetting up and down with a 5ml pipette. Next, cells were centrifuged at 300xg for 5 minutes. Cells were then re-suspended gently in Red Blood Cell Lysis Solution (10×) (Miltenyi, Germany) (prepared by mixing 500ul of the buffer with 4.5ml of dH_2O) to remove red blood cells then incubated on ice for 5 minutes. Cells were then passed through a 40µm cell strainer (Greiner®, Austria) before lysis buffer was quenched with complete DMEM. The centrifuge process was repeated, and the cells were re-suspended in complete DMEM and counted in a hemacytometer. The desired number of cells was placed in relevant tissue culture flasks or plates. Cells were cultured at 37°C at 5% CO_2 in DMEM (Gibco™, USA) supplemented with 10% FBS (Gibco™ USA), 1% Pen/Strep (Gibco™ USA), 1% Glutamax (Gibco™ USA), 0.1% Gentamicin (Sigma-Aldrich® USA) and 10ng/ml M-CSF (PeproTech/Gibco^{TM}, USA). After 3 days (on day 4), cells' media was removed and replaced with fresh DMEM (Gibco™, USA) containing 10ng/ml M-CSF (PeproTech/Gibco™, USA) to ensure the concentration of M-CSF remains sufficient to support the monocytes differentiation into macrophages, 10% FBS (Gibco™ USA), 1% Pen/Strep (Gibco™ USA), 1% Glutamax (Gibco™ USA) and 0.1% Gentamicin (Sigma-Aldrich® USA). On day 7, macrophages became ready to use.

2.3.1.3 Flt3-Ligand Dendritic Cells

Bone marrow was processed as in the M-CSF macrophages protocol to get a single-cell suspension. The desired number of cells was placed with complete DMEM with 200 ng/ml Flt3L (PeproTech/Gibco™, USA) in relevant tissue culture flasks or plates and these cells were in suspension form, not adherent. Cells were kept at 37°C with 5% CO_2 for 6 days. On that day, fresh DMEM with 200 ng/ml Flt3-Ligand was then added to the cells. On day 10, mature pDCs can be used.

2.3.2 Infection of Fibroblast-Like Cells, M-CSF Macrophages, and Flt3-Ligand Dendritic Cells In Vitro with TOSV +/- Sandfly Salivary Gland Extract (SGE)

Primary fibroblasts-like cells and M-CSF macrophages were detached using Trypsin-EDTA (0.25%) (Gibco™, USA) and suspension form FIt3L DCs were taken, and centrifuged at 300xg for 5 minutes. Fibroblasts-like cells were then placed in a 24-well plate (500µl suspension of cells in each well) while M-CSF macrophages and FIt3L DCs were put into a 96-well plate, incubated overnight. Before using SGE on cells, it was UV-sterilised. In addition, TOSV stock was diluted in 0.75% PBSA [prepared with Bovine Serum Albumin Fraction V (Roche®, USA) and PBS (-Ca/Mg) (Sigma-Aldrich® USA)] to relevant MOIs. Tissue culture plate was gently agitated for 1 hour at 37°C 5% CO_2 with virus, then complete media added. Cells were then incubated for a further 24 hours at 37°C 5% CO_2. Media were collected and cell pellets/monolayers lysed with 350µl Lysis buffer (Invitrogen™ PureLink™ RNA Mini Kit, USA) with 10µl β-mercaptoethanol (Gibco™, USA) per ml lysis. Lysates were transferred to QIAshredder columns (QIAGEN, Germany) and spun at 12,000xg for 2 minutes. The samples were stored at -80°C until RNA extraction.

2.4 MICE

Wild type C57BL/6 mice were bred in the SBS facility at the University of Leeds. *Ifnar1-/-* mice were purchased from the Jackson Laboratory and bred in-house at the SBS at the University of Leeds. Mice were maintained at the SBS under specific pathogen free conditions. All mice were between 4-12 weeks old at time of use and were age and sex matched for experiments. All *in vivo* procedures were undertaken following local ethical (AWERB) and Home Office (HO) approval (Personal License I83228479, Project Licences PP0258562).

2.4.1 *In Vivo* Model

This thesis utilised a well-established mouse model for SFV infection (Lefteri et al., 2022). In addition, a unique mouse model for TOSV infection has been established here and used.

2.4.1.1 Obtaining Sandfly Salivary Gland Extracts

Four-day-old female sandflies (non-blood-fed) from the cage using an aspirator and were transferred into a small container placed on ice to immobilize them. Under a dissecting microscope, use a glass slide with bottom lighting and add drops of phosphate-buffered saline (PBS) onto the slide. Using tweezers, hold the sandfly by its

wings to prevent it from escaping during the dissection process if it wakes up. Carefully remove the legs, then position the body near the edge of a PBS drop. Hold the sandfly by the abdomen with tweezers and gently pull the head away from the body using a hooked pin. The salivary glands will remain attached behind the head. Remove the headless body from the slide. Next, carefully separate the salivary glands from the head by placing a needle between the eyes or grasping the mouthparts with tweezers and gently pulling. Ensure that no other tissues or hairs remain attached to the glands. The glands were disrupted by sonication for 10 seconds, followed by centrifugation at 10,000xg for 2 minutes, after which the supernatant was collected (Giunchetti et al., 2008; Bowles et al., 2015).

2.4.1.2 Sandfly Salivary Gland Extract and TOSV (strain 1812), TOSV Expressing mCherry Fluorescent Protein and SFV Infection

Mice were anesthetised using isoflurane (Henry Schein®, UK) administered via inhalation. 1µl of SFV4, TOSV (strain 1812), TOSV (ΔNSs:mCherry) in PBSA was injected subcutaneously (s.c.) at certain concentration into the dorsal aspect of left foot skin, with or without the equal volumes of sandfly salivary gland extract (SGE), which was kindly supplied us by Prof. Petr Volf at Charles University in Prague. Injections were carried out using custom-made point 4 style 33-gauge microneedles (Hamilton®, Switzerland) and a 5µl volume glass 75 RN Hamilton syringe (Hamilton®, Switzerland). Immediately following injections, mice were placed in their cages and monitored carefully until they had regained consciousness.

2.4.1.3 Sandfly Biting of Mice and TOSV (strain 1812) and TOSV Expressing mCherry Fluorescent Protein Infection

Mice were anaesthetised with 0.1ml/10g of Sedator®/Ketavet via intraperitoneal (i.p.) injection. The mice were placed in a specially prepared box that would protect their entire bodies and allow them to breathe easily. Then, they were placed in the cage in a way that only the dorsal side of left or right foot skin of their feet remained exposed. Notably, the toes were covered with tape to prevent sandfly biting. At least two up to 4 sandflies were allowed to bite each foot. Sandflies were left to feed until fully engorged. 1µl of virus of either 10^5 PFU/µl TOSV (strain 1812) or 10^5 PFU/µl TOSV (ΔNSs:mCherry) were then made subcutaneously accordingly directly at the bite site using Hamilton® needles. Mice were then kept warm and monitored every hour until recovery or injected with 0.1ml/10g of Revertor® reversal agent.

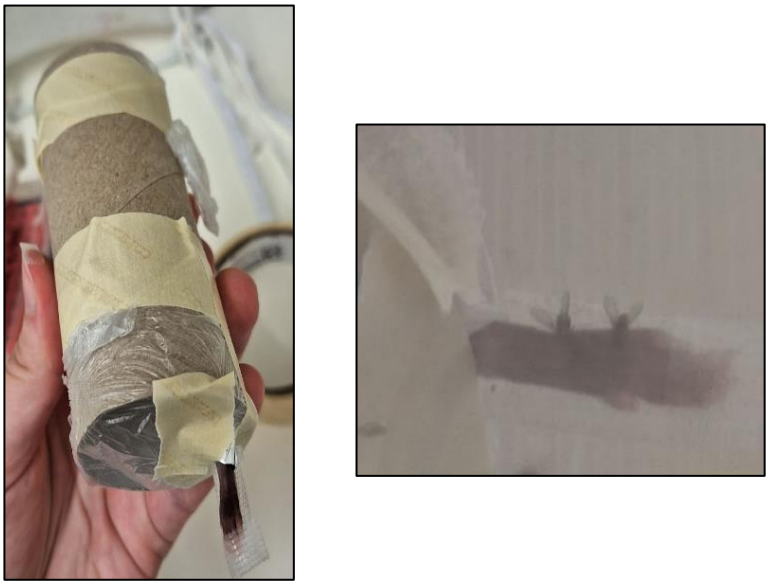


Figure 2.1: A specially designed box that ensures adequate airflow for mice during blood feeding (left). Two female sandflies were taking blood on the upper foot skin of a mouse (right).

2.4.1.4 Mosquito Biting of Mice and TOSV (strain 1812) Infection

Mice were anesthetised as above and placed on aluminium foil on top of the mosquito cages in such a way as to only allow the dorsal aspect of left foot to be bitten (by no more than 5 mosquitoes) until the insects were engorged. Particularly, the toes were covered with tape to prevent mosquitoes biting. 1µl of TOSV (strain 1812) was then injected subcutaneously, using Hamilton® microneedle, into the mosquito bitten area. Mice were allowed to recover from the anaesthesia in a warm room (28°C) with access to food and water and monitored every 30 minutes. In some cases, wild type C57BL/6

mice were administered with the InVivoMab anti-mouse IFNAR-I, clone - MAR1-5A3 (2BScientific, UK) by subcutaneous administration (into the loose skin over the neck), 24 hours prior to infection.

2.4.2 Survival and Mice Monitoring

Ifnar1-/- mice infected with TOSV (strain 1812), a neurotropic virus in humans, were monitored 4 times daily and weighed once a day for the entire duration of the experiment. Mice exhibiting two or more of the symptoms listed in Table 2.1 were promptly culled. All remaining mice were culled via Schedule 1 on days 15 or 21 post-infection.

Moderate	Severe
Loss of body weight of up to 20%	Loss of body weight greater than 25%
Reduction in food and water consumption of up to 40% less than normal for 72 hours	Reduction in food and water consumption of up to 40% less than normal for 7 days or anorexia (complete inappetence for 72 hours)
Staring coat-marked piloerection	Marked piloerection accompanied with other signs of dehydration including skin tenting
Subdued even when provoked. Limited peer interaction	Unresponsive to activity and provocation
Hunched intermittently	Hunched persistently
Vocalisation if provoked	Distressed – persistent vocalisation
Persistent oculo-nasal discharge	Persistent and abundant oculo-nasal discharge
Intermittent abnormal breathing	Laboured respiration
Intermittent tremors	Persistent tremors
Intermittent convulsions	Persistent convulsions
No self-mutilation	Self-mutilation
Intermittent prostration (< 1 hour)	Prolonged prostration (> 1 hour)

Table 2.1: Typical Signs in Mice Infected with a Neurotropic Virus and Their Corresponding Severity Categories.

(Bryden et al., 2020)

2.4.3 Collection and Storage of Mice Tissue Samples

Mice were culled via the Schedule 1 Method (rising carbon dioxide inhalation). Tissues taken depended on the experiment aims and included skin from the foot, popliteal lymph node, spleen and brain. Blood samples were also collected from the lung cavity following rupture of heart ventricles. Tissue samples were stored in 0.5ml RNAlater (Sigma Aldrich®, USA) in 1.5ml tubes. However, spleen and brain samples were halved and stored in 1 ml of RNAlater (Sigma Aldrich®, USA) to ensure thorough permeabilization of the tissue by the RNAlater (Sigma Aldrich®, USA). All samples

were stored at 4°C for the short term or at -80°C for the long term until RNA extraction. Blood samples also were centrifuged (8000-10,000xg, 10 minutes), serum collected and then stored at -80°C until use in plaque assays (unless stated otherwise).

2.5 PLAQUE ASSAYS

Plaque assays were performed to quantify titre of infectious virus in viral stocks and for the quantification of viremia following virus infection of mice. The assays consist of three main stages, which are cell preparation, infection with the virus stock or blood serum samples, and read-out of plaque numbers. BHK-21 cells were exposed to 200µl of sample containing virus to be quantified at an 80% confluency in a 12 well plate, diluted in 0.75% PBSA (PBS with 0.75% bovine serum albumin), in duplicate. Typically, each sample was diluted tenfold for several orders of magnitude, and each dilution was titred for plaques. Diluted samples were added to each well and left for an hour at 37°C, 5% CO2, swirling every 20 minutes to infect BHK-21 cells. Following infection, 2ml of a 1:1 mixture of 2X MEM (Temin's modification) (Gibco™, USA) supplemented with 5% foetal bovine serum (Gibco™, USA) and 2% Pen/Strep (Gibco™, USA) and 1.2% Avicel (FMC Biopolymer, UK), which is a low-viscosity overlay medium used in virus plaque assays, were then added to the cells. Cells were incubated for either two days (SFV), or three days (TOSV) at 37°C with 5% CO_2. Media was then removed, and cells were then fixed in 10% PFA (ThermoFisher, USA) for an hour and dyed with 0.1% Toluidine Blue (Sigma-Aldrich®, USA) or 1% Crystal Violet (Fluka™, Switzerland), which stains cellular matter, for at least 30 minutes. Following staining, the bottom surface of a 12 well plate was washed in pressurised tap water, then plaques were counted. PFU was calculated per ml using the following equation:

PFU/ml = average number of plaques (in duplicate)÷(Dilution Factor x Inoculation Volume)

2.6 SERUM NEUTRALISATION ASSAY

The assay protocol has been designed to evaluate the efficacy of serum from infected *ifnar1-/-* mice in neutralising TOSV. BHK-21 in 96-well plates (Figure 2.2) were incubated overnight at 37°C in a 5% CO_2 incubator. On the day of infection, serial dilutions of the serum samples were made using 0.75% PBSA (PBS with 0.75% BSA) in an empty plate on ice, lacking cells. The TOSV stock with 1000 PFU/ml was added into all dilutions of serum and incubated at 37°C for 1 hour. During this process, if the

serum contained antibodies against TOSV, they would bind to the virus. These mixtures of virus/serum were then added to the cells in duplicate and incubated again for 1 hour at 37°C, 5% CO_2. Complete DMEM was then added, incubated and monitored for cytopathic effect (CPE) over the next 1-3 days. After observing CPE, the supernatants were removed, and 100 µl of 10% PFA (ThermoFisher, USA) was added to each well , preserving their morphology and neutralising any remaining virus, and incubated at least 30 minutes, removed, and 100 µl of 1% crystal violet dye (Fluka™, Switzerland) added for at least 30 minutes, washed to remove excess dye. ImageJ software (NIH) was used to measure the integrated density (IntDen) for each well regarding the serum dilution folds. The IntDen is the sum of pixel values in the selected area, corresponding to the staining intensity. In this context, a lower integrated density would indicate a higher number of plaques (more viral activity) and vice versa.

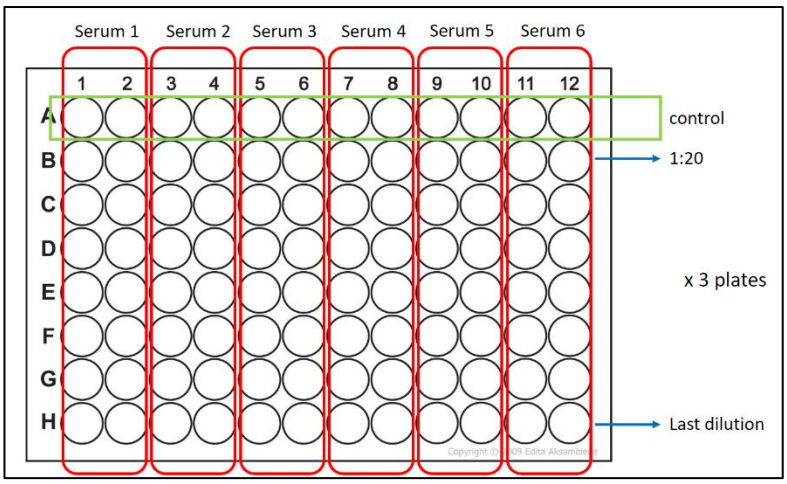


Figure 2.2: The Neutralisation Assay 96-Well Plate Template

2.7 RNA EXTRACTION

RNA extraction protocols aim to isolate RNA from biological samples such as tissues and cells. RNA extraction is a fundamental step in molecular biology, which allows various analyses to be performed. In this thesis, the extracted RNA was used for gene expression analysis and detection of viral RNA by Reverse Transcription and Quantitative PCR (RT-qPCR). RNA extractions were carried out using the Invitrogen™ PureLink™ RNA Mini and Micro Kits for tissue and cell samples, respectively, following the manufacturer's protocol.

2.7.1 From Tissue Samples

Tissue samples in RNAlater solution (Sigma Aldrich®, USA) were transferred to 2ml microcentrifuge tubes which contain 1ml TRIzol® reagent (QIAGEN, UK) and single stainless-steel bead (QIAGEN, UK). A single stainless-steel bead was used for spleen,

brain, or lymph node samples, whereas two beads were utilised for tougher skin samples. Tissue samples were then homogenised by shaking at 50Hertz (Hz) for 10 minutes in a TissueLyser LT (QIAGEN, UK). 200µl of chloroform was added to each sample, and the tubes were inverted 15 times to gently mix the solutions. The samples were then centrifuged at 12,000xg for 15 minutes at 4°C, resulting in separation into three phases: a lower phenol-chloroform phase, an interphase, and an upper aqueous phase containing RNA. A volume of 400 µl from the upper aqueous phase containing the RNA was transferred to a new 1.5ml RNase-free microcentrifuge tube along with an equal volume of 70% ethanol. The samples were then vortexed for at least 10 seconds. 650µl of the mixture was placed into silica-based spin cartridges with collection tubes and centrifuged at 12,000xg for 30 seconds at room temperature to bind RNA to the column. Two wash steps were performed using wash buffer I and wash buffer II. For each wash, either 350µl (for wash buffer I) or 500µl (for wash buffer II) was added to the column, followed by centrifugation at 12,000xg for 30 seconds, and the flow-through was discarded. Using the RNase-free DNase set (QIAGEN, UK), an on-column DNA step was incorporated between the initial and subsequent washes with wash buffer I. This step aimed to degrade any genomic DNA contamination, preventing SYBR Green from binding. Each spin column received 80µl of DNase mixture, consisting of 10µl of DNase and 70µl of reaction buffer, and was then incubated at room temperature for 15 minutes. Following the washes, the column underwent a 1-minute centrifugation to ensure it was dry before RNA elution. RNA was eluted in RNase-free water, with 30µl added for lymph node samples, 60µl for skin samples, and 100µl for brain and spleen samples. After incubating at room temperature for 1-2 minutes, centrifugation was performed at 12,000xg for 2 minutes. The purified RNA was subsequently stored at -80°C.

2.7.2 From Cells

The lysis buffer from Invitrogen™ PureLink™ RNA Micro Kit was mixed with 1% β-mercaptoethanol (Gibco™, USA), and added to the cell samples. Cell lysate was transferred to a QIAshredder column (QIAGEN, UK) and centrifuged at 12,000xg for 2 minutes to ensure complete lysis of cells. RNA extractions were conducted using on-column purifications, following the same procedure as for tissue samples. However, 20µL of DNase master mix was added to each column in the relevant stage of the protocol. In the final step, all cell samples RNA was eluted in 30µl of RNase-free water.

2.7.3 Measurement of RNA Quality and Degradation

The RNA quantification and qualification were evaluated by nano-dropping 1µl of the sample onto a NanoDrop ND-1000 Spectrophotometer (ThermoFisher, USA) blanked with 1µl of nuclease free water. The instrument measures the absorbance of the sample at specific wavelengths. Good quality RNA samples typically have high concentrations, indicating successful RNA extraction and minimal sample loss during processing. In addition to RNA concentration, the NanoDrop can also provide information about the purity of the RNA sample by measuring the absorbance ratio at 260/280 nm and 260/230. These ratios indicate the presence of contaminants such as proteins or phenol. A ratio of ~2.0 is generally accepted as "pure" for RNA (Desjardins and Conklin, 2010; Matlock, 2015).

2.8 COMPLEMENTARY (C)DNA SYNTHESIS

Acquired RNA was converted to cDNA using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems™, USA). The kit contained 20X Enzyme mix and 2X RT Buffer Mix and was kept at -20°C when not used. For each reaction, 1µl of 20X RT Enzyme Mix was combined with 10µl of 2X RT Buffer Mix in a 2ml microcentrifuge tube. Additionally, 6µl of RNase-free water was added to the enzyme-buffer mix per reaction for skin samples, while 8µl of RNase-free water was added per reaction for spleen and brain samples. Next, up to 2µg of total RNA in distinct volumes depending on the sample per 20-µL reaction was used. The volume of RNA (9µl, 3µl, and 1µl, for lymph node, skin, and spleen/brain, respectively) was placed in a 96-well plate; the enzyme-buffer mix was then added to them, giving a final reaction volume of 20µl. After briefly centrifuging the plate to remove bubbles, it was sealed with a StarSeal® aluminium foil cover (StarLab, UK). Following, the plate was then transferred to the GeneAmp® PCRsystem2700 (Applied Biosystems™, ThermoFisher, USA) PCR machine, where the programme was ready: incubated at 37°C for 60 minutes, then stopped by heating to 95°C for 5 minutes and held at 4°C. After the RT reaction, the final cDNA was diluted 1 in 5 with nuclease-free water and became ready for use in real-time PCR applications or long-term storage in a -20°C freezer.

2.9 REAL-TIME QUANTITATIVE PCR (RT-qPCR)

Quantitative Real-Time PCR (qPCR) was performed to measure the relative expression of diverse transcripts throughout this thesis. A list of all primers targeting these transcripts can be found in Table 2.2.

This technique amplifies and simultaneously quantifies a targeted DNA molecule. It combines the principles of traditional PCR with fluorescent probe (SYBR green used in this thesis) technology to measure the accumulation of DNA products during the amplification process in real time. By detecting fluorescence intensity in real time during the PCR reaction, we can establish a relationship between the DNA concentration in the PCR product and the fluorescence intensity. This relationship is represented by the cycle threshold (Ct), which is the number of PCR cycles needed for the fluorescence to surpass the background level. Essentially, a higher DNA concentration results in reaching the cycle threshold more quickly, meaning fewer PCR cycles are required.

2.9.1 Primer design

PCR primers for all genes of interest Table 2.2 were designed on the Primer3 software, version 4.0 according to the following parameters:

1. To be between 18 and 23 base pair (bp) long
2. Melting temperatures (Tm) of primers are between 59.5°C * and 61°C
3. Primer GC% have between 40% and 60%
4. Ideally self-complementarity should be max 2.00**
5. Max 3' prime end self-complementarity should be 1.00
6. To be amplicons range from 60 – 150 base pairs
7. In the last five primer bases, more than two of G, C bases should be avoided to the stable primer (two or below is better)

*Minimum Tm was lowered to 59°C if no primers were suggested by the software

**Maximum self-complementarity could be increased to 3 if no primers were suggested by the software.

For each gene, two sets of primers were designed: one set for quantitative PCR and another set to produce PCR products that were subsequently used to create standard templates. Such standards enabled semi-quantitative qPCR to be undertaken. Primers for standards were designed to amplify section where qPCR primers could bind. Primers were purchased from Integrated DNA Technologies (IDT®, USA) and Sigma Aldrich® (USA) and reconstituted to a final concentration of 0.1 nm/µl upon arrival.

Gene Name	Orientation	Sequence	Product size (bp)	NCBI Reference
18S	Forward	gactcaacacgggaaacctc	124	NR_003278. 1
	Reserve	taaccagacaaatcgctccac		
18S Standard	Forward	cgtagttccgaccataaacga	443	NR_003278. 1
	Reserve	acatctaagggcatcacagacc		
TOSV Ns	Forward	gaaccagactttacgagccaac	102	X53794.1
	Reserve	gccacctgagagcagacaa		
TOSV Ns Standard	Forward	ggttcaggccacaagaggt	519	X53794.1
	Reserve	agcagtcaatccgtgctttt		
SFV E1	Forward	cgcatcaccttcttttgtg	173	DQ_189086
	Reserve	ccagaccacccgagatttt		
SFV E1 Standard	Forward	aagtgaagacagcaggtaaggtg	446	DQ_189086
	Reserve	tatgagttgccccgagtttc		
CXCL10	Forward	tgccacgatgaaaaagaatg	182	NM_021274
	Reserve	aggggagtgatggagagagg		
CXCL10 Standard	Forward	atccctgcgagcctatcc	524	NM_021274
	Reserve	aaacttagaactgacgagcctga		
IFN-β	Forward	cacagccctctccatcaact	152	NM_010510
	Reserve	gcatcttctccgtcatctcc		
IFN-β Standard	Forward	ggcttccatcatgaacaaca	399	NM_010510
	Reserve	tcccacgtcaatctttcctc		
Rsad2	Forward	tgaagcgtggcggaaagtat	73	NM_021384.4
	Reserve	tccttcccatctcagcctca		
Rsad2 Standard	Forward	ctgtgcgctggaaggttttc	583	NM_021384.4
	Reserve	cactggaccttgctcctctg		
ISG15	Forward	cgcagactgtagacacgctta	80	NM_015783.3
	Reserve	ctcgaagctcagcagaact		
ISG15 Standard	Forward	gtccgtgactaactccatgac	504	NM_015783.3
	Reserve	tcccaaaagtcctccatacc		
CXCL2	Forward	aagtttgccttgaccctgaa	129	NM_009140
	Reserve	tctctttggttcttccgttg		
CXCL2 Standard	Forward	cgcccagacagaagtcatag	484	NM_009140
	Reserve	actcaccctctccccagaaa		
IL-1β	Forward	cgctcagggtcacaagaaac	67	NM_008361.3
	Reserve	gaggcaaggaggaaaacaca		
IL-1β standard	Forward	aaagtatgggctggactgtttc	410	NM_008361.3
	Reserve	atgtgctggtgcttcattca		
TBP	Forward	tgctgttggtgattgttggt	99	NM_013684
	Reserve	aactggcttgtgtgggaaag		
TBP Standard	Forward	gagttgcttgctctgtgctg	274	NM_013684
	Reserve	atactgggaaggcggaatgt		
CCL2	Forward	ctcacctgctgctactcattca	153	NM_011333.3
	Reserve	ccattccttcttggggtca		
	Forward	caccagcaccagccaact	519	NM_011333.3

CCL2 standard	Reserve	gcatcacagtccgagtcaca		
TNF-α	Forward	caccaccatcaaggactcaa	96	NM_013693
	Reserve	gaggcaacctgaccactctc		
TNF-α Standard	Forward	tctgtgaagggaatgggtgt	420	NM_013693
	Reserve	ggctggctctgtgaggaa		
IL-6	Forward	ttccatccagttgccttctt	171	NM_031168
	Reserve	atttccacgatttcccagag		
IL-6 Standard	Forward	tccagaaaccgctatgaagt	370	NM_031168
	Reserve	ctccagaagaccagaggaaa		

Table 2.2: Primer List. List of primers, primer orientation, sequences, product size and NCBI reference for all qPCR primers used in this thesis.

(Pingen et al., 2016; Bryden et al., 2020)

2.9.2 Generating DNA Standards to Establish Standard Curves for Absolute Quantitative qPCR

Generating DNA standards to establish standard curves for absolute quantitative qPCR involves creating a series of known concentrations of DNA samples that will be used as reference points in qPCR experiments. The standards employed were created by performing a PCR reaction using a randomly selected sample known to possess the gene of interest, along with primers explicitly designed for the target gene. 2µl of cDNA sample were mixed with 1µl each of the forward and the reverse primers as well as 25µl of REDTaq® PCR Reaction Mix (ReadyMix™, Sigma-Aldrich®, USA) containing Taq DNA polymerase, 99% pure DNTPs, reaction buffer, and an inert red dye in a 2X concentrate, and 21µl of Nuclease-free water. The mixture was then added to 0.2ml PCR tube. A PCR reaction was then undertaken using the GeneAmp® PCRsystem2700 (Applied Biosystems™, ThermoFisher, USA) using the following programme:

1. 3 minutes at 94°C
2. 15 seconds at 94°C for denaturing of DNA
3. 10 seconds at 60°C for annealing of primers
4. 50 seconds at 72°C for extension

x40 cycles

5. 7 minutes at 72°C
6. Held at 4°C

The PCR product underwent electrophoresis on a 2% agarose gel containing ethidium bromide, running at a voltage between 80 to 100V for 1 hour. The 50 bp DNA Ladder (Invitrogen™, USA) served as a reference to determine the product's molecular weight and the resulting bands were visualised using the ChemiDoc XRS+ gel imager (BioRad, USA). Primers were purified only when distinct single bands appeared at the expected molecular weight. In instances where no bands or multiple bands were observed, new primers were designed, and the previous ones were discarded.

The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Germany). The QIAquick system employs an easy bind-wash-elute process. Five volumes of Buffer PB were added to 1 volume of the PCR sample and then mixed. The mixture was transferred to the spin cartridge containing a silica-based column and centrifuged at 17,900xg for 30–60 seconds at room temperature. 750µl buffer PE (contains ethanol) was then added before a second centrifugation. The flow-through was discarded, and the QIAquick column was placed back into the same tube. The column was then centrifuged for an additional 1 minute to dry. Purified PCR product was eluted in 50µl of elution buffer containing 10 mM Tris-Cl. Lastly, the purified standards were subsequently diluted 100-fold, labelled as 10^{-2}, and stored at -20°C until needed.

2.9.3 qPCR with SYBR Green

All RT-qPCR reactions in this thesis, were done using PerfeCTa SYBR® Green FastMix (Quantabio, USA) for fluorescent labelling of DNA. SYBR Green I binds non-specifically to double-stranded DNA (dsDNA). It shows minimal fluorescence when free in solution, but its fluorescence significantly intensifies upon binding to dsDNA. The benefits of using dsDNA-binding dyes include straightforward assay design (requiring only two primers and no probe design), the capability to test multiple genes rapidly without needing multiple probes, affordable (since probes are more costly), and the ability to conduct melt-curve analysis to verify the specificity of the amplification reaction (Navarro et al., 2015).

cDNA was diluted 1 in 5 in RNAse free water to prevent the RT buffer from interfering with the PCR reaction, and reducing DNA concentration, which at higher levels can inhibit Taq and thereby PCR efficiency. Subsequently, a master mix was prepared consisting of cDNA, primers, RNAse free water, and PerfeCTa SYBR® Green FastMix (Quantabio, USA). 5µl of SYBR Green I, 4µl of RNAse free water, and 0.15µl of primer mix (forward + reverse) were mixed up for each sample. Of the master mix, 9µl

was then transferred to a 384 well plate. A triplicate technical replicate was made for each sample. A standard curve was created by performing serial 10-fold dilutions of the 10^{-2} PCR standard. Moreover, a non-template control (NTC) containing RNAse free water was included on the plate with the master mix. Once all samples were loaded into the 384-well plate, it was briefly centrifuged at 400xg for 15-30 seconds to eliminate any air bubbles. After centrifugation, the plate was sealed with clear Polyolefin StarSeal (PCR) (StarLab, UK) and stored at 4°C in the dark until the reaction commenced.

The 384 well plate were run on QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™, USA) and the protocol was composed of the following steps:

1. 95°C for 3 minutes
2. 95°C for 3 seconds } x25-40 cycles
3. 60°C for 30 seconds
4. Disassociation and melt curve (records fluorescence between the final temperature increase of 60°C to 95°C)

The Ct value was automatically computed by the QuantStudio software, identifying the logarithmic phase of the PCR reaction. The threshold was determined at the cycle where fluorescence surpassed background levels. Semi-absolute quantities of each sample were determined based on their placement on the standard curve. The standard curve's efficiency required close to 100%, as indicated by a coefficient $R^2 \geq 0.998$ and a slope of 3.3 (indicating a doubling every PCR cycle). Melt curve analyses were performed to assess PCR product specificity, with a single peak indicating one specific amplicon had been generated.

2.9.4 Normalisation with Reference Genes

To address potential disparities in the absolute quantities of nucleic acid among samples, normalization to a reference/housekeeping gene was performed. Samples can show variations in nucleic acid quantities. This difference can result from variations in tissue size and degradation of RNA/cDNA throughout the freeze-thaw cycle. Unless specified otherwise, the 18S gene was mostly utilised as the housekeeping gene for RT-qPCR data analysis in this thesis. Most other housekeeping genes have been shown to be differentially expressed following virus infection and are not suitable (Pingen et al., 2016). The 18S gene comprises ribosomal RNA (rRNA) from the 18S ribosomal subunit. 18S exhibits stability and ubiquitous expression across all tissues during arbovirus infections, including bunyaviral. The relative expression levels of the genes of

interest were normalised by dividing the quantity of each gene by the quantity of 18S in every sample. Finally, this result was multiplied by the arbitrary value of 1 x 10^7 to obtain final values on an appropriate scale.

2.9.5 Analysis

Data analysis was carried out using Microsoft Excel by calculating the median quantity of technical replicates and normalising values to the housekeeping gene, which was similarly quantified using a standard curve.

2.10 FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

The fluorescence-activated cell sorting (FACS) technique was frequently used to investigate the response of skin cellular composition, either leukocytes or stromal, during virus infection with or without sandfly factors.

2.10.1 List of Antibodies, Antibody Clones, Fluorophores

The antibodies (Table 2.3) in established different FACS panels were utilised to define different cell populations in the skin depending on the aims of the experiments in this thesis.

Antibody	Clone	Fluorophore	Manufacturer
CD45	30-F11	FITC	BioLegend
CD11b	M1/70	APC	BioLegend
MERTK (Mer)	2B10C42	APC-Cy7	BioLegend
Ly-6G	1A8	Brilliant Violet 421	BioLegend
Ly-6C	HK1.4	PE	BioLegend
MHC II	M5/114.15.2	APC	BioLegend
CD11c	N418	Pe-Cy7	BioLegend
Vimentin	280618	APC	Biotechne
CD326 (Ep-CAM)	G8.8	APC-Cy7	BioLegend
CD326 (Ep-CAM)	G8.8	FITC	BioLegend
CD31	390	PE	BioLegend
CD31	390	Pacific Blue	BioLegend
CD31	390	FITC	BioLegend
CD90.2 (Thy1.2)	30-H12	PE/Cy5	BioLegend
CD90.2 (Thy1.2)	30-H12	Pacific Blue	BioLegend
Podoplanin	PMab-1	Alexa Fluor 647	BioLegend
CD140a (PDGFR-α)	APA5	PE/Cy7	BioLegend
Ly-6A/E (Sca-1)	W18174A	PE	BioLegend

Table 2.3: List of Antibodies, Antibody Clones, Fluorophores, and Manufacturer.

2.10.2 Tissue Digestion

Collected skin samples were immediately transferred and submerged in 500µl of complete DMEM and kept on ice until tissue digestion. Skin samples were moved to a new sterile 1.5ml microcentrifuge tube with 1ml of digestion solution containing 0.9ml of Hanks balanced saline solution (HBSS) (Sigma-Aldrich®, USA), 100µl Collagenase P (Roche®, USA) at a concentration of 1mg/ml), 50µl of Dispase II (Roche®/Sigma Aldrich®, USA) at a concentration of 0.5mg/ml, and 8µl of DNase I (Roche®, USA) at a concentration of 0.1mg/ml). Skin samples were then quickly chopped into small bits using new sharp scissors. Following, samples were incubated at 37°C for 50 minutes, shaking at 1100RPM on an Eppendorf thermomixer F1.5 (Eppendorf, Germany). After incubation, the samples were kept on ice, and 500µl of complete DMEM was added to each sample to protect the viability of the cells. Next, each sample was transferred to a new sterile 50ml tube through a 70µm cell strainer (Greiner®, Austria), using 1ml of complete DMEM to flush any remaining cells from the 1.5ml tube and a further 1ml of complete DMEM to flush the cell strainer. Cells were centrifuged at 300xg for 5 minutes at 4°C and re-suspended in an appropriate volume of DMEM, which was used to plate equal numbers of cells per well in a 96 well round-bottom plate.

2.10.3 FACS Staining

Cells were divided in equal numbers into a 96 well round-bottom plate. 250µl of FACS buffer (100ml PBS (-Ca/Mg), 500µL FBS, 400µL EDTA 0.5M) was added to each well and centrifuged at 300xg for 5 minutes at 4°C. The plate was flicked up and down to discard the supernatant. This stage was repeated one more time. The cells were re-suspended in 50µl of Fc Receptors Block (BD Pharmingen™, USA) prepared with 2µl Fc block reagent and 48µl FACS buffer per well and incubated on ice for 5 minutes. Next, 50µl of the antibody mix containing 0.25µl of each antibody per sample was added and incubated in the fridge in the dark for 45 minutes. At this stage, Anti-Rat/Hamster Ig κ/Negative control compensation beads (BD™ CompBeads, USA) were also stained. This was done by adding 1 drop of positive beads and 1 drop of negative beads to the corresponding wells in the plate, using the same volume and concentration of antibody mixture as used for staining the cells. The beads do not need a fixation step; therefore, after incubation, they were kept at 4°C in a dark condition until used. The cells were washed two times with PBS, centrifuged, and the supernatant was discarded. Next, the cells were re-suspended with 50µl of Live/Dead (L/D) cell stain conjugated with Zombie UV (Biolegend®, USA) containing 1µl L/D dye and 49µl FACS buffer per

sample and incubated at room temperature, in the dark, for 10 minutes. Each well was then washed twice with FACS buffer, with cells pelleted at 300xg for 5 minutes at 4°C and re-suspended in 250µl of FACS buffer to remove any remaining viability dye. In the final step, the cells were fixed in 100µl of 4% paraformaldehyde for 30 minutes at room temperature. They were then re-suspended in 250µl FACS buffer and stored at 4°C in the dark until analysis.

The above protocol is for extracellular staining. However, some antibodies need to be stained intracellularly. This thesis utilised the intracellular flow cytometry staining protocol for the vimentin antibody. Differently, after the L/D staining, cells were re-suspended in 100µl of Cytofix/Cytoperm solution (BD Cytofix/Cytoperm™, USA) for fixation and permeabilization. The cells were then incubated at 4°C in the dark for 20 minutes. Pelleted cells were washed two times with 250µl of 1×BD Perm/Wash™ buffer (1:10 diluted with dH_2O). 50µl of vimentin antibody mix containing 0.25µl of the antibody plus 49.75µl Perm/Wash™ buffer for each sample was added to the wells. Next, cells were incubated at 4°C in the dark for 30 minutes and washed two times with Perm/Wash™ buffer, then one time with FACS buffer. Finally, the cells were re-suspended with 250µl FACS buffer and stored at 4°C in the dark until analysis.

2.10.4 FACS Analysis

The samples were run on the CytoFLEX LX Flow Cytometer (Beckman Coulter, USA), and cell antigen expression data were acquired in FCS file format. All analyses were undertaken either on CytExpert software (Beckman Coulter, USA), which was instrument-provided or on FlowJo software (FlowJo LLC, USA). Data were analysed relying on the principle of gating following data compensation. Gates and regions were defined around cell populations with shared characteristics, typically including forward scatter (FCS), side scatter (SSC), and marker expression (e.g., L/D dye-ve, CD45+ve), to examine and quantify these specific populations. Detailed gate strategies related to experiments will be given in the relative sections of Chapter 5.

2.11 MAGNETIC ACTIVATED CELL SORTING (MACS® Cell Separation)

Immunomagnetic cell separation relies on antibodies attached to magnetic beads. When these beads are incubated with a cell suspension, they bind to cells that express the specific epitope.

Harvested skin samples were processed using the digestion protocol (section 2.10.2), and a single-cell suspension was acquired. Cells were kept cold, and pre-cooled

solutions were used to prevent capping of antibodies on the cell surface and non-specific cell labelling. The number of cells was determined using a haemocytometer. Next, cells were centrifuged at 300xg for 10 minutes and the supernatant was aspirated. Cell pellet was re-suspended in 90 µl of MACS buffer containing PBS (-Ca/Mg) (Sigma-Aldrich® USA), 1% FBS (Gibco™ USA), and 2 mM EDTA (ChemCruz™ biochemicals) per 10^7 total cells. 10 µL of Anti-F4/80 MicroBeads UltraPure (Miltenyi Biotec, Germany) was added per 10^7 total cells, mixed well, and incubated at 4°C in the dark for 15 minutes. F4/80 belongs to the epidermal growth factor (EGF)-transmembrane 7 (TM7) family and is regarded as one of the most specific cell surface markers for identifying murine macrophages. Following, the cells were washed with of MACS buffer, centrifuged, and re-suspended again with 500µl of the buffer. MS column (Miltenyi Biotec, Germany) was placed in the magnetic field of a suitable MACS Separator. The column was rinsed using 500 µl of MACS buffer. Next, the cell suspension was applied to the column, and flow-through containing unlabelled cells was collected. This process was repeated until the volume of cell suspension samples was finished. The column was removed from the magnetic separator and placed in a sterile collection tube. The magnetically labelled cells were immediately flushed out by firmly pushing the syringe plunger into the column. The flow-through consisted of F4/80+ cells in the buffer.

2.12 DECALCIFICATION OF MICE BONE FOR H&E STAINING

Mice's feet were decalcified in an EDTA solution before paraffin embedding. The bone was dissected from mice infected with TOSV alone, TOSV co-injected with sandfly salivary extract or resting mice. The specimens were fixed with 4% paraformaldehyde (PFA) for 48 hours. Following rinsing with dH2O to remove PFA residues, the tissues were placed in a 1.5ml microcentrifuge tube containing 14% EDTA (ChemCruz™ biochemicals) and vortexed briefly. They were then incubated at 4°C. The EDTA solution was changed every 2 days, and the stages were repeated for 10 days. Decalcification was complete when the bone was soft and pliable. The decalcified bone was rinsed three times with dH2O and placed in 70% ethanol prior to sending H&E staining.

2.13 ENDOTOXIN ASSAY

The ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, USA) was used to detect and measure endotoxins in sandfly salivary gland extract (SGE). The kit reagents containing *E. coli* endotoxin standard, LAL powder, chromogenic substrate,

and colour Stabiliser 1-2-3 were prepared according to the manufacturer's instructions. Sandfly SGE was diluted in appropriate volume of LAL reagent buffer. A series of endotoxin standards were prepared (0.1, 0.25, 0.5, 1 EU/mL) by diluting the endotoxin stock solution with endotoxin-free water. 100µl of each standard, sandfly SGE, and LAL reagent water were dispensed into separate endotoxin-free vials. 100µl of reconstituted LAL was added to each vial and mixed well by swirling gently. The vials were incubated at 37°C for 8 minutes. Next, 100µl of reconstituted chromogenic substrate was added to each vial to detect endotoxin levels by changing colour. The substrate reacts with the endotoxin-activated LAL enzyme, resulting in a measurable colorimetric signal proportional to the amount of endotoxin present in the sample. The vials were then incubated at 37°C for 6 minutes. After, 500µl of each reconstituted colour stabiliser (1-2-3) was added to the vials and mixed well, respectively. 200µl of the final solutions were transferred into a 96-well plate and read on a Cytation 5 reader at the absorbance of 545nm within 5 hours. The results were analysed as per the manufacturer's instructions. Briefly, absorbance readings at 545nm (y-axis) were plotted against the known endotoxin concentrations (x-axis) to create the standard curve. The standard curve equation was used to convert the absorbance readings of the test samples into endotoxin concentrations.

2.14 STATISTICAL ANALYSIS

All data was analysed utilizing GraphPad Prism software (Version 10, San Diego, CA, USA). Due to the non-Gaussian distribution of the data, which is typical for *in vivo* virus infection, non-parametric Kruskal-Wallis test with Dunn's multiple comparison test was used for comparisons between more than two groups whereas non-parametric Mann-Whitney was performed for comparisons between two groups. Ordinary-ANOVA was performed for comparisons between more than two groups of normally distributed data. All plots have statistical significance indicated with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = not significant.

CHAPTER 3: DEVELOPMENT AND USE OF MOUSE AND CELL CULTURE MODELS TO ASSESS THE ROLE OF SANDFLY SALIVA IN MODULATING SUSCEPTIBILITY TO ARBOVIRUS INFECTION

3.1 INTRODUCTION

Emerging and re-emerging infectious arthropod-borne viruses have a massive impact on global health, as was discussed in Chapter 1, section 1.2. Indeed, Toscana virus (TOSV) infection causes severe meningitis and encephalitis in humans, which is especially concerning in much of the Mediterranean. The ongoing climate crisis is expanding the geographic range in which the vector lives to more northern European countries. Importantly, like most arboviruses, there is no specific therapeutic approach (e.g., antiviral, or vaccine) for TOSV.

There are few studies that have described TOSV replication cycle or pathogenesis in either the vertebrate host or its invertebrate vector. In particular, there is little known about the relationship between TOSV, its vector, the cutaneous response to vector biting, and how these responses may modulate host susceptibility to TOSV. For mosquito-borne arboviruses, host response to mosquito saliva, deposited during blood feeding, is a key factor responsible for enhancing infection and worsening pathogenesis (Pingen et al., 2017). Interestingly, it is known that the salivary factors from sandfly vector of *Leishmania*, a parasitic disease, can exacerbate the disease outcome (Titus and Ribeiro, 1988). Depositing saliva into the skin throughout blood meal is a common aspect of all arthropod-borne viruses. As such this is a key aspect of infection, and a better understanding the mechanism of salivary factors may help inform the design of novel pan-therapeutic candidates that target arthropod saliva.

In this thesis, we hypothesise that TOSV in the presence of sandfly saliva gland extract (SGE) can infect inflammatory cells recruited to the skin inoculation site by SGE, resulting in enhance the TOSV infection. Previous work, studying the effect of mosquito saliva on mosquito-borne virus infection, has demonstrated the requirement for an *in vivo* model. This is because the effects of saliva on host susceptibility to virus is only observable in animal models and not e.g., using *in vitro* models. Therefore, to undertake experiments that can help test our hypothesis, it was required to firstly optimise a new mouse model of TOSV infection and develop assays for quantitatively defining TOSV quantities in biological samples.

Therefore, the aims of this chapter were:

1. **To optimise plaque assays and a qPCR assay to define titre of TOSV and the quantity of TOSV RNA in infected cells and mouse tissues.**

2. **To develop TOSV infection in the *in vitro* system.**

3. **As proof of principle, to determines whether sandfly SGE enhances SFV infection, a well-established arbovirus mouse model.**

4. **To establish an *in vivo* mouse model for TOSV to test our hypothesis.**

3.2 OPTIMISATION BABY HAMSTER KIDNEY FIBROBLASTS (BHK)-21 CELLS INFECTION WITH TOSV (strain 1812) AND TOSV EXPRESSING MCHERRY FLUORESCENT PROTEIN FOR PLAQUE ASSAYS

TOSV (strain 1812) stocks were first generated by infecting BHK-21 cells in our laboratory, using the original vial Prof. Maria Grazia Cusi, University of Siena, provided. Next, a plaque assay protocol was optimised to define TOSV stock titre. Firstly, BHK-21 cells were grown to 80% confluency and infected with different dilutions of new TOSV stock in 24 well plates as described in the methods chapter. Of note, we found that increasing the incubation period post infection from 48 hours to 72 hours increased the size of plaques and made them easier to count (Figure 3.1). The same protocol was applied to TOSV- mCherry, provided by Prof. Alain Kohl at the University of Glasgow.

This optimised plaque assay protocol was frequently used in this thesis to determine TOSV titers in infected cells or blood serums.

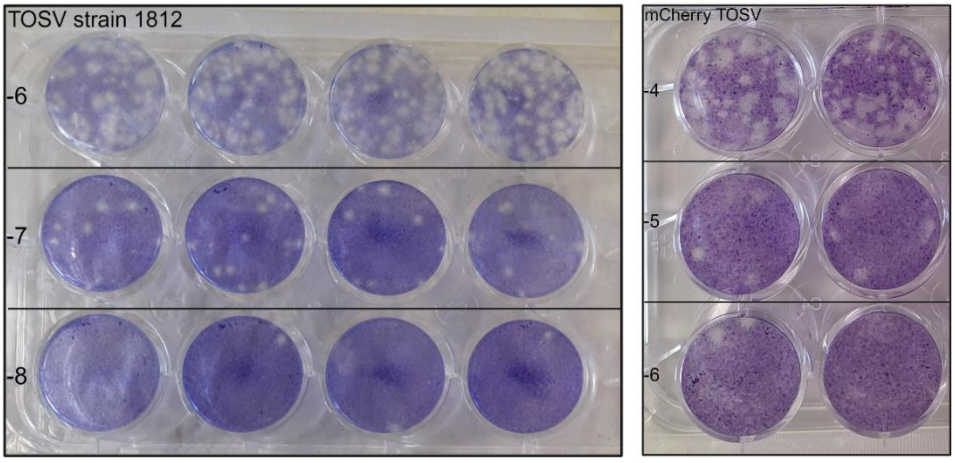


Figure 3.1: Example of TOSV (strain 1812) and TOSV-mCherry Plaque Assay Results

3.3 ESTABLISHING NOVEL TOSV NSs AND N QUANTITATIVE PCR ASSAYS

Here, TOSV primers were designed for use in qPCR assays. These candidate primers (Table 3.1) were designed utilizing the Primer3 software (see Chapter 2, section 2.8). This assay is semi-absolute, with two sets of primers; one set that is used in the qPCR assay itself, and another set of primers that only used infrequently to generate a larger PCR amplicon that is used as a standard.

TOSV cDNA was made from TOSV infected BHK cell RNA and used to test all primers for specificity using standard PCR. The PCR products were characterised by agarose gel electrophoresis. TOSV NSs (A) primers was deemed suitable for qPCR, because the primer combination generated a single, clear, and distinct band of the correct predicted size (Figure 3.2). Subsequently, the TOSV NSs (A) standard primer generated PCR product was purified and used to generate a standard curve for use in qPCR assay, which enabled quantification of TOSV RNA (Figure 3.3A). TOSV NSs (A) primers were then assessed for their ability to quantity a serial dilution of the TOSV PCR standard, using qPCR. This was done to define specificity of the assay and its quantitative dynamic range (Figure 3.3A, B). At the end of each amplification, a melt curve analysis was also performed to confirm PCR specificity (Figure 3.3C). The results show that the qPCR primers could define copy numbers over a broad range of starting copy numbers, with good correlation ($R^2 = 1$) between copy number and Ct. Therefore, due to specificity of TOSV NSs (A) primers to quantify TOSV NSs gene, these primers were selected for determining TOSV RNA level in all subsequent experiments below.

Gene Name	Orientation	Sequence	Product size (bp)
TOSV N	Forward	tcatggctcttgggtaggtt	118
	Reserve	gtgtcagccgcatttgttc	
TOSV N Standard	Forward	cgatacttctcagcactcctcac	540
	Reserve	caggggaaacaagccaga	
TOSV NSs (A)	Forward	gaaccagactttacgagccaac	102
	Reserve	gccacctgagagcagacaa	
TOSV NSs (A) Standard	Forward	ggttcaggccacaagaggt	519
	Reserve	agcagtcaatccgtgctttt	
TOSV NSs (B)	Forward	aaggggacaagagggctaaa	116
	Reserve	ccaaaaggtggaaagagcag	
TOSV NSs (B) Standard	Forward	ggagtttcctgctgtgctg	713
	Reserve	gccactttgtcactctcatgtc	

Table 3.1: List of the TOSV Primers.

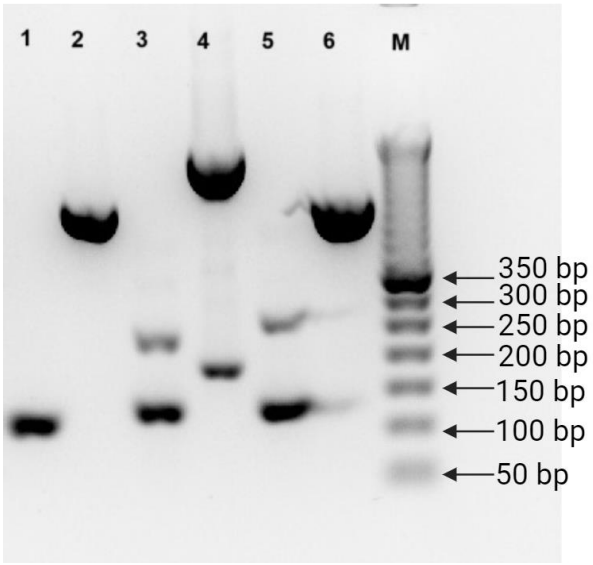
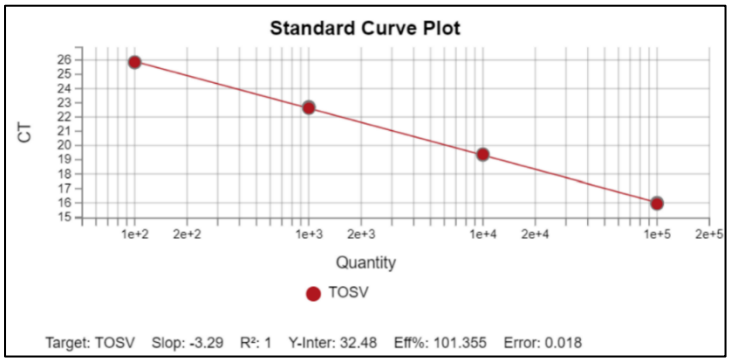


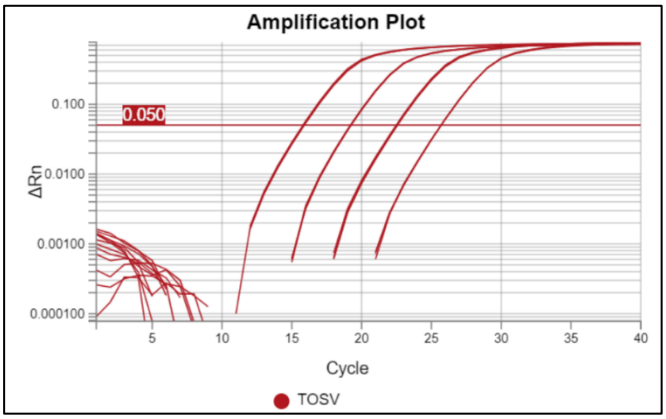
Figure 3.2: The Gel Electrophoresis of the PCR Products Produced

Agarose gel electrophoresis of PCR products of TOSV primers. Lane 1: NSs (A); Lane 2: NSs (A) standard; Lane 3: NSs (B); Lane 4: NSs (B) standard; Lane 5: N; Lane 6: N standard; M: 50bp DNA size marker**.**

A



B



C

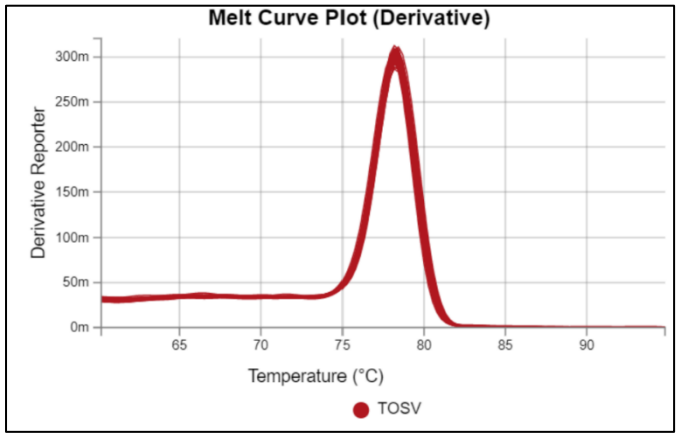


Figure 3.3: TOSV Ns (A) Primer and Standard of qPCR Result

(A) Standard curve with Ct and copy number of TOSV RNA, showing that different starting quantities of TOSV standard could be accurately quantified.
(B) An example amplification plot of these standards, showing good reproducibility (for each dilution n=3).
(C) At the end of each qPCR assay, each well is slowly heated and any loss of fluorescence that is indicative of PCR products melting measured. This melt curve analysis plot shows that the PCR product amplified during the qPCR assay represents one specific product, based on the similarity in melting temperature.

3.4 TOSV INFECTION IN VITRO SYSTEMS

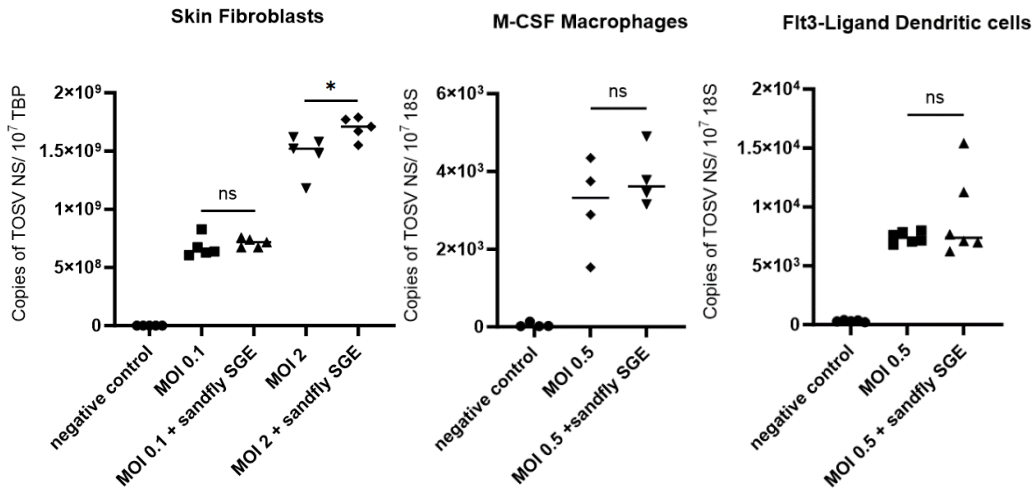
It is well-known that mosquito promotes arbovirus infection (Lefteri et al., 2022), though the effect of sandfly saliva on virus infections remains unclear. To investigate this, we firstly aimed to develop an *in vitro* model system. Such models are highly manageable and reduce the need for animal use. Here, we employed cell types that become infected and replicate other arboviruses in the skin, including macrophages, dendritic cells, and dermal fibroblasts (Pingen et al., 2017). Using this model, we asked whether sandfly SGE modulates infection in isolated cell cultures.

Initially, back skin and femur were removed from mice and the fibroblast-like cells, macrophages and dendritic cells were prepared for infection, as explained in Section 2.3.1 in Chapter 2. Respective groups of cells were treated with sandfly SGE (1 pair gland extract/µl) 20 minutes before infection with TOSV (strain 1812) with an MOI of either 0.1 or 2 for fibroblast-like cells and 0.5 for macrophages and dendritic cells. The cells were then collected at 24hpi and the quantity of TOSV RNA defined by qPCR. Fibroblast-like cells pre-treated with sandfly SGE had a very modest, if significant increase in TOSV RNA, compared to those cells infected with virus alone at MOI of 2 (Figure 3.4A). However, there was no significant difference at lower MOI (Figure 3.4A). The quantity of TOSV RNA in infected macrophages and DCs did not show a

difference in either the presence or absence of sandfly SGE treatment with an MOI of 0.5 (Figure 3.4A).

Next, the experiment was repeated infecting skin fibroblast-like cells with an MOI of 2, to ensure the previous results were reproducible in terms of sandfly SGE effect. This was deemed particularly necessary as the observed difference in mean TOSV RNA quantity was small. However, in this repeat we found that sandfly SGE inhibited TOSV infection of the cells at MOI 2 (Figure 3.4B), which was opposite to the previous experimental result (Figure 3.4A). Thus, our findings with sandfly SGE appears to mirror previous observations using mosquito saliva and SFV infection, where the addition of mosquito saliva *in vitro* failed to replicate the virus-enhanced infection observed *in vivo* (Lefteri et al., 2022). This suggests that sandfly SGE, like mosquito saliva, may require specific *in vivo* processes to modulate host susceptibility to viral infection.

A



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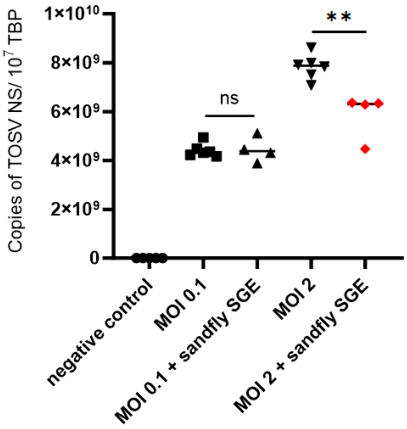


Figure 3.4: Effect of Sandfly SGE on TOSV Infection of Fibroblast-like Cells, M-CSF Macrophages, and Flt3-Ligand Dendritic Cells In Vitro System

(A, B) TOSV (strain 1812) infection with an MOI of either 0.1 or 2 for fibroblast-like cells (n=5) and 0.5 for macrophages (n=4) and dendritic cells (n=6) on its own or following pre-treatment with one pair of sandfly salivary gland per microliter per well for 20 minutes before infection. **(B)** The graph illustrates the infection of skin fibroblast-like cells with an MOI of either 0.1 or 2, either alone or after pre-treatment with one pair of sandflies salivary gland/µl per well for 20 minutes before infection. Red dots represent the group with an MOI of 2 treated with sandfly SGE.
The cells were collected 24hpi and TOSV RNA (Ns gene) copy number was determined by qPCR assay. Data is presented as dot plots with each dot representing a separate biological sample with a line at the population median. ns=not significant, significant *P < 0.05, **P < 0.01.

Additionally, we aimed to examine the impact of sandfly SGE on the expression of inflammatory genes (*ccl2*, *cxcl2*), which are linked to host skin responses, as well as *ifn-β*, a crucial interferon in the type I IFN system. Here, in our initial experiment, sandfly SGE significantly downregulated the expressions of both *ccl2* and *cxcl2* at an MOI of 0.1 during TOSV infection (Figure 3.5A), suggesting SGE modulates cellular immune response to TOSV infection. However, in the repeated experiment, fibroblast-like cells pre-treated with sandfly SGE before MOI 0.1 of TOSV showed either no difference in expression (for *ccl2*) or a slight significant upregulation of expression (for *cxcl2*) compared to the virus-only group (Figure 3.5B). For *ifn-β*, in the first experiment, the expression level of *ifn-β* showed no significant difference at either MOI 0.1 or MOI 2 of TOSV, regardless of the presence of sandfly SGE. However, in the repeated experiment, *ifn-β* expression was modestly, if significantly, higher in the presence of sandfly SGE at an MOI of 0.1 compared to the virus alone (Figure 3.5C).

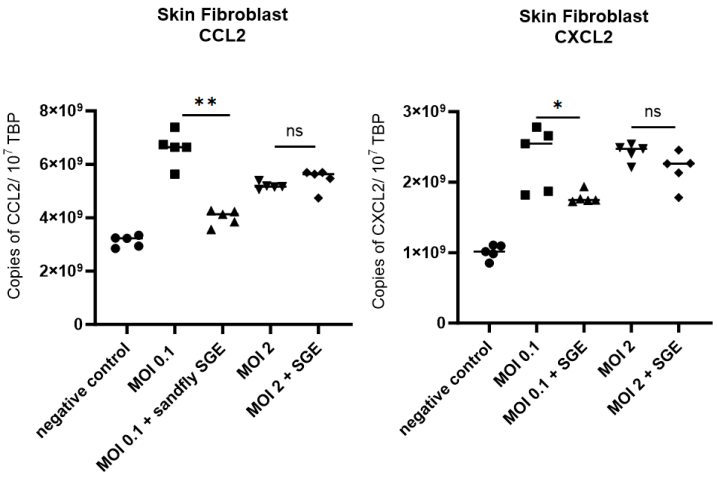
Interestingly, for all samples with a MOI of 2, there was no difference in gene expression between the two groups for any of the genes assessed. This is likely due to the higher amount of virus infection and the inability of SGE to modulate this response (Figure 3.5A, B, C).

Lastly, in TOSV-infected macrophages at an MOI of 0.5, sandfly SGE did not significantly modulate *ccl2* or *ifn-β* expression levels (Figure 3.5D, E). Similarly, dendritic cells also did not significantly express *ifn-β* at an MOI of 0.5 in the presence of sandfly SGE compared to its absence.

Together, this shows that sandfly SGE does not reproducibly alter the ability of fibroblasts, macrophages or dendritic cells to become infected with TOSV. Neither does

SGE reproducibly alter the gene expression of key immune genes in response to TOSV infection. Those examples where statistical difference between control and SGE treated cells were observed, demonstrated only marginal differences in mean gene expression of either TOSV RNA or host cytokines. We propose that an *in vivo* model should be developed to better assess whether SGE modulates host susceptibility to TOSV.

A



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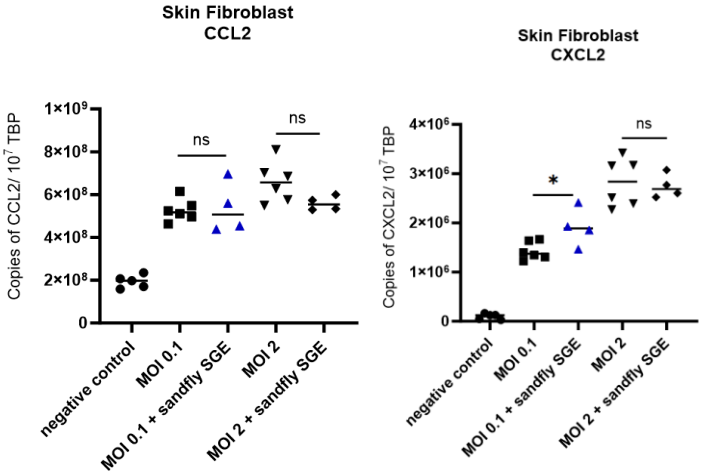

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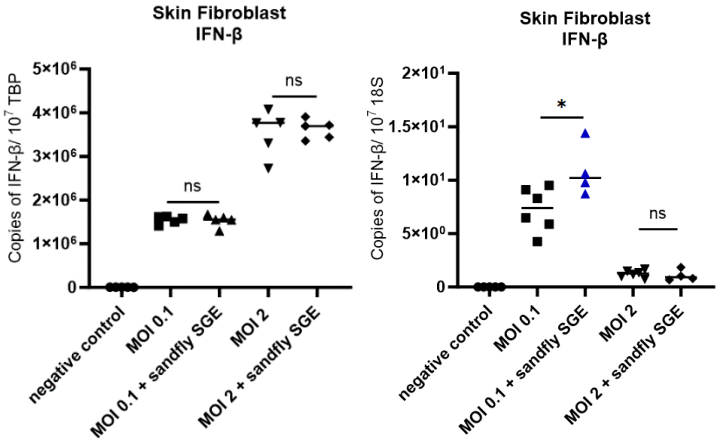


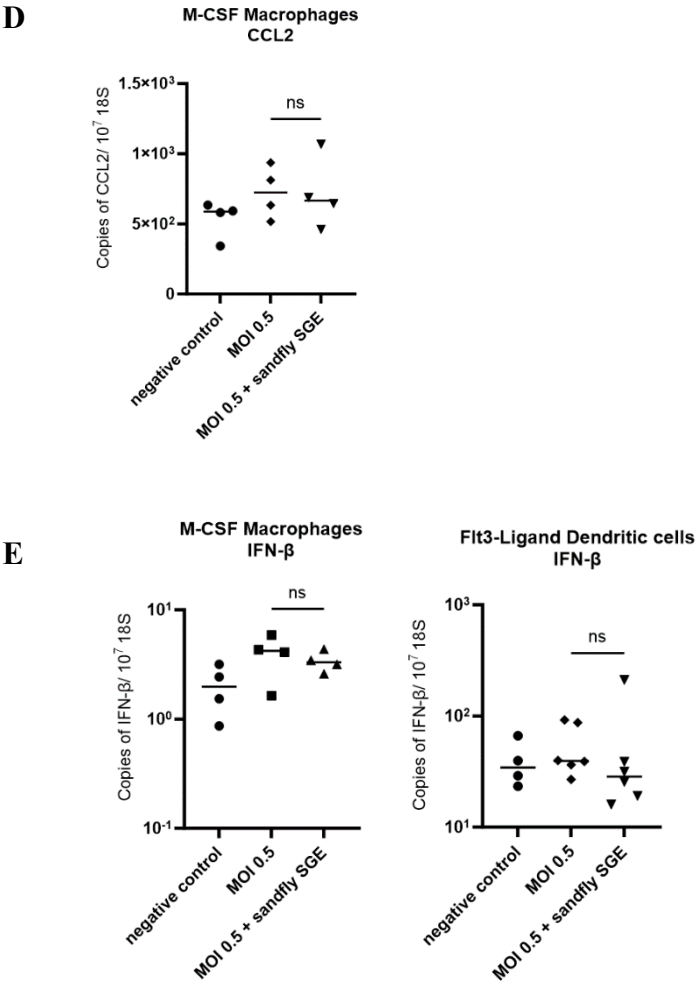


Figure 3.5: Sandfly SGE on TOSV Infection of Primary Cell Cultures Modulate the Induction of Genes Related to Immune System

(A)-(B) *ccl2, cxcl2* expression on fibroblast-like cells (n=5) **(C)** the levels of *ifn*-β on fibroblast-like cells (n=5) **(D)** *ccl2* levels in macrophages (n=4) **(E)** the expression of *ifn*-β on macrophages, dendritic cells (n=6). The cells were collected at 24hpi, and the gene copy numbers were determined by qPCR assay. Data is presented as dot plots, representing a separate biological sample with a line at the population median. Blue dots show infected skin fibroblast-like cells with an MOI of 0.1 + sandfly SGE from the repeated experiment. ns=not significant, significant, *P < 0.05, **P < 0.01.

3.5 SFV INFECTION OF MICE INCORPORATING SANDFLY SALIVARY GLAND EXTRACT WITH INOCULUM

3.5.1 Sandfly Salivary Gland Extract Enhance Arbovirus Infection in SFV Mouse Model

In this thesis, we wanted to preliminarily define whether SGE from sandflies has the capacity to modulate host susceptibility to infection with other arboviruses. Therefore, before investigating the relationship between TOSV and vector saliva, we chose to assess whether sandfly SGE could influence the severity of infection with Semliki

Forest virus (SFV), a well-characterised neurotropic arbovirus that replicates efficiently in immunocompetent C57BL/6 mice. Infection of mice with SFV is enhanced if co-injected with mosquito saliva, as already shown by previous group members (Pingen et al., 2016). Here, wanted to determine whether sandfly SGE also modulated SFV infection in mice. In doing so, we sought to define whether sandfly SGE has any ability to modulate host susceptibility to infection with a model arbovirus.

Mice were culled at 24hpi because the quantity of SFV RNA and viremia peaks at this time point (Pingen et al., 2016). There were significant differences between SFV alone and SFV with sandfly SGE group in skin inoculation site and popliteal lymph nodes samples (Figure 3.6A), with SFV RNA significantly higher in mice co-inoculated with sandfly SGE. There was no significant difference in virus titres in blood serum between SFV alone and SFV co-injected with SGE groups. (Figure 3.6B).

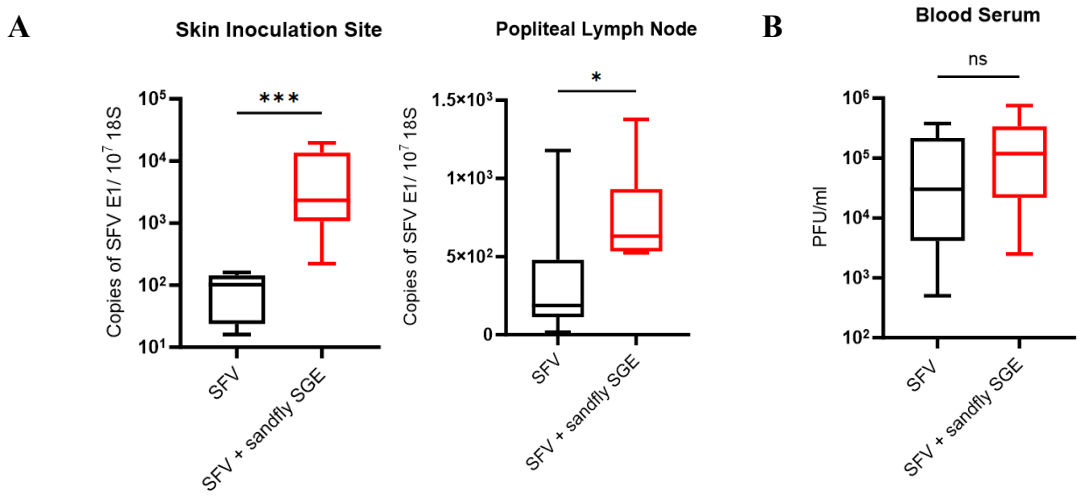


Figure 3.6: Sandfly SGE enhance Arbovirus Infection in a SFV Mouse Model

C57BL/6 mice were infected with 10,000 PFU of SFV4 with or without sandfly salivary gland extract. Tissues and blood samples were taken from mice at 24hpi. SFV RNA (E1 gene) copy number was determined by qPCR. Infectious units in serum were determined by plaque assay. Plots show the median value ± interquartile range. ns=not significant, significant *P < 0.05, ***P < 0.001.

(A) The graphs indicate that the quantity of viral RNA in the SFV-alone group and the sandfly SGE-treated group in both skin and popliteal lymph nodes at 24 hours post-infection (n=8 mice).

(B) The graph shows PFU/ml of virus in blood serum samples belonging to SFV-alone and SFV for the SGE group at 24hpi (n=8 mice).

To define whether this finding could be repeated, and because there was a trend in the blood PFU quantities that suggested an increase for mice co-inoculated with SGE and SFV, we undertook a second independent experiment to further assess whether sandfly

SGE could modulate infection of mice with SFV. In the skin inoculation site, the expression of TOSV RNA in the mice co-inoculated with sandfly SGE was 10-fold higher compared to SFV alone group (Figure 3.7A). Moreover, there was a clear 10-fold increase and a significant difference in the titre of blood virus for mice co-inoculated with virus and SGE, compared to virus alone. (Figure 3.7B). Together, this suggests that sandfly SGE can enhance arbovirus infection in the mammalian host.

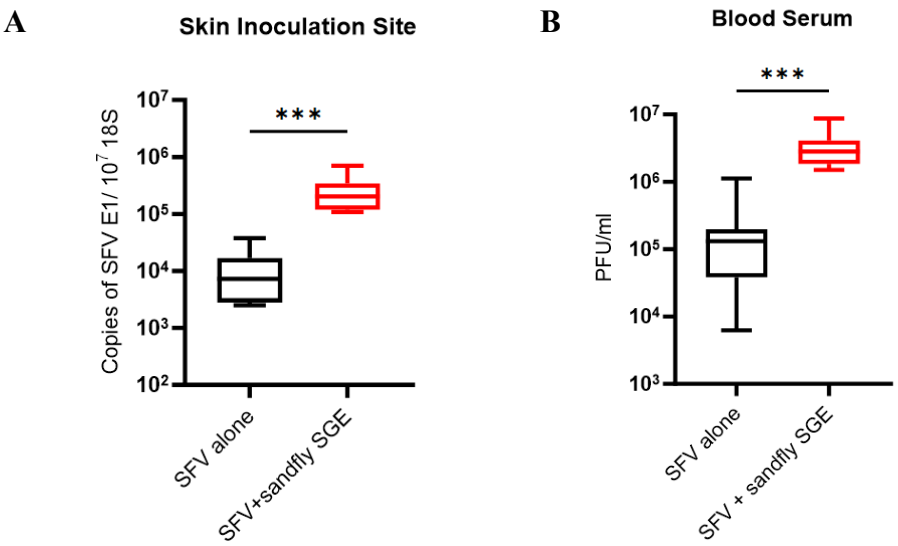


Figure 3.7: Sandfly SGE enhance Arbovirus Infection in a SFV Mouse Model
C57BL/6 mice were infected with 10,000 PFU of SFV4 with or without salivary gland extract. Skin and blood samples were taken from mice at 24hpi. SFV RNA (E1 gene) copy number was determined by qPCR. Infectious units in serum were determined by plaque assay. Plots show the median value ± interquartile range. significant ***P < 0.001.
(A) The graph shows quantities of virus RNA in the sandfly SGE group and the SFV-alone group in the skin at 24hpi (n=8 mice).
(B) The graph demonstrates PFU/ml of virus in blood serum samples between SFV-alone and SFV + sandfly SGE groups at 24hpi (n=8 mice).

3.5.2 Sandfly Salivary Gland Extract Enhances the Induction of Innate Immune Genes to SFV

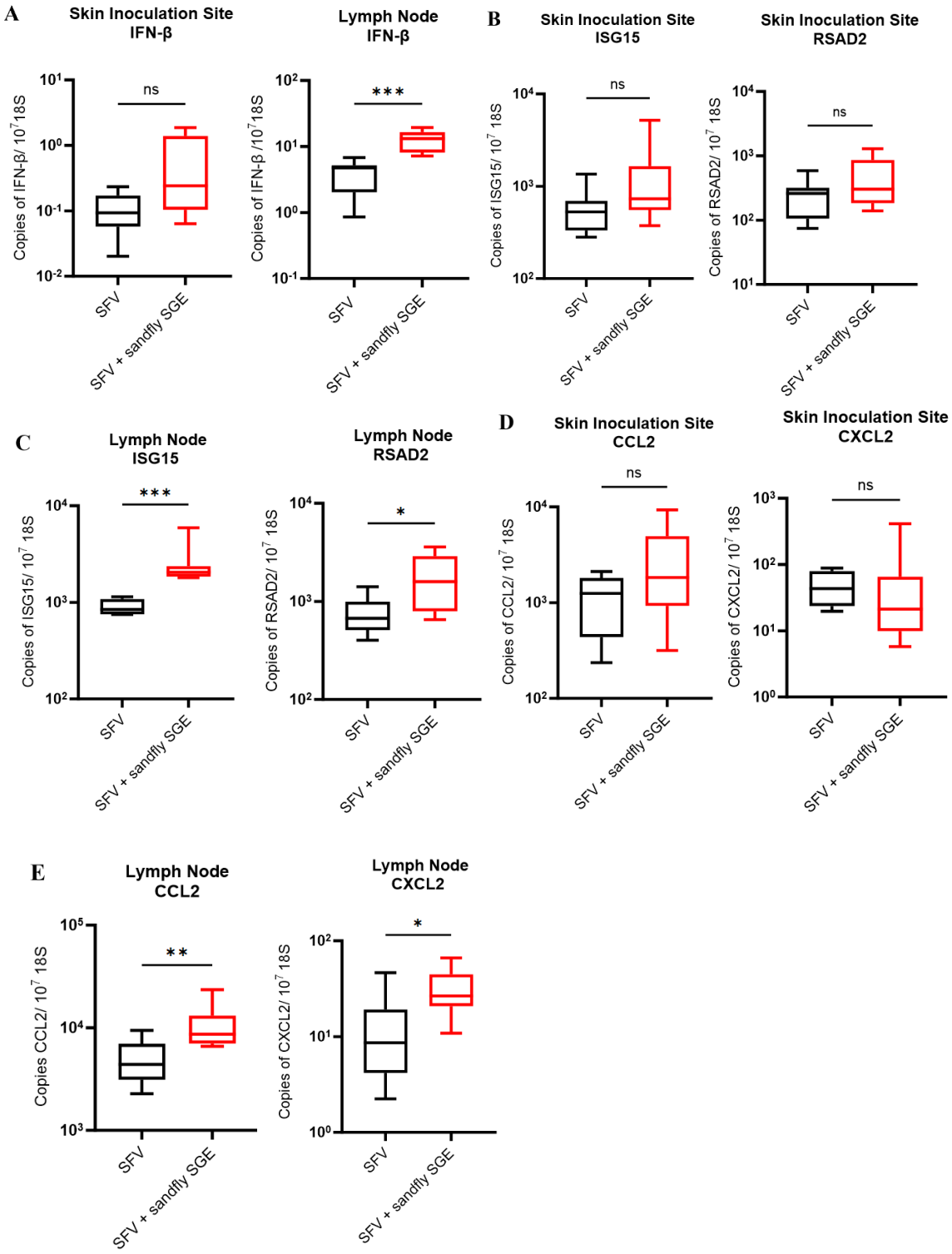
Mosquito saliva enhances arbovirus infection, likely by the modulation of early host responses to virus, including innate immune responses (Pingen et al., 2016). Consequently, we suggest that putative modulation of TOSV infection by arthropod saliva could occur due to similar modulation of innate immune pathways. Because we demonstrated above a significant difference increase in quantity of SFV when inoculated with sandfly saliva, compared to injection with virus alone, in skin and popliteal lymph node and blood serum (Figure 3.7)

To explore the potential impact of sandfly saliva on the induction of innate immune response genes, we assessed gene transcript expression in the skin and the popliteal

lymph node samples at 24hpi. As sandfly SGE enhanced SFV infection, we initially hypothesised that this could be explained the ability of sandfly SGE to **inhibit** expression of anti-viral type I IFNs.

Here, the expression of *ifn-β*, one of the key interferons in the type I IFN system, was low but there was a significant increase in *ifn-β* in the lymph node of mice inoculated with SFV in the presence of sandfly SGE (Figure 3.8A). Type I IFNs potently induce the expression of many anti-viral genes, known as IFN stimulated genes (ISG). Interestingly, expression of ISGs in response to SFV were not significantly modulated by presence of SGE in the skin (Figure 3.8B), although expression of *isg15* was significantly higher in the draining lymph node in mice infected with SFV in presence of sandfly SGE (Figure 3.8C). Overall, there was the increased induction of type I IFNs by sandfly saliva during the arbovirus infection. This is the opposite of our hypothesis, and preliminarily suggests sandfly SGE does not enhance virus infection through suppression of IFN function.

We also assessed the expression of inflammatory genes that are associated with host skin responses to arthropod biting and their saliva (Pingen et al., 2016). *Ccl2*, which functions to recruit monocytes, and *cxcl2* that functions to recruit neutrophils, respectively into tissues, is upregulated following mosquito biting (Pingen et al., 2016). Here, when compared to the virus alone group, the difference in expression of these two chemokines in the skin was not significantly different when infected in presence of sandfly SGE (Figure 3.8D). However, there was a statistically significant increase in *ccl2* and *cxcl2* gene expression following infection in presence of sandfly SGE in the lymph node (Figure 3.8E). Similarly in the LN, the expression of both *il-1β* and *il-6* (potent pro-inflammatory cytokines), were also significantly increased in the SFV-sandfly saliva group, while the expression of *il-6* was also significantly increase in the skin by sandfly SGE (Figure 3.8F). It is also noteworthy that several other innate immune genes assessed here had a trend for increased expression in the skin when infected with SFV in presence of SGE; however, these changes did not reach statistically meaning difference (Figure 3.8B, D). Together, this suggests SGE induced a more robust pro-inflammatory responses following SFV infection.


A
Skin Inoculation Site
IFN-β
Lymph Node
IFN-β
B
Skin Inoculation Site
ISG15
Skin Inoculation Site
RSAD2
C
Lymph Node
ISG15
Lymph Node
RSAD2
D
Skin Inoculation Site
CCL2
Skin Inoculation Site
CXCL2
E
Lymph Node
CCL2
Lymph Node
CXCL2
Copies of IFN-β/ 10^7 18S
Copies of ISG15/ 10^7 18S
Copies of RSAD2/ 10^7 18S
Copies of CCL2/ 10^7 18S
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ns
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*
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SFV
SFV + sandfly SGE

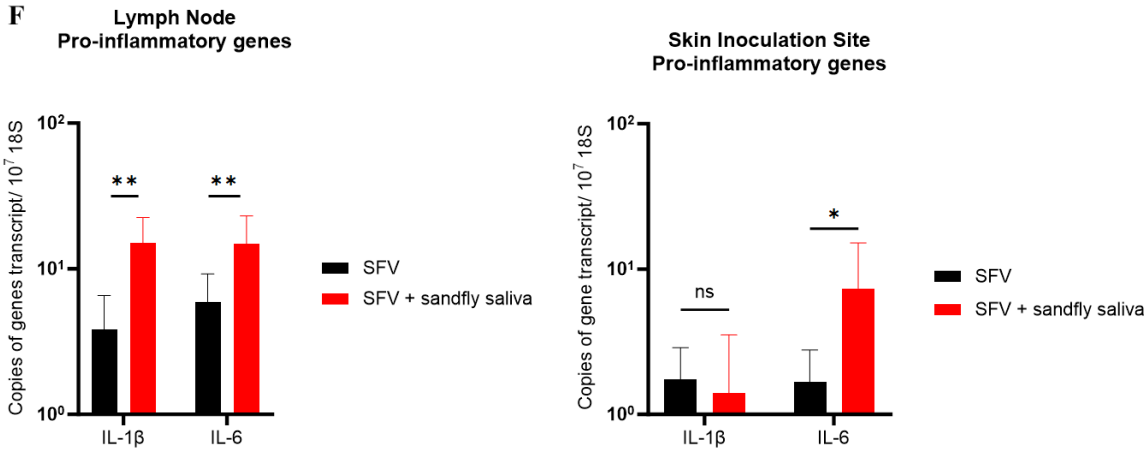


Figure 3.8: Arbovirus Infection with Vector-Arthropod Saliva Modulate the Induction of Innate Immune Genes

(A) *ifn-β*, **(B)-(C)** ISGs (*isg15* and *rsad2*) **(D)-(E)** Chemokines, **(F)** Pro-inflammatory genes, all in the skin and lymph nodes (LN) samples taken from mice at 24hpi. Gene expression was measured in these tissues by qPCR (n=8 mice). Plots show the median value ± interquartile range. ns=not significant, significant, *P < 0.05, **P < 0.01, ***P < 0.001.

3.5.3 Sandfly Salivary Gland Extract Enhance Infection of SFV Independently of The Type I IFN Response

Because we had shown above that sandfly saliva did have some ability to alter host IFN response to virus, we next wanted to determine more comprehensively whether the enhancement of SFV infection is due to the suppression of Type I IFN responses, as interferon responses are crucial during the first few hours of infection. Therefore, *ifnar1-/-* mice (which lack all type I IFN signalling) were infected with 10,000 PFU of SFV4 with or without sandfly SGE (1 pair gland extract/µl) subcutaneously into the skin on the upper side of the left foot. The quantity of virus was compared between mice subjected to virus injection in the presence and absence of sandfly SGE at 24 hours post-infection. Titration of the virus in the serum (Figure 3.9A) and quantification of SFV RNA at the skin inoculation site and spleen revealed a significant increase in virus quantity when the virus was injected with sandfly SGE, compared to when it was injected alone without sandfly SGE. Although both groups showed high viral RNA loads in the lymph nodes, there was no significant difference between them (Figure 3.9B). Moreover, the SFV RNA quantity was similar to the results of the SFV infection in immunocompetent mice experiment. This further suggests that the mechanism behind sandfly SGE viral enhancement, occurs independently of the host type I IFN response.

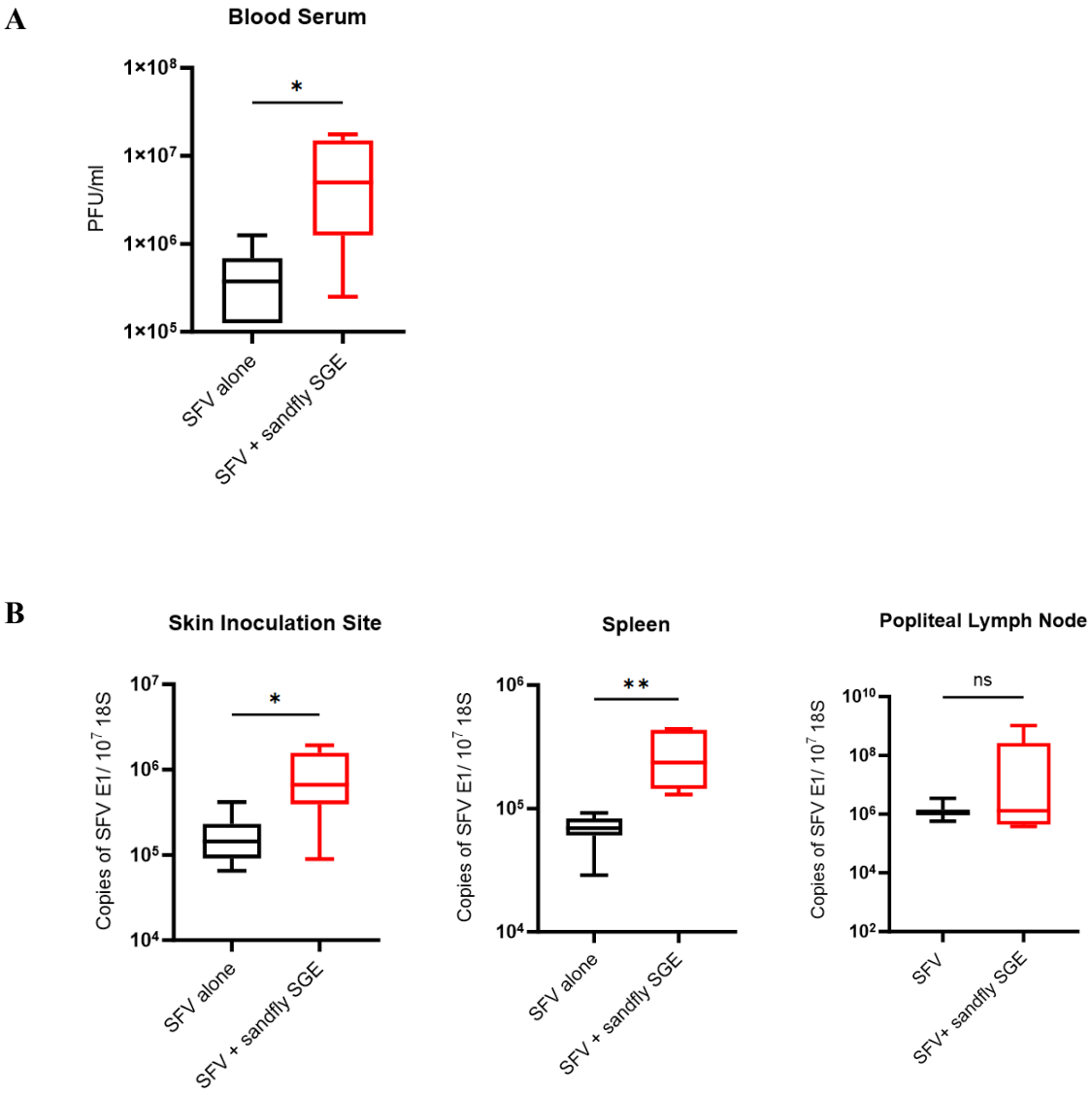


Figure 3.9: Sandfly SGE Mediated Viral Enhancement Independent of Type I IFN Response

Ifnar1-/- mice were infected with 10,000 PFU of SFV4 with or without sandfly SGE. Skin, spleen, popliteal lymph node (PLN), and blood samples were taken from mice at 24hpi. SFV RNA (E1 gene) copy number was determined by qPCR. Infectious units in serum were determined by plaque assay. Plots show the median value ± interquartile range. ns=not significant, significant *p < 0.05, **p < 0.01.
(A) PFU/ml of virus in blood serum samples at 24hpi (n=8 mice).
(B) The graphs show the quantity of virus RNA in skin, spleen and PLN at 24hpi in the groups of SFV and SFV + sandfly SGE (n=8 mice).

In addition, we investigated the expression of inflammatory genes that are associated with host skin responses to arthropod biting which are *ccl2*, *cxcl2*. Although *ifnar1-/-* mice, which lack the type I interferon receptor (IFNAR1), have a significantly impaired immune response to viral infections, these mice can still respond to viral infections

through other components of the immune system, such as cytokines, chemokines, and other signalling pathways, albeit less effectively. Here, when compared to the virus-only group, the expression of *cxcl2* chemokine in the skin was significantly higher when infected with sandfly SGE, although this was not the case for *ccl2* (Figure 3.10A). There was no statistically significant increase in *ccl2* and *cxcl2* gene expression in the lymph node following infection with sandfly SGE (Figure 3.10B). Together this suggests that SGE modulates host response to virus infection in skin. However, this may not necessarily be a direct effect, as e.g. this may reflect higher virus titre in the skin.

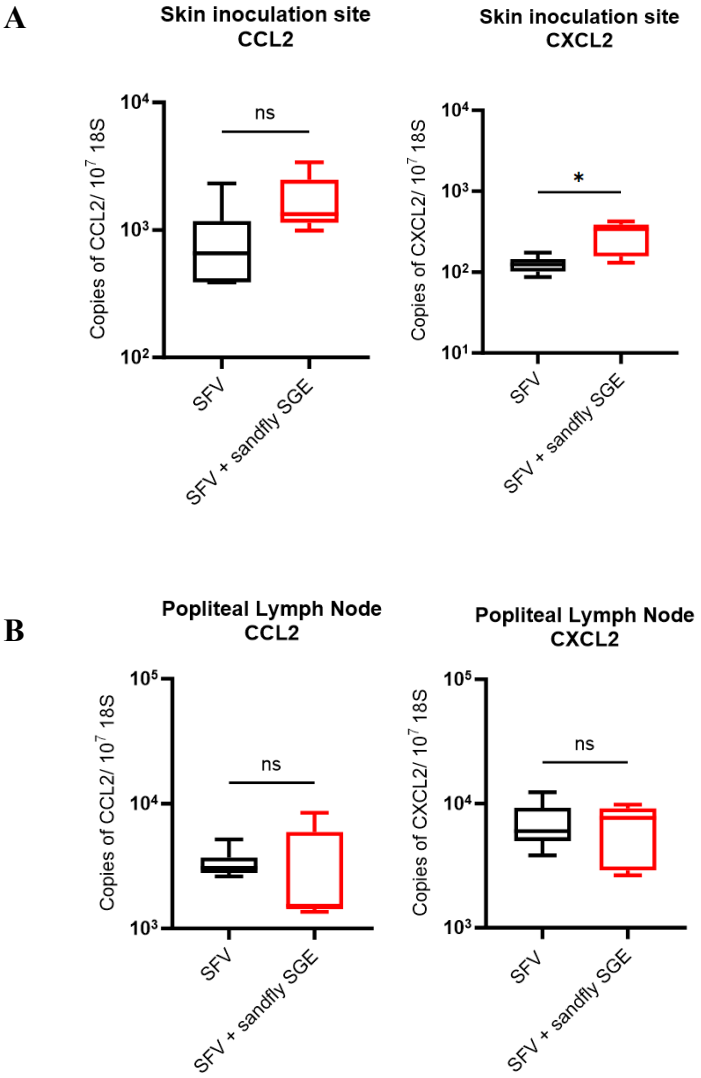


Figure 3.10: SFV Infection with Sandfly SGE Modulate the Induction of Inflammatory Chemokines

(A) *ccl2* **(B)** *cxcl2*. The skin and popliteal lymph nodes (PLN) samples taken from *ifnar1-/-* mice at 24hpi. Gene expression was measured in these tissues by qPCR (n=8 mice). Plots show the median value ± interquartile range. ns=not significant, significant, *P < 0.05.

3.6 ESTABLISHING A NOVEL TOSV ANIMAL MODEL

3.6.1 Optimisation of a Novel TOSV Infection Mouse Model That Can Incorporate TOSV-Competent Vector Saliva

Here, we wanted to establish and use a new *in vivo* model of TOSV infection that uniquely combines salivary components derived from the sandfly, its natural vector. We attempted to optimise a protocol for infecting mice with TOSV. Initially, TOSV stocks were grown in BHK-cells, a common mammalian cell line frequently used to generate arbovirus stocks, in 20% (low) and 80% (high) BHK-21 cell confluency in T150 cm^2 flasks. Then, the titre of TOSV produced by low and high confluency cultures were quantified as 1 x 10^8 PFU and 4.5 x 10^8 PFU, respectively via plaque assay. We used the high concertation stock (4.5 x 10^8 PFU of TOSV) in the experiments; this is relevant for mouse studies that mimic natural transmission, as high concentration enables one to inject very small volumes of virus into mice. This better mimics natural infection by arthropod bites that typically deposit high titre/low volume inoculum into the skin (Styer et al., 2007).

3.6.1.1 Optimising TOSV Dose and Sampling Times Following TOSV Infection in Immunocompetent Mice

To ascertain whether the TOSV stock could infect immunocompetent mice, C57BL/6 mice were infected at three different doses: 1000, 10,000, and 100,000 PFU of BHK-derived TOSV in the dorsal skin of the left foot. Unexpectedly, although TOSV RNA could be detected at the inoculation site, the expression of viral RNA in tissues remote from the inoculation site was low (Figure 3.11). Interestingly, mice could rapidly clear virus replication, as levels of virus RNA decreased by 48hpi. From these data, it was unclear whether the detected virus RNA represented a newly replicated virus or measured residual virus inoculum. However, it suggested that mice receiving 100,000 PFU had a relatively higher viral load in skin inoculation site, lymph node and spleen tissues at 24hpi (Figure 3.11). Nonetheless, these data showed reproducible evidence of TOSV RNA at 24 hours. Thus, all remaining TOSV optimisation experiments in this chapter assessed TOSV RNA expression at the 24-hour time point.

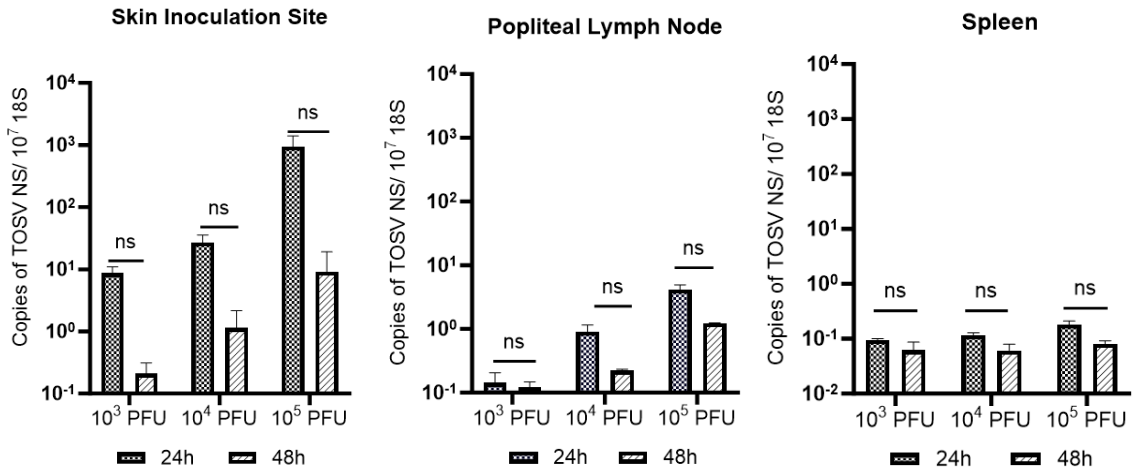


Figure 3.11: First TOSV Infection Optimisation Experiment, Three Doses, Two-Time Points

C57BL/6 mice were infected with either 1000, 10,000, or 100,000 PFU of TOSV (BHK cell-derived). Tissues were taken from infected mouse at 24hpi and 48hpi. TOSV RNA (Ns gene) copy number was determined by qPCR. Blood was also collected to assess quantity of infectious units by plaque assay (no detectable plaque). Plots show the median value ± interquartile range. ns=not significant. The graphs show the quantity of viral RNA each for tissues (skin, draining popliteal LN, and spleen) separately with 1000, 10,000, 100,000 PFU of TOSV at 24hpi and 48hpi (n=2 mice).

3.6.1.2 Pre-treatment of mice with Anti-Mouse IFNAR-I Antibodies

Because quantity of TOSV was low and was rapidly cleared in immunocompetent mice, we hypothesised that mouse type I IFN responses might have been preventing efficient replication of TOSV. To determine whether type I interferon (IFN) responses play an important role in controlling arbovirus infection, we pre-treated mice with anti-mouse IFNAR-I antibodies to block IFN signalling. We hypothesised temporary immunosuppression of IFN signalling might allow more robust replication of TOSV. Notably, when compared to either the first optimisation experiment (Figure 3.11), or the IFN-sufficient mice used here, the levels of viral RNA were higher in mice that received IFNAR-blocking antibodies (Figure 3.12). Together, this suggests that the TOSV could not successfully disseminate *in vivo* in immunocompetent mice. However, this result suggests that utilizing anti-mouse IFNAR-I could be useful if further optimised. Because mice receiving 10,000 PFU exhibited more reproducible levels of TOSV RNA, this dose was chosen for further experiments reported below (Figure 3.12).

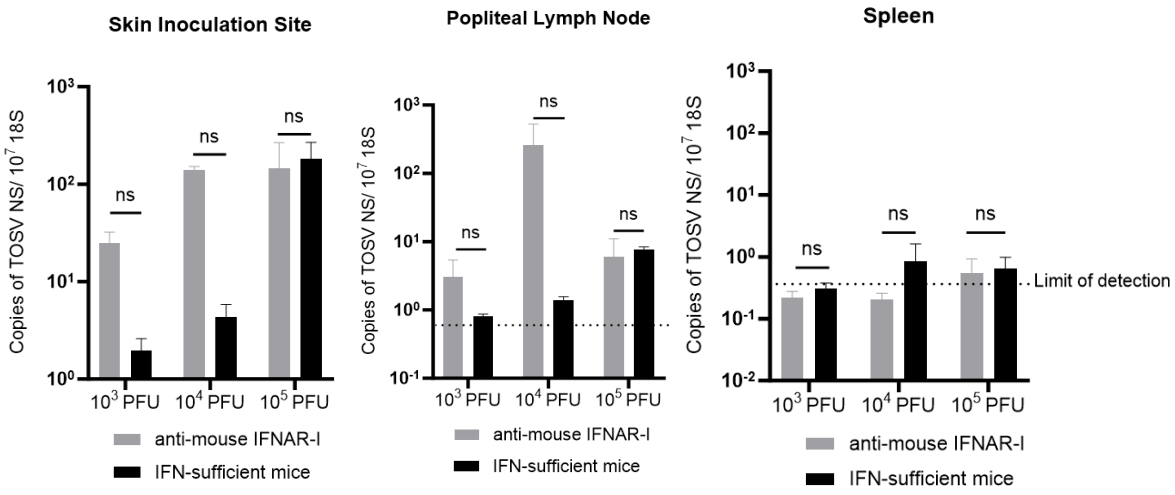


Figure 3.12: Second TOSV Infection Optimisation Experiment, Three Doses, One-Time Point

C57BL/6 mice were either left resting or administered subcutaneously with 499.5 µg of InVivoMAb anti-mouse IFNAR-blocking antibody 24 hours prior to infection and then infected with either 1000, 10,000, 100,000 PFU of TOSV (BHK cell-derived). Tissues were taken from infected mouse at 24hpi and TOSV RNA (Ns gene) copy number determined by qPCR. Blood was also collected to assess quantity of infectious units by plaque assay (no detectable plaque). Plots show the median value ± interquartile range. Dotted lines represent the assay's limits of detection based on qPCR values. ns=not significant. The graphs depict the quantity of TOSV RNA in various tissues (draining popliteal lymph nodes, skin, and spleen) following infection with 1000, 10,000, and 100,000 PFU of the virus at 24hpi (n=2 mice).

3.6.1.3 Combining A Higher Dose of Anti-Mouse IFNAR-I Antibodies and Pre-exposed Mice to Arthropod Bites

Because quantity of TOSV was still quite low, even with IFNAR1 antibody blocking, e.g., compared to e.g. following SFV infection, we next investigated two distinct approaches for optimising infection of mice with TOSV. The first was to use higher amounts of anti-mouse IFNAR-blocking antibody to determine whether, this enables higher replication of virus in the mammalian host. The second approach was to determine whether virus replication and dissemination could be further enhanced by pre-exposing mice to an arthropod bite, in this case a mosquito bite at the inoculation site. We decided to use mosquitoes as they were available, can be easily made to bite mouse skin, and because mosquito biting is known to enhance infection of mice with many genetically different arboviruses such as SFV and Bunyamwera virus (BUNV) (Pingen et al., 2016).

The results showed that in draining popliteal LN and skin samples, the quantity of viral RNA was somewhat higher in mice administered higher doses of the IFNAR-I blocking

antibody compared to those given lower doses, those given higher doses combined with mosquito bites, and IFN-sufficient mice (Figure 3.13B). Therefore, we can say that when the type I IFN response is suppressed, the virus is able to exhibit higher levels of replication. In contrast, mice are exposed to biting mosquitoes did not exhibit any notable effect increase in TOSV RNA quantities in inoculation site, LN and spleen, suggesting that this is not a limiting factor that is preventing efficient infection of mice with TOSV in wild type mice (Figure 3.13A). Importantly, in all experiments the virus could not disseminate to the blood or the spleen. These results led us to hypothesise that infecting mice that are genetically deficient in IFNAR expression might be a more reliable approach for establishing *in vivo* TOSV infection (which we report below in 3.6.2).

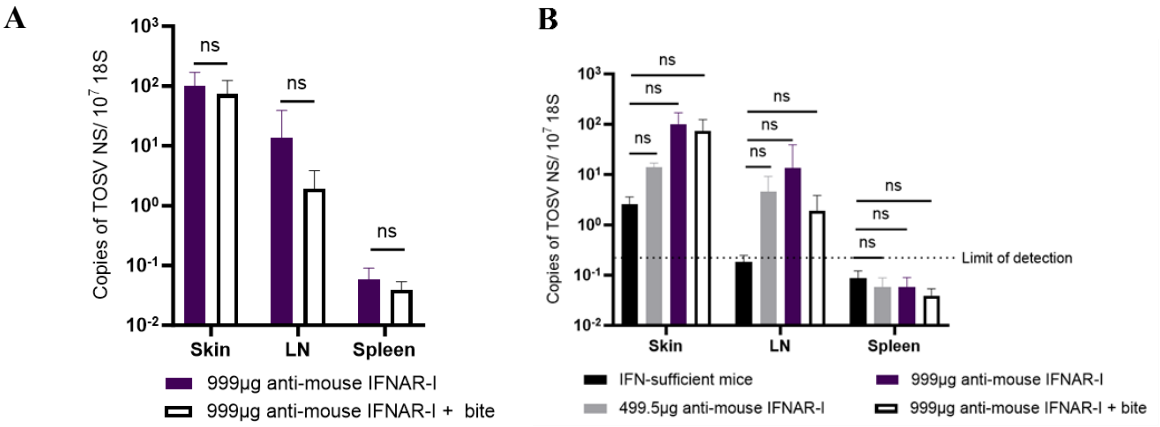


Figure 3.13: Third TOSV Infection Optimisation Experiment, One Dose, One-Time Point

C57BL/6 mice treated with IFNAR-1 a day before infection were infected with 10,000 PFU of TOSV subcutaneously on their own or following exposure to up to 5 mosquito bites. Tissues were taken from infected mice at 24hpi. TOSV RNA (Ns gene) copy number was determined by qPCR. Blood was also collected to assess quantity of infectious units by plaque assay (no detectable plaque). Plots show the median value ± interquartile range. Dotted line represents the assay's limits of detection based on qPCR values. ns=not significant.

(A) The graph demonstrates quantities of viral RNA in skin, draining popliteal lymph nodes (LN) and spleen at 24hpi in two groups: one pre-treated with 999µg of an IFNAR-blocking antibody and the other with 999µg of the IFNAR-blocking antibody followed by exposure to mosquito bites (n=6 mice).

(B) The plot shows the quantity of TOSV Ns present in draining popliteal LN, Skin, and Spleen in IFN-sufficient mice (n=2), 499.5µg anti-mouse IFNAR-I (n=2), and 999 µg anti-mouse IFNAR-I with or without mosquito bites at 24hpi (n=6 mice).

3.6.1.4 Examining Different Cell Line-Derived TOSV Infections in Immunocompetent Mice

Before investigating TOSV in *ifnar1-/-* mice (below), we lastly wanted to assess whether TOSV derived from different cell lines affects the virus infection on the IFN-sufficient mice. Previous work that demonstrated infection of mice with TOSV, used TOSV that was derived from Vero cells (Grazia Cusi et al., 2005b). Moreover, it has been shown that TOSV RNA was detected in lymph nodes and spleen at later post infection time points when using Vero-derived TOSV (Grazia Cusi et al., 2005b). However, we found that when mice were infected with TOSV derived from Vero cell, the TOSV RNA quantity was lower in the skin, lymph nodes and spleen than that following inoculation with BHK cell derived TOSV, as done in the previous optimisation experiments (Figure 3.14). The results suggest that immunocompetent mice eliminate the virus replication; therefore, BHK- or Vero- derived TOSV could not disseminate *in vivo* sufficiently to initiate a viremia.

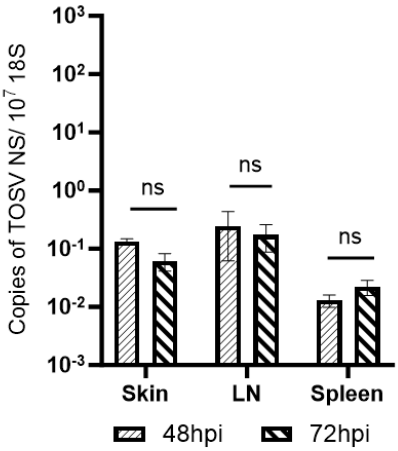


Figure 3.14: Vero-derived TOSV Experiment

Tissues were taken from infected C57BL/6 mice at 48hpi and 72hpi. TOSV RNA (Ns gene) copy number was determined by qPCR. Blood was also collected to assess quantity of infectious units by plaque assay (no detectable plaque). Plot shows the median value ± interquartile range. ns = not significant. The plot shows the quantity of viral RNA present in skin, draining popliteal LN and spleen 6000 PFU (Vero-derived TOSV) at 48hpi and 72hpi (n=2 mice).

3.6.2 *Ifnar1-/-* Mouse Model for TOSV Infection

The literature only describes a mouse model for TOSV (strain 1812) that uses the intracranial route to generate infection in newborn and adult BALB/c mouse brain (Grazia Cusi et al., 2005; Gori Savellini et al., 2008). However, intracranial injection

bypasses the natural replication strategy of arthropod-borne viruses, even for neurotropic viruses such as SFV and TOSV.

In the absence of a mouse model for TOSV that incorporates the natural transmission route, we above attempted to establish one using C57BL/6 mice. However, our optimization experiments (discussed in Section 3.6) indicated that TOSV could not effectively replicate in immunocompetent mice when injected subcutaneously into the skin on the upper side of the foot. Injecting a high-titer, low-volume inoculum into the skin better mimics sandfly bites under BSL2 laboratory conditions. In addition, our previous optimisation experiments showed that when anti-mouse IFNAR-I blocking antibody used before TOSV infection, the quantity of viral RNA was higher than IFN-sufficient mice (Figure 3.13). Although this increase was inefficient in disseminating TOSV infection in the bloodstream, it encouraged us to investigate the infection further in *ifnar1*-/- mice that fully deficient in IFN signalling.

To establish this new *in vivo* model of TOSV infection (Figure 3.15) we injected 100,000 of PFU TOSV per µl into the dorsal foot skin of *ifnar1*-/- mice under inhalation anaesthesia. Thus, we raised the TOSV titre tenfold compared to the virus titre in the optimization experiments above. Foot skin, spleen, lymph nodes and blood were taken at 24hpi and 72hpi.

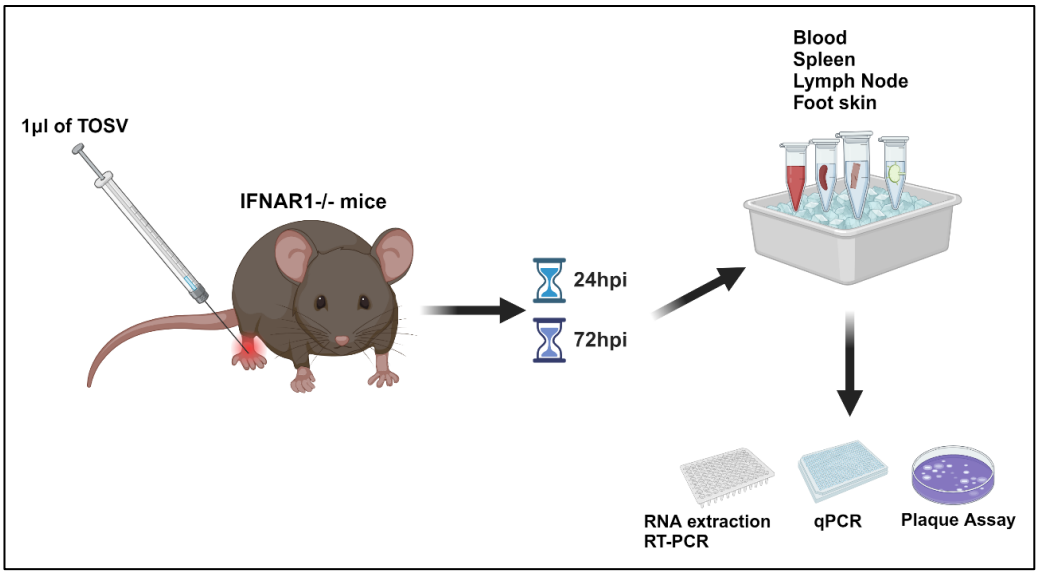


Figure 3.15: In Vivo Model for TOSV Infection

A cartoon to summarise the infection of *ifnar1* null mice with TOSV.

Here, TOSV RNA level was 10-fold significantly higher at 72hpi than at 24hpi in the skin samples (Figure 3.16A), suggesting virus has replicated over time. This is different to the above *in vivo* experiments in wild type mice, in which TOSV quantity fell over time and did not reach such high quantities. Moreover, the viral RNA was also significantly higher in the spleen at 72hpi (Figure 3.16A), indicative of systemic dissemination of virus. Interestingly, the quantity of TOSV RNA was low in both lymph nodes, popliteal (draining) and inguinal (non-draining), and there was no significant difference between the two-time points (Figure 3.16B), suggesting that TOSV did not replicate in lymph nodes. Plaque assays on blood serum were negative, suggesting that the quantity of infectious virus in blood was still below the level of detection.

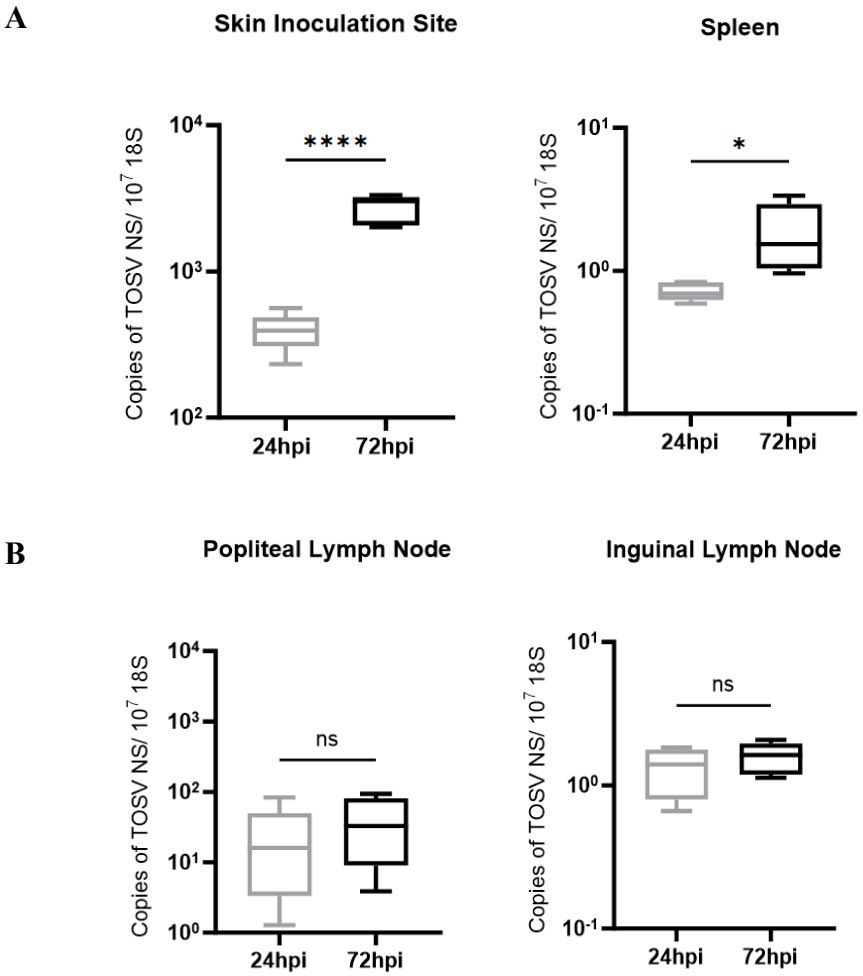


Figure 3.16: TOSV Infection in *Ifnar1-/-*, One Dose, Two-Time Points

Ifnar1-/- mice were infected with 100,000 PFU of TOSV (BHK cell-derived). Tissues were taken at 24hpi and 72hpi and TOSV RNA (Ns gene) copy number determined by qPCR. Blood was also collected to assess quantity of infectious units by plaque assay (no detectable plaque). Plots show the median value ± interquartile range. ns=not significant, significant *P < 0.05, ****P < 0.0001.

(A) The graphs show TOSV Ns quantities in skin and spleen samples at 24hpi and 72hpi (n=5 mice).

(B) The graphs indicate the viral RNA quantities in popliteal and inguinal lymph nodes at 24hpi and 72hpi (n=5 mice).

To assess whether infection in these mice also resulted in increased immune gene expression with increase in virus RNA quantity, we assayed *ccl2* and *cxcl2* expression. The results showed that the expression of these chemokines was significantly higher at 72hpi compared to resting skin (Figure 3.17A). Notably, *cxcl2* expression was more upregulated at 72hpi than at 24hpi (Figure 3.17A). The quantity of *isg15*, a prototypic interferon-stimulated gene activated by viral sensing, was significantly elevated, showing a 10-fold increase at the later time point (Figure 3.17B), presumably in response to IFNAR1 independent signalling. Additionally, the pro-inflammatory gene *il-6* exhibited significant differences in expression between the time points (Figure 3.17B). Together, these findings suggest that TOSV infection upregulates the expression of host inflammatory genes.

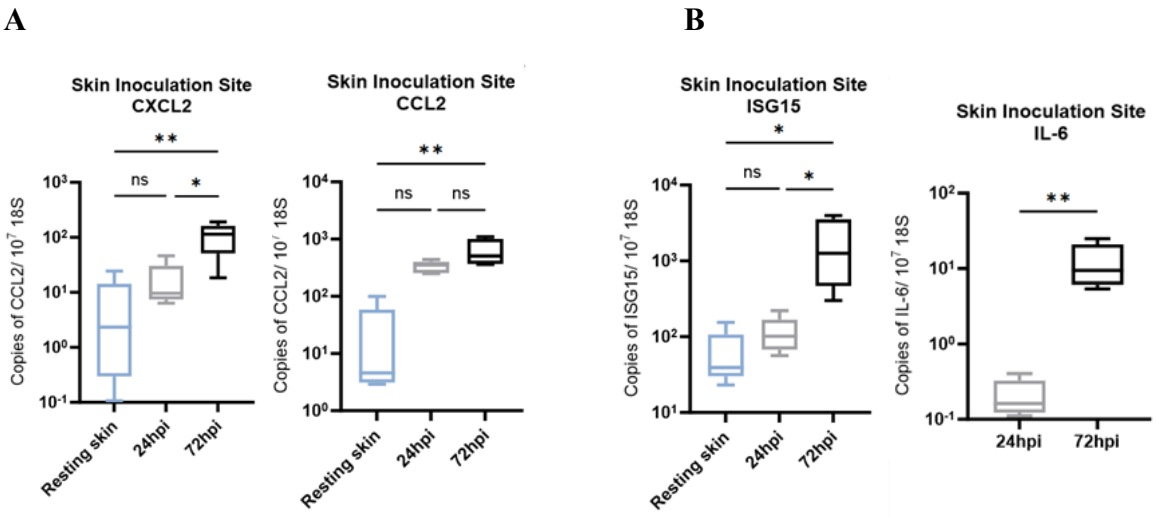


Figure 3.17: TOSV infection in *Ifnar1-/-* Mice with Modulate the Induction of Innate Immune Genes

(A) Chemokines, (B) *isg15, il-6*. The skin samples taken from *ifnar1-/-* mice at 24hpi and 72hpi. Resting skin samples were taken uninfected *ifnar1-/-* mice. Gene expression was measured in these samples by qPCR (n=5 mice). Plots show the median value ± interquartile range. ns=not significant, significant, *P < 0.05, **P < 0.01.

3.7 SUMMARY AND CONCLUSIONS

In this chapter, we generated BHK-cell-derived TOSV (strain 1812) stocks, optimised plaque assays to determine the titre of the wild-type and genetically modified TOSV, and optimised a qPCR assay for TOSV RNA, to enable us to quantify the amount of TOSV RNA in mouse tissues.

We developed an *in vitro* model system for the study of sandfly SGE dependent TOSV infection enhancement. However, utilising murine dermal fibroblasts, macrophages, and dendritic cells we demonstrated that, *in vitro*, sandfly SGE does not enhance virus infection reproducibly. In addition, there was no significant effect of sandfly SGE on the susceptibility of macrophages and dendritic cells to TOSV. Furthermore, IFN response and the inflammatory gene expression during TOSV infection with or without sandfly SGE on those cells demonstrated variable results that were not parallel between experiments. These findings suggest that if sandfly saliva can enhance arbovirus infection, it may require an *in vivo* model, similar to the mechanism by which mosquito saliva facilitates arbovirus enhancement (Lefteri et al., 2022).

In our hypothesis, we stated that sandfly SGE will be investigated in terms of its ability to enhance TOSV infection. Before testing this aspect, we wanted to describe whether sandfly SGE enhanced arbovirus infection in the mammalian host. In the light of a prior study, that has noted the SFV infection enhancement by mosquito saliva (Pingen et al., 2016), we decided to use SFV infection model. The findings showed that sandfly SGE can enhance arbovirus infection in the mammalian host. Furthermore, we identified that the mechanism by which sandfly SGE enhances SFV occurs independently of the host's type I IFN response. These promising results encourage us to also define whether sandfly SGE can enhance TOSV infection in mice, once we have established our *in vivo* model.

This chapter also interrogated the induction of type I IFN response, and other genes associated with the host response to virus infection, and whether this was altered if in absence/presence of co-inoculated sandfly SGE. There was an upregulation of *ifn-β* and ISGs following infection with SFV in the presence of sandfly SGE. We also explored the expression of *ccl2*, *cxcl2*, *il-6*, *il-1β*, inflammatory genes that are associated with host skin responses to arthropod biting (Pingen et al., 2016). In general, injection of SGE induced enhanced upregulation of inflammatory genes to SFV infection, compared to SFV alone. Together this suggests that sandfly saliva may enhance host susceptibility to SFV infection through enhanced inflammatory responses, as previously shown for mosquito saliva (Pingen et al., 2016).

To establish a novel TOSV animal model, we conducted several optimisation experiments. In the first *in vivo* optimisation experiment, the expression of virus RNA was low, although reproducible, but likely represented residual virus inoculum, as it was

quickly cleared and did not disseminate to the spleen, which is distal from the skin inoculation site.

Type I IFN responses are one of the most important anti-viral innate immune mechanisms, and we hypothesised here that, the limitation of replication of TOSV in the wild type mice may be due to the inability of TOSV to efficiently inhibit the IFN response in mice, similar to ZIKV infection in mice (Gorman et al., 2018). In the second experiment, we identified that when IFN signalling in mice is temporarily repressed through administration of a blocking antibody, the expression of TOSV RNA showed an increase in LN and skin compared to IFN-sufficient mice.

Next, we hypothesised that the limited TOSV replication observed above could also reflect the absence of host response to arthropod bites. Indeed, arboviruses are not inoculated into resting skin, but rather arthropod bites. Therefore, we wanted to seek whether mosquito biting has an infection-enhancing effect on TOSV infection in mice given the IFN-receptor blocking antibody. Mosquito bites enhance infection of mice by at least two genetically distinct arboviruses (Pingen et al., 2016). However, here, it was shown that mosquito biting was not sufficient to enable efficient TOSV infection in wild type mice. Instead, it is more likely a reflection of inefficient IFN signalling blockage by TOSV in mouse cells.

During optimisation studies, BHK-derived TOSV was used to infect the mice. However, the replication success of the virus remained low in the mice, although the stock of TOSV could infect the BHK-21 cell line successfully. Previous work that demonstrated infection of mice with TOSV, used TOSV that was derived from Vero cells (Grazia Cusi et al., 2005). Thus, as part of optimization experiments, here, Vero cells were infected with TOSV to see if virus derived from this cell line was better able to infect mice. Because previous work has suggested that TOSV infection may peak at later time points (Grazia Cusi et al., 2005) that 24 hours in mice, we therefore assessed TOSV level at 48hpi and 72hpi. Here, irrespective of the cell line used to generate TOSV, the quantity of virus expressions was again low and did not increase with time. None of the mice in any experiments become viraemic. Together, we have identified that IFN-sufficient mice do not allow replication of TOSV efficiently.

In the final part of this chapter, we developed a unique TOSV mouse model using *ifnar1-/-* mice. These mice, homozygous for the *ifnar1*-null allele, lack a functional type-I interferon receptor, leading to a diminished immune response and heightened

susceptibility to viral infections. Here, we found that at 72 hours post-infection, we detected high quantities of TOSV RNA in the skin and spleen tissues. This phenomenon is also observed in other bunyavirus infections. For example, Rift Valley fever virus (RVFV) was detected in tissues at 5 days post-infection (dpi) following intraperitoneal or intradermal infection in wild-type mice (Le Coupanec et al., 2013). Similarly, in an *ifnar1-/-* mouse model of Crimean-Congo hemorrhagic fever (CCHFV), mice met endpoint criteria 5–6 days after subcutaneous infection (Welch et al., 2019). Since the 72-hour post-infection time point yielded higher viral RNA levels and gene expression in tissues, all subsequent infections were conducted at this time point unless otherwise specified. However, we found low quantities of TOSV RNA in the lymph nodes. This is quite different to most other arboviruses spread by either mosquitoes, ticks, or midges where prominent LN infection is evident. This finding suggests that TOSV may not be able to infect cells in the lymph node. Additionally, our results demonstrated that TOSV can modulate the host inflammatory response by upregulating the expression of innate immune genes.

In summary, we have now developed a new mouse model for TOSV infection which can be used to assess the impact of sandfly saliva on host susceptibility to infection, as reported in chapter 4.

CHAPTER 4: DEFINING WHETHER SANDFLY SALIVA MODULATES HOST SUSCEPTIBILITY TO TOSV INFECTION

4.1 INTRODUCTION

The factors predisposing the host to more severe arbovirus disease are poorly understood, but they are most likely due to a combination of viral, host, and environmental factors. One such host factor known to enhance infection with many medically important arboviruses is the host inflammatory response to mosquito bites at the cutaneous inoculation site (Michael J Conway et al., 2014; Pingen et al., 2017). Our group and others have shown that inflammatory responses to mosquito bites significantly enhance infection with Flaviviruses, e.g. DENV (McCracken et al., 2014; Michael J. Conway et al., 2014; Schmid et al., 2016) and West Nile virus (Styer et al., 2011c; Moser et al., 2016), Alphaviruses, e.g. Semliki Forest virus (SFV) (Pingen et al., 2016), and Chikungunya virus (CHIKV) (Agarwal et al., 2016) and Bunyavirales (Edwards et al., 1998; Pingen et al., 2016). In addition, our group have shown that mosquito saliva enhances arbovirus infection in the absence of a mosquito bite (Lefteri et al., 2022). Importantly for this thesis, sandfly salivary factors have been shown to exaggerate at least one vector-borne disease, Leishmaniasis (Titus and Ribeiro, 1988).

In the previous chapter, it was demonstrated that sandfly saliva extract modulates SFV infection *in vivo*. Therefore, in this chapter, we wanted to investigate whether sandfly saliva extract and additionally a sandfly bite can enhance susceptibility to TOSV infection in an animal model, newly established in the previous chapter. In addition, the pathogenesis of TOSV in a murine model has been poorly studied. It is not known whether sandfly bites and/or saliva are capable of enhancing sandfly-vectored virus infection.

Finally, we wanted to determine whether non-sandfly encoded gene products (i.e. microbiota) that are also pro-inflammatory, has an ability to modulate TOSV infection *in vivo.* This is possible, as deposition of bacteria by sandflies can modulate outcome to sandfly-vectored *Leishmania* infection (Dey et al., 2018). The microbiota in sandfly saliva may play a role in activating immune responses to saliva. Consequently, these immune responses to the microbiota could also indirectly modulate outcome to virus infection.

Thus, the aims of this chapter were:

1. **To investigate whether sandfly salivary gland extract (SGE) and sandfly bite enhances TOSV infection.**

2. **To study the pathogenesis of TOSV infection in a mammalian host, and whether this is modulated by inclusion SGE in the inoculum.**

3. **To define a potential role of salivary microbiota in modulating TOSV infection *in vivo.***

4.2. SANDFLY SALIVARY GLAND EXTRACT ENHANCES TOSV INFECTION IN MAMMALIAN HOST

To determine whether sandfly saliva modulates host susceptibility to TOSV infection, we utilised our mouse model developed in chapter 3. Briefly, we injected 100,000 PFU of TOSV per µl into the skin on the upper side of the left foot of *ifnar1-/-* mice under inhalation anaesthesia, either in the presence or absence of sandfly salivary gland extract (1 pair gland extract/µl) (Figure 4.1). The salivary gland extract (SGE) from *Phlebotomus perniciosus,* the most important vector for TOSV, was kindly provided by Prof. Petr Volf at Charles University in Prague. TOSV was first identified in *Ph. perniciosus,* which is recognised as the primary vector of the virus (Charrel, 2012). Foot skin, spleen, lymph nodes, and blood samples were collected 24 hours post-infection (hpi) and 72hpi.

Tissue samples were then analysed via RT-qPCR for gene expression analysis of the TOSV viral NS, a gene encoded by TOSV that we have previously established as a good indicator of virus RNA quantities. Similarly, blood serum was analysed for infectious virus titres via plaque assays.

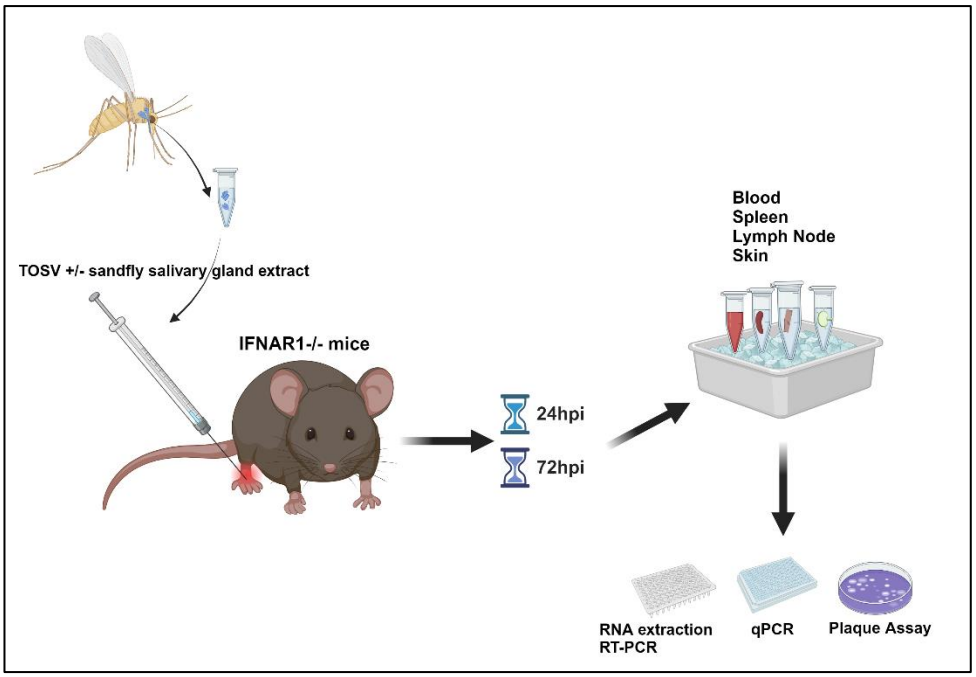
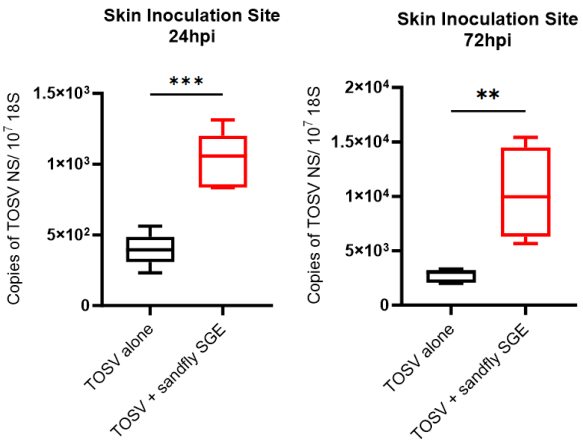


Figure 4.1: *In Vivo* Model for TOSV Infection with Sandfly Salivary Gland Extract

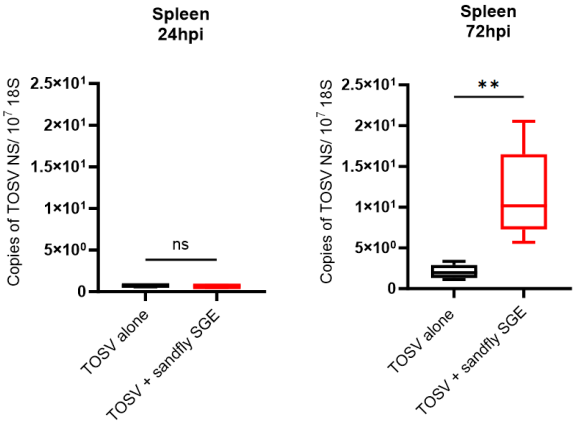
An illustration summarizing the infection process of *ifnar1* null mice with TOSV, with or without the addition of sandfly salivary gland extract.

As a result, we showed that the quantity of TOSV RNA was significantly higher by 10-fold in mice receiving SGE in the virus inoculum, compared to the virus alone group at both time points (Figure 4.2A). In addition, TOSV RNA quantities were higher at the later time point (72hpi), indicating virus had replicated over time (Figure 4.2A). Similarly, the viral load in spleen was also significantly higher by 10-fold in the SGE group, compared to the virus alone group at 72hpi (Figure 4.2B). However, the quantity of viral RNA in inguinal lymph node (ILN) (Figure 4.2C) and spleen (Figure 4.2B) samples in both groups at 24hpi was under the threshold of detection, suggesting virus does not become systemically disseminated until after 24hpi. The quantity of TOSV RNA was uniformly low in all lymph node samples. The addition of SGE in the inoculum did not significantly increase replication in these lymphoid tissues at either time points (Figure 4.2C). This result supports the suggestion that TOSV does not replicate efficiently in lymph nodes, as was shown in Section 3.6.2, in Chapter 3. Blood was also collected to define the number of infectious units (PFU) by plaque assay, and a few plaques were detected at 72hpi in some mice that received the sandfly SGE (Figure 4.2D).

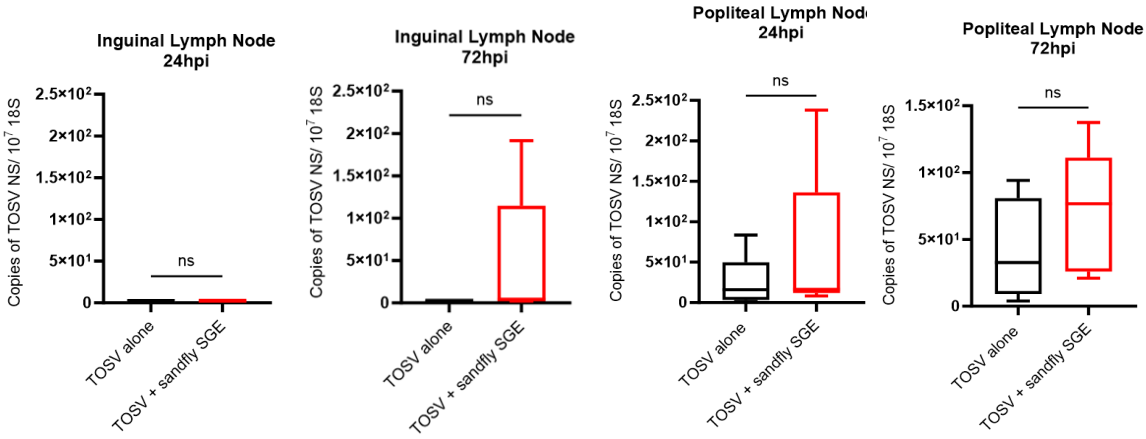
A


Skin Inoculation Site
24hpi
Copies of TOSV NS/ 10^7 18S
1.5×10^3
1×10^3
5×10^2
0
***
TOSV alone
TOSV + sandfly SGE
Skin Inoculation Site
72hpi
Copies of TOSV NS/ 10^7 18S
2×10^4
1.5×10^4
1×10^4
5×10^3
0
**
TOSV alone
TOSV + sandfly SGE

B


Spleen
24hpi
Copies of TOSV NS/ 10^7 18S
2.5×10^1
2×10^1
1.5×10^1
1×10^1
5×10^0
0
ns
TOSV alone
TOSV + sandfly SGE
Spleen
72hpi
Copies of TOSV NS/ 10^7 18S
2.5×10^1
2×10^1
1.5×10^1
1×10^1
5×10^0
0
**
TOSV alone
TOSV + sandfly SGE

C


Inguinal Lymph Node
24hpi
Copies of TOSV NS/ 10^7 18S
2.5×10^2
2×10^2
1.5×10^2
1×10^2
5×10^1
0
ns
TOSV alone
TOSV + sandfly SGE
Inguinal Lymph Node
72hpi
Copies of TOSV NS/ 10^7 18S
2.5×10^2
2×10^2
1.5×10^2
1×10^2
5×10^1
0
ns
TOSV alone
TOSV + sandfly SGE
Popliteal Lymph Nod
24hpi
Copies of TOSV NS/ 10^7 18S
2.5×10^2
2×10^2
1.5×10^2
1×10^2
5×10^1
0
ns
TOSV alone
TOSV + sandfly SGE
Popliteal Lymph Node
72hpi
Copies of TOSV NS/ 10^7 18S
1.5×10^2
1×10^2
5×10^1
0
ns
TOSV alone
TOSV + sandfly SGE

D

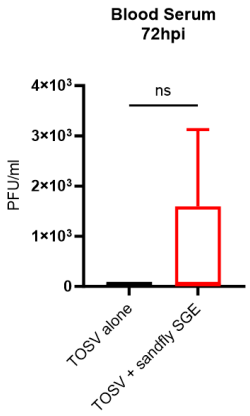

Blood Serum
72hpi
PFU/ml
4×10^3
3×10^3
2×10^3
1×10^3
0
ns
TOSV alone
TOSV + sandfly SGE

Figure 4.2: Sandfly Salivary Gland Extract Enhance TOSV Infection in a Mammalian Host

Ifnar1-/- mice were infected with 100,000 PFU of TOSV with or without sandfly salivary gland extract (SGE) (1 pair gland extract/µl). Tissues were taken at 24hpi and 72hpi, with TOSV RNA (Ns gene) copy number determined by qPCR. Infectious units in serum were determined by plaque assay. Plots show the median value ± interquartile range. ns=not significant, significant **p < 0.01, ***P < 0.001.

(A) The graph illustrates the quantity of TOSV Ns detected in the skin at 24 and 72hpi. Mice were infected with either TOSV alone or TOSV in combination with sandfly SGE (n=5 mice).

(B) The graph shows the quantity of TOSV Ns in the spleen at 24 and 72hpi, comparing TOSV alone and co-inoculation with sandfly SGE (n=5 mice).

(C) The graph depicts the quantity of viral RNA in the popliteal and inguinal lymph nodes in TOSV alone and TOSV with sandfly SGE groups at 24 and 72hpi (n=5 mice).

(D) The graph presents PFU/ml of virus in blood serum samples belonging to TOSV-alone and TOSV for the SGE group at 72hpi (n=5 mice).

We also investigated host immune gene expression in skin of these samples (Figure 4.3), to define whether SGE modulated their expression. Compared to the uninfected mice skin samples, those infected with TOSV + sandfly SGE displayed a significant increase in *ccl2*, *cxcl2*, and *isg15* genes at 24hpi and 72hpi. The transcript level of *isg15* showed a significant difference between the virus-only group and the sandfly SGE group at 24hpi but not at 72hpi. *Il-6* expression was significantly higher in the sandfly SGE group compared to the virus-only group at 72hpi, although this gene's transcription level was below the detection threshold at 24hpi. Notably, the absolute copy numbers of gene transcripts were around ten times higher at 72hpi than at 24hpi in both experimental groups, indicating their expression was being driven by increase in virus quantity/infection.

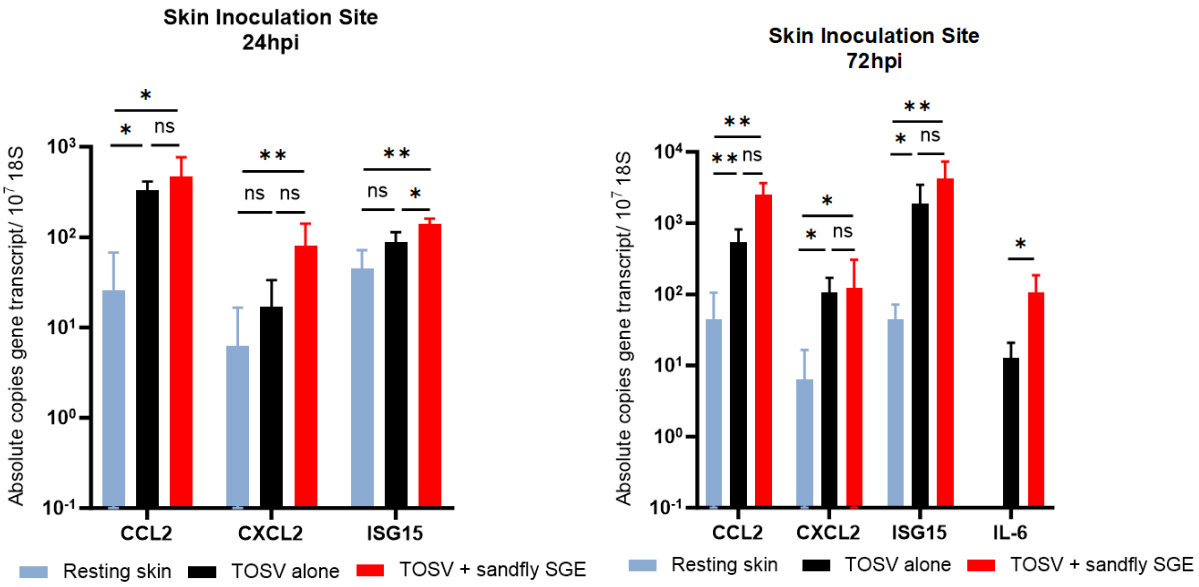


Figure 4.3: Sandfly Salivary Gland Extract and TOSV Infection Combine to Induce Gene Salivary Factor-Associated Gene Expression

The expression levels of Chemokines (*ccl2*, *cxcl2*), *isg15*, *il-6*. These skin samples were the same ones analysed above in Figure 4.2. Resting skin samples were taken from uninfected *ifnar1-/-* mice. Gene expression was measured in these samples by qPCR (n=5 mice). Plots show the median value ± interquartile range. ns=not significant, significant *P < 0.05, **P < 0.01.

4.2.1 The Enhancement Effect of Sandfly SGE is Dose-Dependent

We wanted to assess the dose dependence of the saliva enhancement phenotype after showing that sandfly SGE augmented TOSV infection in our *in vivo* model. For instance, this phenomenon has been shown with mosquito salivary SGE in WNV infection before (Moser et al., 2016). Here, we tested two doses of SGE by inoculating mice skin on the upper side of the left foot with TOSV plus equivalent to 1 pair gland extract and 3 pair glands extracts. The level of TOSV RNA at 72hpi was significantly elevated in all groups in skin inoculation site and spleen that received SGE compared to that in the control group (Figure 4.4). In addition, a dose response was observed. Mice who received three times higher doses of SGE showed a 10-fold significant increase in viral load in both tissues compared to those infected with equivalent to one pair of glands (Figure 4.4). Together these data illustrated that SGE has a strong, dose-dependent impact on TOSV replication enhancement.

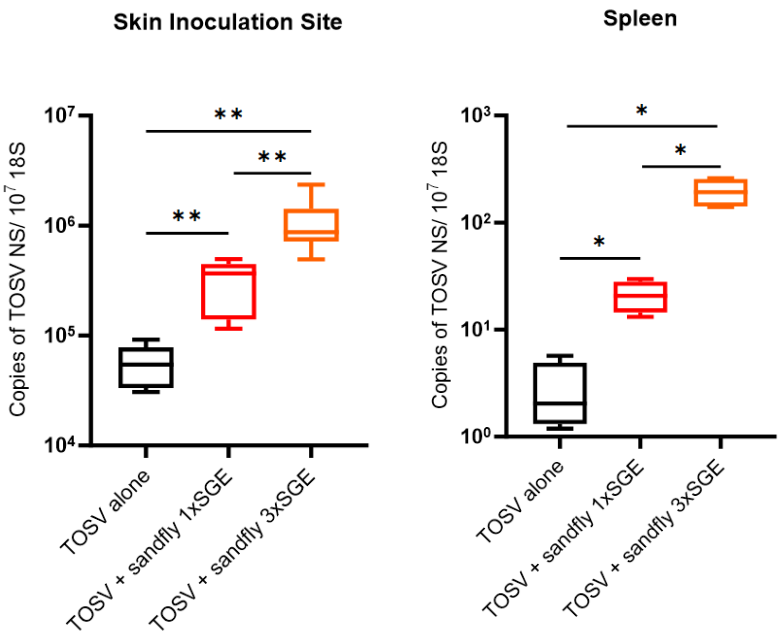


Figure 4.4: Sandfly SGE Enhances TOSV Infection in a Dose-Dependent Manner

Ifnar1-/- mice were inoculated subcutaneously in mice skin on the upper side of the left foot with either 100,000 PFU of TOSV alone or mixed with two different sandfly SGE doses (1-3 pair glands/µl). Skin and spleen samples were taken at 72hpi and TOSV RNA (Ns gene) copy number determined by qPCR (n=6). Blood was also collected to assess quantity of infectious units by plaque assay, but no plaques were detected. Plots show the median value ± interquartile range. Significant *P < 0.05, **p < 0.01.

4.2.2 Pathogenesis of TOSV Infection in *Ifnar1*-/- Mice

TOSV is responsible for causing CNS disease including meningitis and encephalitis in humans. TOSV infection is also associated with notable muscle pain (myalgia), along with fever and headache. Additionally, asymptomatic infections and those without CNS involvement have been reported and discussed in Section 1.2.5.2.2.4, in Chapter 1.

The pathogenesis of TOSV has previously been partially investigated using an immunocompetent, four-week-old murine model infected intracerebrally (i.c.) (unknown volume), and via subcutaneously (s.c.) with high volume (200µl of TOSV per injection) (Grazia Cusi et al., 2005; Cusi et al., 2016). In the intracerebral model, the mice fell ill, and all died within 10 days, whereas those infected through the subcutaneous route showed no clinical or pathological symptoms.

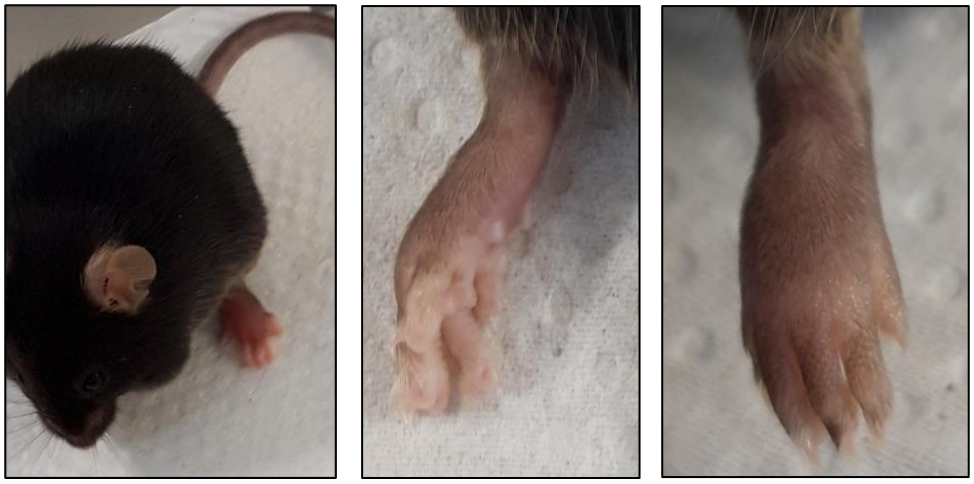
4.2.2.1 TOSV Infection Causes Pathology and Clinical Signs in *Ifnar1*-/- Mice, Which Is Enhanced by Co-inoculation With Sandfly SGE

Adult *ifnar1-/-* mice were injected with TOSV into the skin as described above, with or without the presence of *Ph. pernicious* sandfly SGE. Total inoculum volume was ~1-2µl. Two separate pathogenesis experiments were conducted, in which mice were

monitored for either two weeks or three weeks post-infection. Mice were monitored for development of clinical signs and weighed daily until two weeks after infection. After two weeks they were checked three times a day during the third week after infection, as some mice at this stage developed neurological signs.

In the first experiment, at 7-8 days post-infection, mice co-inoculated with TOSV and sandfly SGE (n=10) all developed inflamed and swollen hind foot joints, whereas only two (2/10) in the TOSV-alone group exhibited the same signs (Figure 4.5). After observing foot joint inflammation, one mouse belonging to the sandfly SGE group, became partially paralysed and was culled at 9-day post-infection. Importantly, the joint swelling lasted at least 4 days for mice that received SGE with TOSV. In contrast, for those few mice that received TOSV alone and exhibited joint swelling; this quickly resolved and was inapparent just one day later after initial observation. Interestingly, at 12-13 days post-infection, a small number of mice that had received SGE with TOSV, and a fewer number of mice that had received TOSV alone, established atypical neurological signs (e.g. hyperactivity). Together, the number of clinical signs suggested a worse infection outcome if SGE was present in the inoculum (Figure 4.6A).

A



B

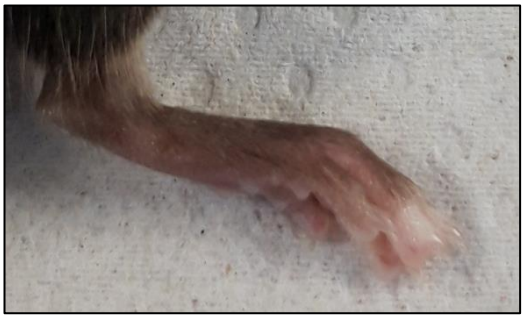


Figure 4.5: *Ifnar1-/-* Mice Had Inflamed Hind Left Foot at 7 dpi

(A) Either TOSV-only or with sandfly SGE inoculated representative mice samples demonstrating significant joint swelling of the inoculated feet, **(B)** an example of uninfected control mice with non-inflamed foot joint

When we looked at the quantity of TOSV NS copies in the brain, skin inoculation site and spleen samples at the end of two weeks post-infection, viral load was significantly higher in the SGE group than the virus-alone group in brain samples (Figure 4.6B). Furthermore, there was a statistically significant increase in virus quantities in mice that had received SGE with TOSV in skin (inoculation site) and spleen (Figure 4.6B). Therefore, TOSV disseminated to the brain from the skin inoculation route, and that this was more efficient in mice receiving sandfly SGE. It should also be noted that quantities of virus RNA in skin are much lower than those seen above at 24 and 72hpi, suggesting the virus was cleared from peripheral tissues at this two-week timepoint. Furthermore, there was a trend for less weight gain in infected mice that also received SGE (Figure 4.6C).

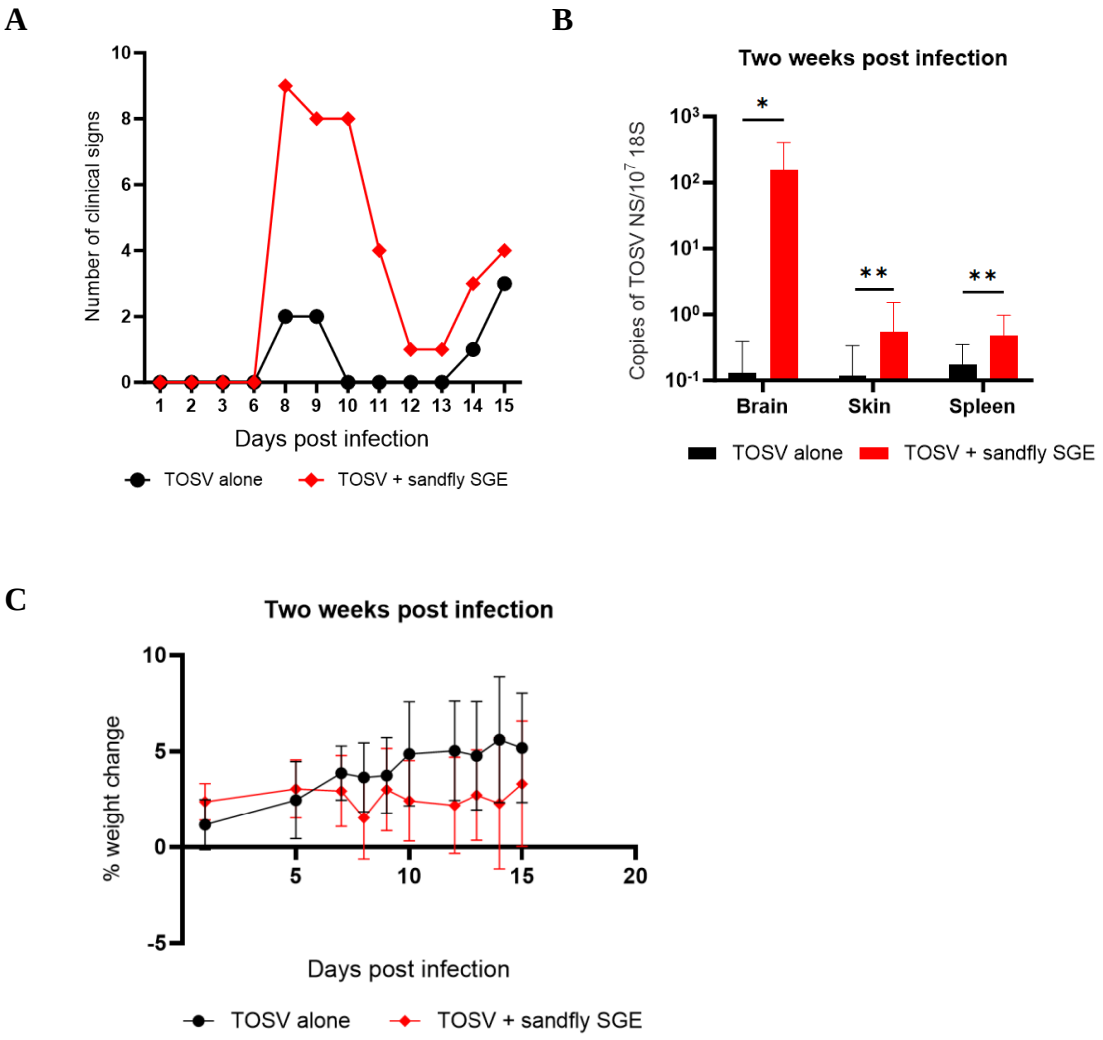


Figure 4.6: Sandfly Salivary Gland Extract Causes More Severe Clinical Outcomes of TOSV Infection in a Mammalian Host

Mice were inoculated as before with either 100,000 PFU of TOSV alone or mixed with sandfly salivary gland extract (SGE) (1 pair gland extract/µl). Half brain, skin and spleen samples were taken from infected *ifnar1-/-* mice at 15dpi and TOSV RNA (Ns gene) copy number determined by qPCR (n=10 mice).

(A) The graph demonstrates the number of clinical signs during TOSV alone and TOSV + sandfly SGE infection (n=10 mice). The two main clinical signs observed were joint swelling, which peaked at day 8, while neurological signs were evident from day 14 onwards.

(B) The quantity of TOSV RNA in brain, skin, and spleen samples at 15dpi. Plots show the median value ± interquartile range. Significant *P < 0.05, **p < 0.01.

(C) The graph shows the weight change kinetics of TOSV-infected *ifnar1-/-* mice in the presence or absence of sandfly SGE for two weeks of infection (n=10 mice).

Cytokines, chemokines, and interferon-stimulated genes are either expressed at low levels or completely absent in the healthy CNS. In symptomatic SFV and WNV infections, inflammatory cytokine, interferon response gene and importantly several chemokines including *ccl2*, *cxcl2*, *cxcl10* are upregulated in the brain (Michlmayr et al., 2014). Thus, in our mouse model for TOSV infection, we aimed to also assess gene expression in the brains of mice two weeks post infection and examine the effect of

sandfly SGE on their transcription. We observed an upward trend in gene expression levels in both the TOSV alone and SGE groups compared to the resting brain (Figure 4.7). Notably, *ifn-β1* and some ISG expressions were higher in the sandfly SGE group, compared to mice that received virus alone. The level of the inflammatory cytokine *il-6* was also low in brain samples from both groups during TOSV infection. Notably, *cxcl10* (inducible by IFN) was the most upregulated chemokine, with significantly higher levels of *cxcl10* and *ccl2*, and an increased level of *cxcl2* in the SGE group compared to the virus alone group. Overall, sandfly SGE could contribute to a more severe outcome of TOSV infection by expressing these critical chemokines in CNS tissue. The chemokine-mediated influx of leukocytes into the brain is an essential key factor in CNS disease caused by neurotropic arboviruses.

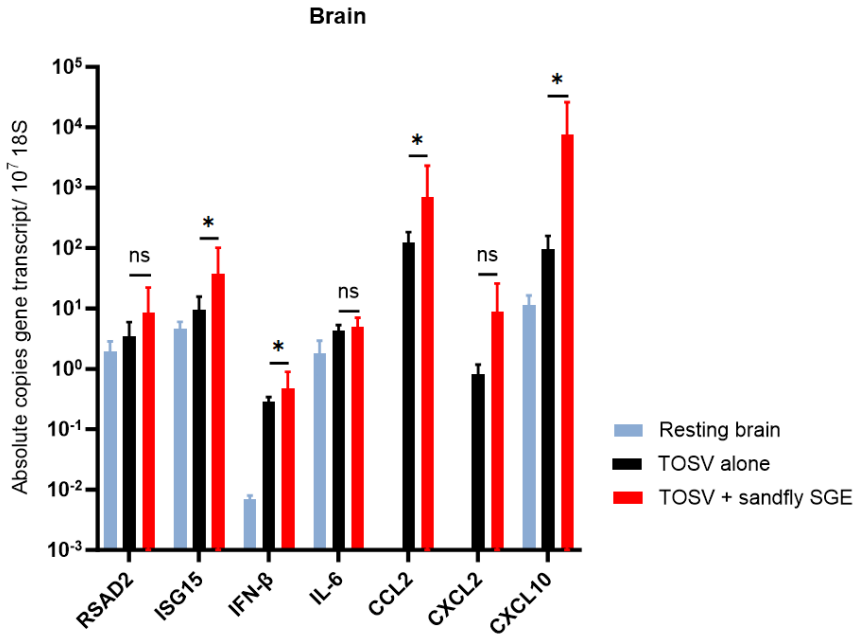


Figure 4.7: The Cytokine, Chemokine and Interferon Stimulated Genes Response to TOSV Infection in The Presence/Absence of Sandfly SGE in The Brains of Mice

The expression levels of Chemokines, *rsad2*, *isg15*, *il-6* and *ifn-β* either TOSV alone or with sandfly SGE or resting brain. The half brain samples taken from *ifnar-/-* mice at 15dpi. Resting half brain samples were taken from uninfected *ifnar1-/-* mice. Gene expression was measured in these samples by qPCR (n=10 mice). Plots show the median value ± interquartile range. ns=not significant, significant, *P < 0.05.

Because we noted an uptick in the number of clinical signs towards the end of the second week (Figure 4.6A), we next asked whether these signs continue into the third week of infection and sought to further validate the findings observed in the first two weeks of infection. Here in this separate experiment, mice co-infected with SGE had a

trend toward less weight gain (Figure 4.8B), which was consistent with their display of other clinical signs of disease. As a side, only one mouse, (belonging to TOSV alone group), experienced a 21% reduction in total weight and was culled before the experiment concluded. Moreover, the same mouse suffered joint inflammation and then left hindlimb paralysis at 10 days post-infection, which resolved one day later.

Importantly, approximately 5-6 days post-infection, all mice (n=8) in the sandfly SGE group developed inflamed and swollen foot joints. In contrast, fewer mice in the TOSV alone group showed symptoms like those in the SGE group. Although some mice showed atypical neurological signs such as hyperactivity and repetitive paw movements from 15dpi onwards, these symptoms were self-resolving a couple of days later. In summary, when sandfly SGE was mixed with TOSV inoculum, a higher number of clinical signs were observed, compared to mice in the virus-alone group (Figure 4.8A).

At the end of this experiment, at 21dpi, tissues were assessed for TOSV RNA quantity by qPCR. The quantity of TOSV RNA in the brain and spleen was significantly higher in the sandfly SGE group compared to the TOSV-alone group (Figure 4.8C). However, the viral load in the brain was tenfold lower at 21dpi in the SGE group compared to 15dpi in the same group (Figure 4.6C), which could be explained by the self-resolving neurological symptoms observed at 21dpi. Conversely, the viral RNA in the spleen showed a significant increase compared to the first experiment's results, indicating a potential systemic infection or inability to efficiently clear virus RNA from this tissue.

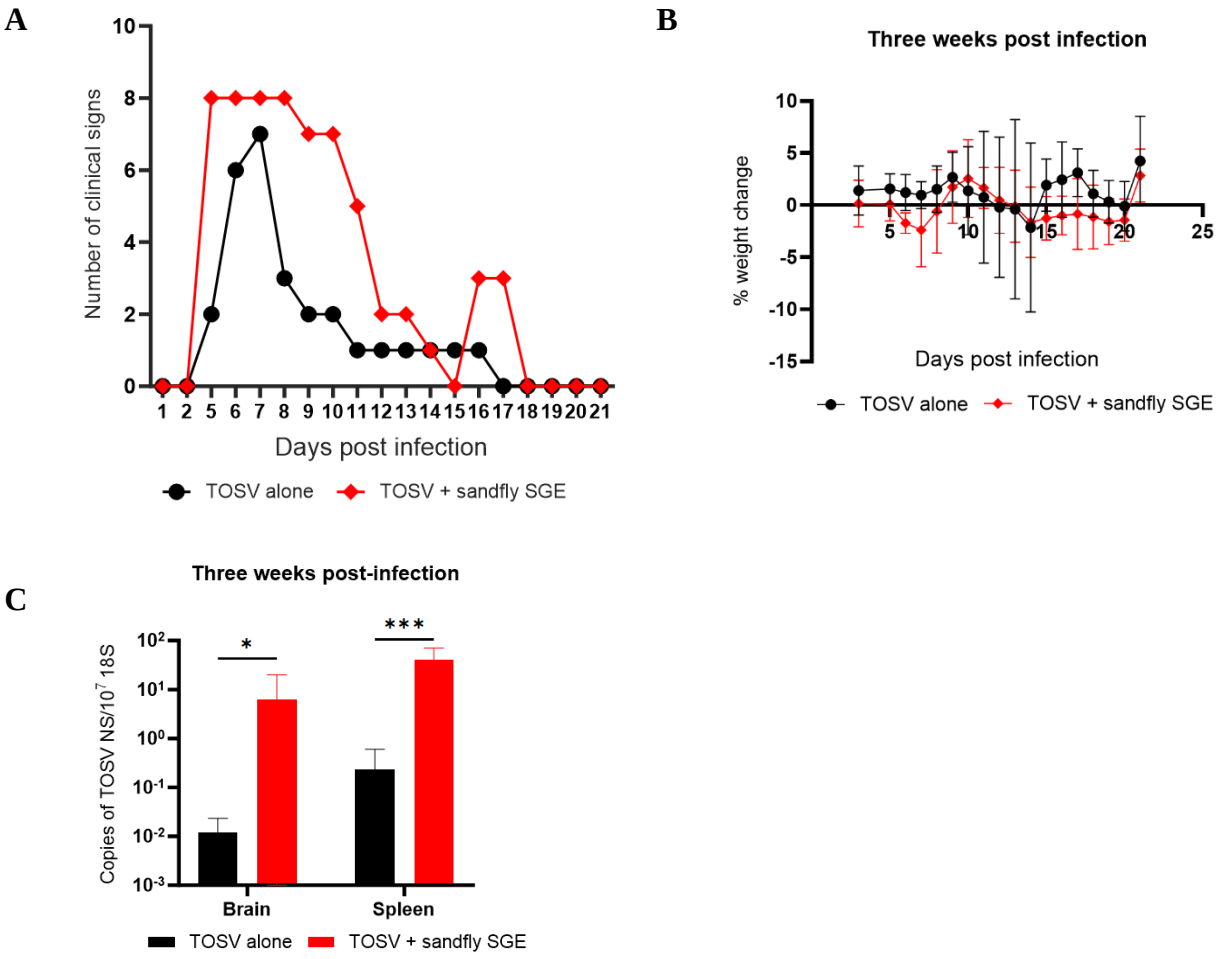


Figure 4.8: The Number of Clinical Symptoms and The Quantity of TOSV RNA in Brain and Spleen Samples at The Three Weeks Post-Infection

Mice were inoculated as before with either 100,000 PFU of TOSV alone or mixed with sandfly salivary gland extract (SGE) (1 pair gland extract/µl). Half brain and spleen samples were taken from infected *ifnar1-/-* mice at 21dpi and TOSV RNA (Ns gene) copy number determined by qPCR (n=8).
(A) The graph demonstrates the number of clinical signs (n=8 mice).
(B) The graph shows the weight change kinetics of TOSV-infected *ifnar1-/-* mice in the presence or absence of sandfly SGE for three weeks of infection (n=8 mice).
(C) The quantity of TOSV RNA in brain and spleen samples at infection of TOSV alone and TOSV + sandfly SGE at 21dpi. Plot shows the median value ± interquartile range. Significant *P < 0.05, ***p<0.001.

We also assessed the chemokine expression, as in the first experiment. The findings indicated a significant elevation in *cxcl10* expression within the SGE groups of mouse brain samples compared to those infected solely with TOSV (Figure 4.9). However, this increase compared to resting tissue was notably lower, around 20-fold, compared to the results from the first experiment. Furthermore, no significant variance was observed in the levels of *ccl2* and *cxcl2* among the different groups in the brain.

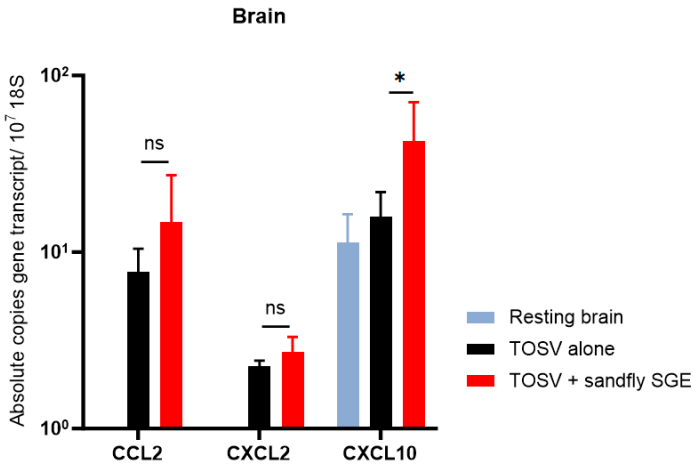


Figure 4.9: The Chemokine Response to TOSV Infection in the Presence/Absence of Sandfly SGE in the Brains of Mice

The expression levels of Chemokines (*ccl2*, *cxcl2*, *cxcl10*) either TOSV alone or with sandfly SGE or resting brain. The half brain samples taken from *ifnar1-/-* mice at 21dpi. Resting half brain samples were taken uninfected *ifnar1-/-* mice. Gene expression was measured in these samples by qPCR (n=8 mice). Plot shows the median value ± interquartile range. ns=not significant, significant, *P < 0.05.

4.2.2.2 TOSV Infection Can Result in Acute Foot Joint and Muscle Inflammation, Which Is Worsened by Inclusion of Sandfly SGE in Inoculum

TOSV infection manifests as a mild feverish condition, headaches, muscle pain, and nausea in humans. More severe infections can cause neurological signs, including meningitis and encephalitis (Papa, Mallias, et al., 2014). In addition, some case reports also indicated arthralgia in the patients (Papa, Kesisidou, et al., 2015), although this is not as common as following infection with some other bona fide arthritogenic arboviruses, including Ross River virus (RRV) and Chikungunya virus (CHIKV). There are several mouse models for investigating severe incapacitating arthralgia that occurs with these infections (Morrison et al., 2006; Goupil et al., 2016; Mostafavi et al., 2022). In CHIKV models, mice infected with the virus showed moderate to significant swelling in the feet where they were inoculated. Histopathologic evaluation revealed moderate mononuclear inflammation in the deep layers of the skin and subcutaneous tissue, extensive necrosis and inflammation of muscle cells, as well as tendonitis and synovitis (Goupil et al., 2016).

We found that TOSV infection of *ifnar1-/-* mice invariably resulted in swollen and inflamed hind feet in mice receiving both TOSV and SGE, and only occasionally in mice receiving TOSV alone. Importantly, this clinical sign was more severe and lasted longer in mice receiving sandfly SGE than those receiving the virus alone (Figure 4.10).

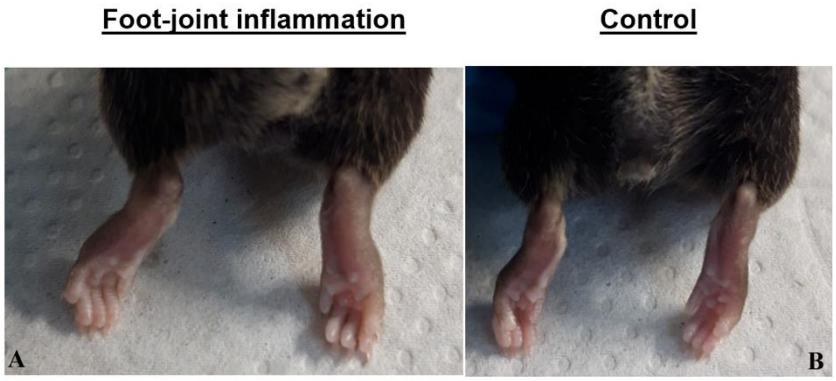


Figure 4.10: *Ifnar1-/-* mice sacrificed at 7dpi

TOSV + sandfly SGE inoculated mouse demonstrating significant swelling of the feet **(A)**, TOSV alone infected mouse illustrating no swelling **(B)**.

Therefore, we wanted to define the pathology of TOSV infection in mice. Firstly, it was not clear whether virus had disseminated to the joint tissue itself, therefore we assessed whether TOSV could be detected in joints at 7dpi, when swelling was most extensive, by qPCR. The number of copies of TOSV NS in the skin and foot joint samples was approximately ten times greater significantly in the sandfly SGE group compared to the virus alone group (Figure 4.11A, B). The results suggested that the inflammation of the foot joint may be associated with the enhanced quantity of virus in joint.

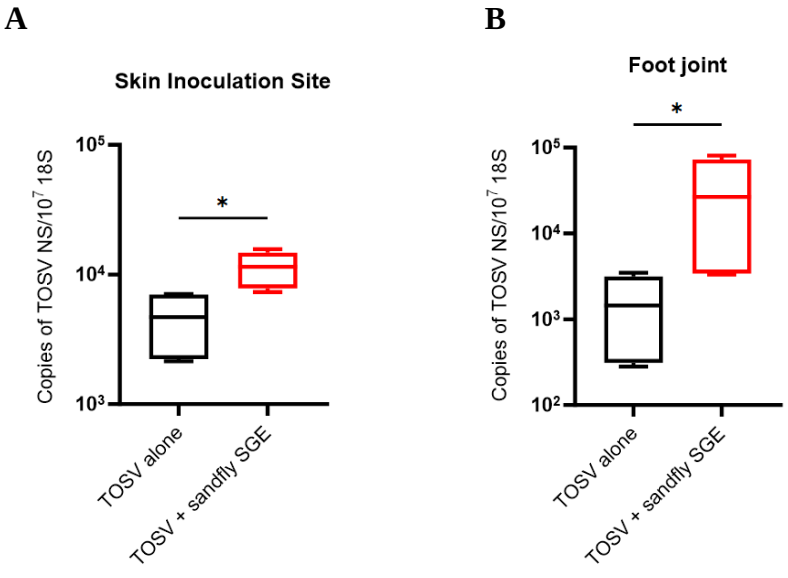


Figure 4.11: Sandfly SGE Enhanced TOSV Replication in Skin and Foot Joint of Mice at 7dpi

Mice were inoculated subcutaneously in the upper foot skin with either 100,000 PFU of TOSV alone or mixed sandfly SGE (1 pair gland extract/µl). Skin and foot joint samples were collected from infected *ifnar1-/-* mice at 7 days of post-infection (dpi) and TOSV RNA (Ns gene) copy number determined by qPCR (n=5 mice). Plots show the median value ± interquartile range. Significant *P < 0.05, for skin **(A)** and foot joint **(B)**.

To characterise whether pro-inflammatory meditators, associated with other inflammatory joint diseases, were expressed in TOSV infected joints, we assessed the expression of chemokines/cytokines. Specific cytokines are linked to pro-joint inflammation in diseases including rheumatoid arthritis, the blockade of which is used in the clinic to treat joint inflammation. This includes *tnf-α* and *il-6* cytokines, whose joint expression here were significantly increased for mice receiving sandfly SGE with TOSV (Figure 4.12A). *Cxcl10* is a potent chemoattractant for immune cells, particularly CD8 T cells and NK cells, both of which can contribute towards tissue damage (Liu et al., 2011), was also upregulated in the foot joints of mice that received SGE compared to the virus alone group (Figure 4.12A). *Ccl2* and *cxcl2*, which recruit inflammatory monocytes and neutrophil respectively to the affected area, were significantly elevated in mice that received sandfly SGE (Figure 4.12A, B). As such, the underlying cause of this pathological joint swelling could be due to direct damage from the virus infection, virus replication in the joints, and/or the result of immune response induced by TOSV.

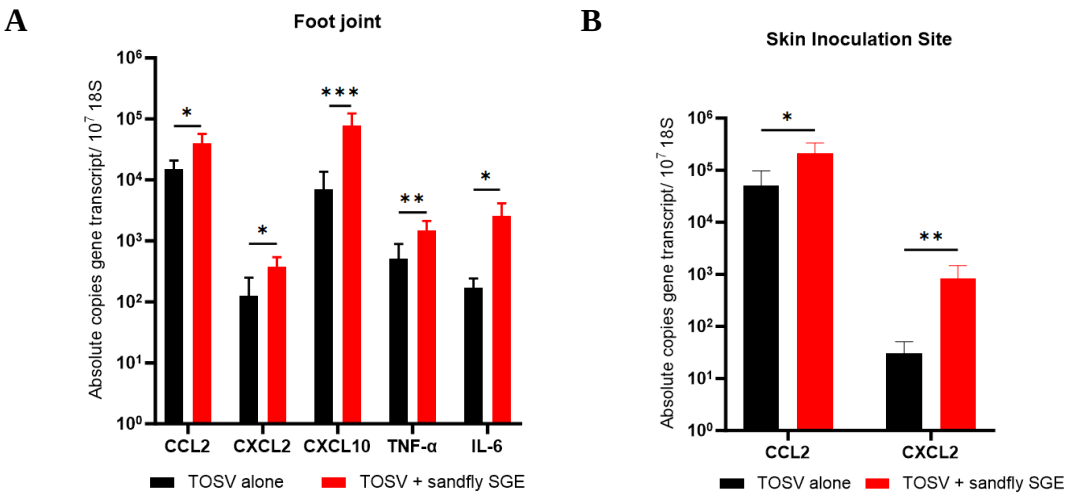


Figure 4.12: Sandfly SGE Induce Inflammatory, Pro-inflammatory Cytokines, Chemokines likely Associated with Foot Joint Inflammation

The expression levels of Chemokines and Cytokines either TOSV alone or with sandfly SGE. The foot joint **(A)** and skin samples **(B)** were taken from *ifnar1-/-* mice at 7dpi. Gene expression was measured in these samples by qPCR (n=5 mice). Plots show the median value ± interquartile range. Significant, *P < 0.05, **p < 0.01, ***p<0.001.

To help characterise TOSV-joint inflammation, we conducted a histopathologic evaluation of TOSV infected mice feet. We collected skin and foot samples, including joint tissues, from three groups: resting, infected with TOSV, and co-infected with sandfly SGE (1 pair gland extract/µl). After fixing the skin samples with 4% PFA, we processed the foot samples by decalcification, as previously described in Section 2.11 of Chapter 2. All samples were then sent for H&E staining.

For the skin that was adjacent to the inflamed joints, we observed significant infiltration of inflammatory cells in the dermis of inflamed skin co-infected with TOSV and sandfly SGE, as indicated by the presence of numerous monocytic-like and polymononuclear cells (Figure 4.13C, F). This contrasted with skin of mice that received either no inoculum (resting) (Figure 4.13A, D) or TOSV-alone (Figure 4.13B, E).

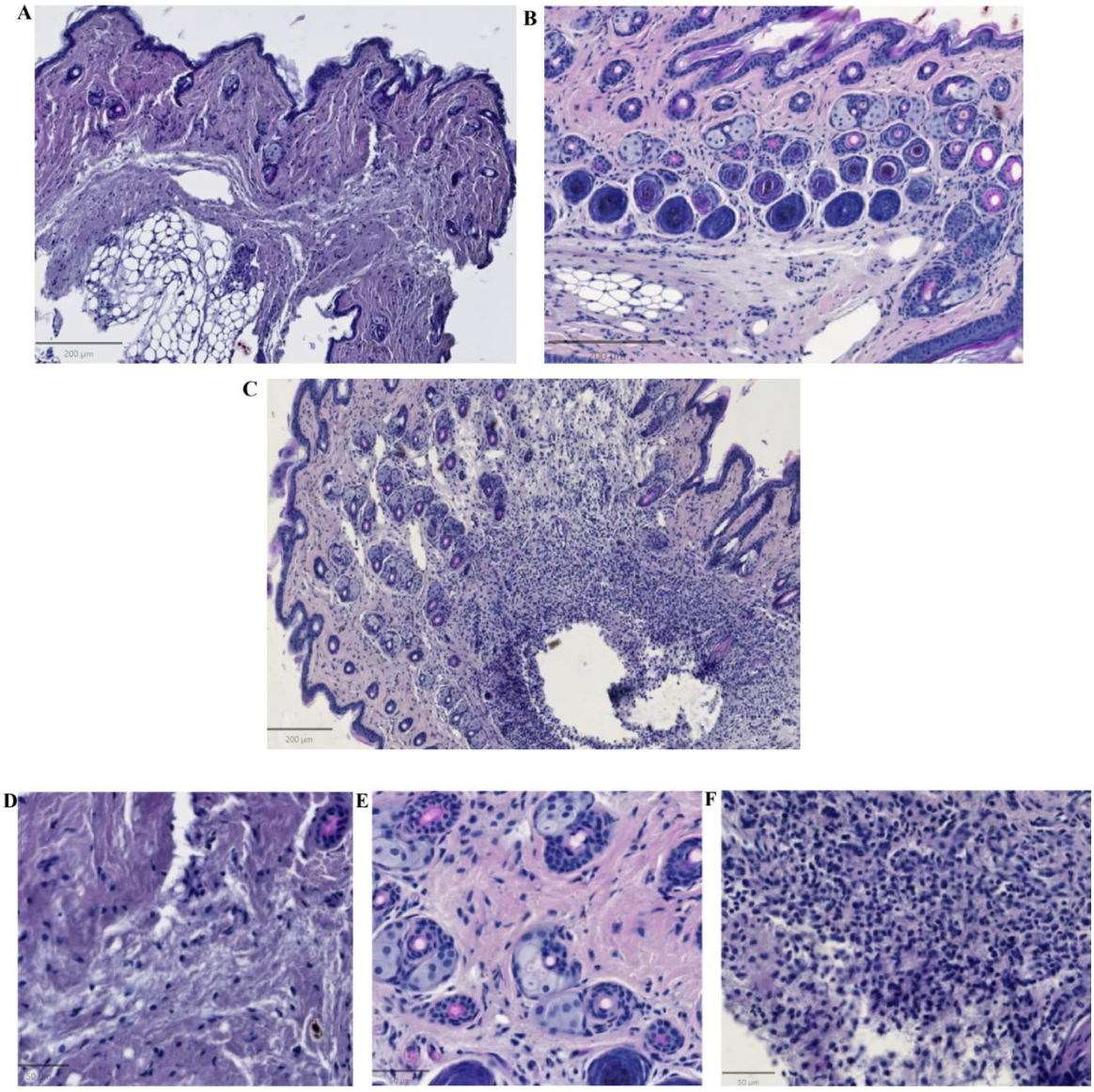


Figure 4.13: Haematoxylin and Eosin (H&E) Staining of the Mouse Foot Skin

(A) Representative Haematoxylin and Eosin (H&E) staining of resting foot skin, **(B)** TOSV-alone infected *ifnar1-/-* mice at 7dpi, **(C)** TOSV with sandfly SGE infected *ifnar1-/-* mice at 7dpi. Note that the lower dermis is enlarged with numerous inflammatory cells present, such that the epidermis is only partially visible. Images (magnification, x2) are representative of two mice per group. **(D)** H&E staining of resting foot skin, **(E)** TOSV-alone infected *ifnar1-/-* mice at 7dpi, **(F)** TOSV with sandfly SGE infected *ifnar1-/-* mice at 7dpi. Images (magnification, x6) are representative of two mice per group.

Next, we investigated the histopathology of joint skeletal muscle associated with the foot joint. In those mice with inflamed foot joints, TOSV caused gaps to appear in the muscle tissue, indicating myofiber degeneration and/or oedema (Figure 4.14B). Additionally, the presence of sandfly SGE in the original TOSV inoculum led to a more substantial accumulation of inflammatory cells in the subcutaneous tissue, extending into the muscular layer (Figure 4.14C), than observed with TOSV alone or in resting foot joint samples (Figure 4.14B, A). In summary, TOSV causes an acute inflammatory response in the foot proximal to the inoculation site. The presence of sandfly saliva increases the efficiency of virus dissemination to foot tissue, resulting in more exaggerated inflammatory response, increased leukocyte influx (Figure 4.15) and muscle fibre damage.

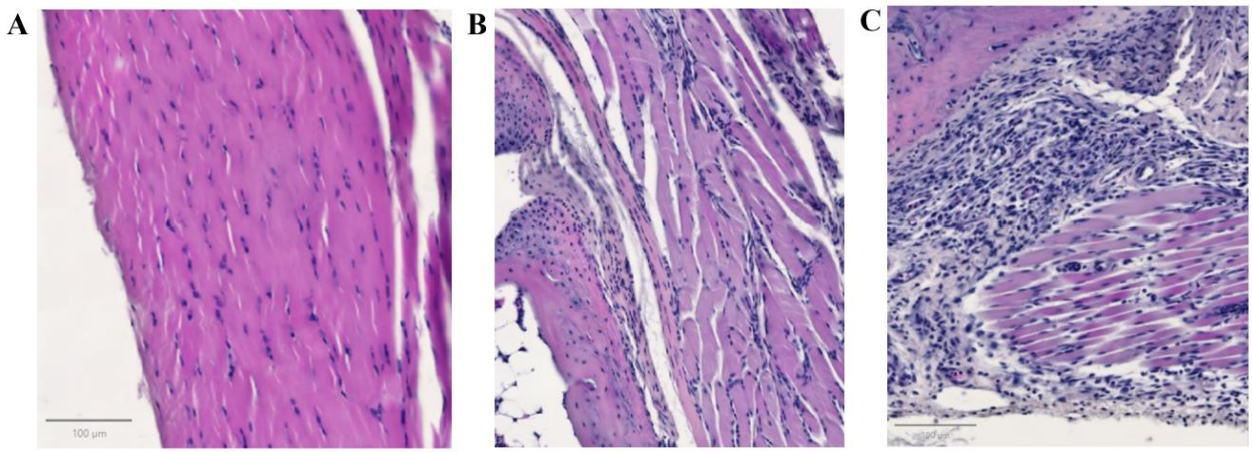


Figure 4.14: Haematoxylin and Eosin (H&E) Staining of the Mouse Foot
(A) Representative Haematoxylin and Eosin (H&E) staining of longitudinal section of skeletal muscle (uninfected), **(B)** representative skeletal muscle from TOSV-alone infected mice at 7dpi, **(C)** representative skeletal muscle from mice infected with TOSV + sandfly SGE at 7dpi. Images (magnification, x4) are representative of two mice per group.

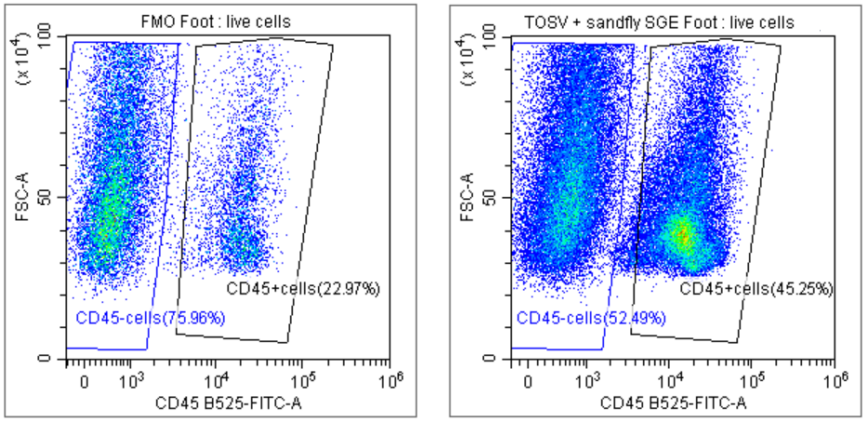


Figure 4.15: Sandfly Salivary Gland Extract Causes the Influx of Leukocytes into Foot Tissue

Ifnar1-/- mice were left uninfected or infected with 100,000 PFU of TOSV, the inoculum of which includes sandfly SGE. Foot joint tissues were digested at 7dpi after observing foot joint inflammation and stained with CD45. The gating strategy defines the population of stromal cells (CD45-) and leukocyte cells (CD45+) in uninfected Fluorescence Minus One (FMO) control and inflamed foot joint tissue, respectively.

4.3 TOSV INDUCES NEUTRALISING ANTIBODY RESPONSES IN *IFNAR1-/-* MICE

After identifying an increase in TOSV RNA in spleen and brain samples at 21dpi, we conducted a serum neutralization assay to assess potential serum protection against TOSV and whether exposure to SGE in the mice influenced the generation of neutralizing antibodies in the serum. Three weeks after infection, blood samples were taken from mice either infected with TOSV or TOSV with sandfly SGE, and an assay was performed and analysed as described in Section 2.6 of Chapter 2. We found that the serum from mice infected with TOSV combined with sandfly SGE provided significantly higher protection than the TOSV-only group at one specific dilution (Figure 4.16A). However, this enhanced protection was not consistent across other serum dilutions (Figure 4.16B). Overall, the serum from mice infected with TOSV alone or with SGE showed similar levels of protection. This shows that mice seroconvert and replicate this core aspect of TOSV infection in humans. Of note, despite the host generating a similar protective virus-neutralising response in the serum, the addition of sandfly SGE still caused worse disease (Figure 4.8).

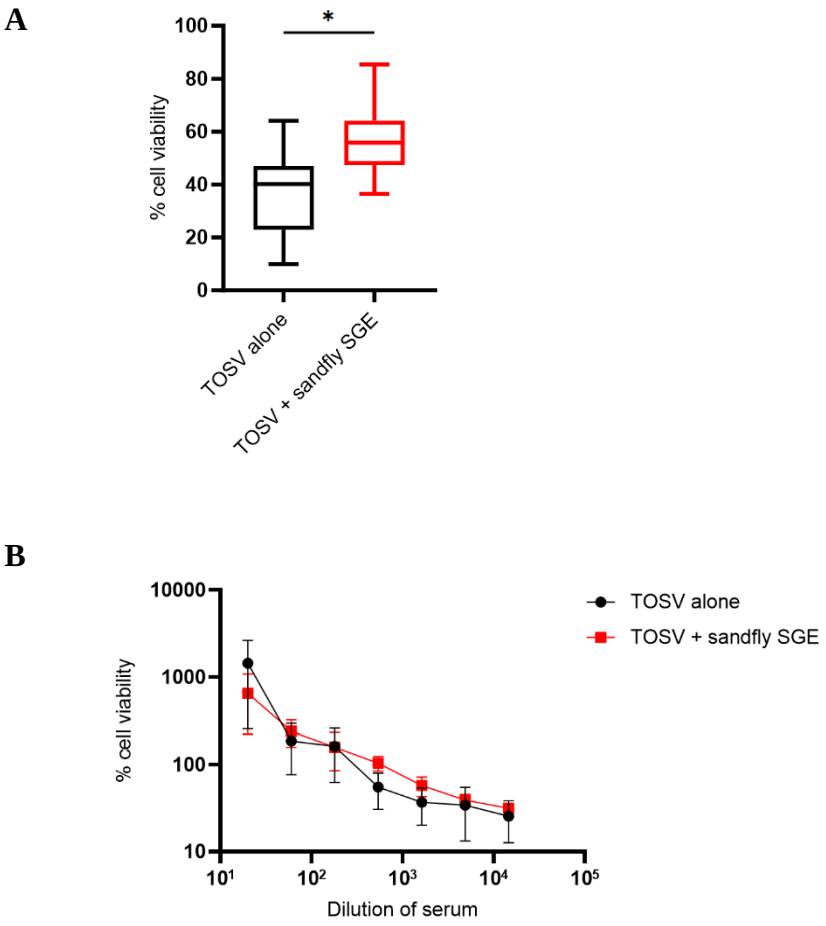


Figure 4.16: The Generation of Neutralising Antibodies to TOSV in Serum
1000 PFU of TOSV was pre-treated with serum collected from *ifnar1-/-* mice infected with TOSV-only or with sandfly SGE at 3 weeks post-infection at dilutions ranging from 1:20 to 1:14580. One hour later, the cells were infected with these mixtures of virus/serum and observed over the next few days. After observing cytopathic effects (CPE), the cells were stained with crystal violet to identify live cells, indicating the percentage of cells protected by the serum. The plates were then scanned and analysed using ImageJ to calculate the percentage of viable cells, serving as a measure of the serum's protective capacity. All cell viability measurements were normalised against background readings from uninfected cells. Plots show the median value ± interquartile range. Significant, *P < 0.05.
(A) The graph shows percentage of viable cells when treated with serum diluted 1:1620.
(B) The graph illustrates percentage of cell viability in a range of dilutions between 1:20 and 1:14580 according to TOSV alone and TOSV + sandfly SGE groups.

4.4 THE SALIVARY GLAND EXTRACT FROM THE *LUTZOMYIA LONGIPALPIS* SANDFLY SPECIES CAN ALSO ENHANCE TOSV INFECTION

To complement these studies using SGE, we wanted to also assess TOSV infection following inoculation at sandfly bites. For this, we decided to conduct *in vivo* bite studies using a colony of the *Lutzomyia longipalpis* species. The colonies and sandfly

salivary gland extract (SGE) were kindly supplied by Dr. Matthew Rogers at The London School of Hygiene & Tropical Medicine. The rationale for this was because *Lu. longipalpis* colonies, which are often raised in laboratories to bite mice, and are well-adapted for this purpose. Unfortunately, *Ph. perniciosus* sandflies do not feed efficiently on rodents in laboratory conditions (although will bite rabbits and humans). Indeed, such colonies can require several generations to fully adapt to a different blood-feeding source (Volf and Volfova, 2011).

Several studies demonstrated that, *Lu. longipalpis* bite/SGE augments the severity of *Leishmania* infection in murine models (Theodos et al., 1991; Lestinova et al., 2017; Teixeira et al., 2018). In addition, a putative, non-insect specific, new *Phlebovirus* (Viola Phlebovirus) has been isolated from *Lu. longipalpis* recently (De Carvalho et al., 2018). This suggested that this species can also be infected with viruses, and likely can transmit them to vertebrate hosts. Although *Lu. longipalpis* is not a natural vector of TOSV, mainly due to their geographic separation, with TOSV residing in the Old world, and *Lu. Longipalpis* specific to the Americas, this sandfly species readily adapts to new habitats. For example, its adaptation to the urban environment is intimately linked to the emergence of visceral leishmaniasis in formerly unaffected locations (Rego and Soares, 2021). Therefore, with increasingly globalisation and climate change, there is a possibility that viruses such as TOSV, and this sandfly species, may co-exist in the same environment in the future due to climate change.

Lastly, it has been shown that the saliva/bite of *Lu. longipalpis* sandflies can induce a potent inflammatory response, including attracting macrophages to the bite site and modulating their functions (Teixeira et al., 2005; Peters et al., 2008; Teixeira et al., 2018). Thus, it serves as a useful tool for investigating our hypothesis that inflammatory responses to sandfly salivary factors enhance infection with TOSV, supported by more frequent infection of inflammatory cells recruited to the skin and worsening clinical outcomes of the virus.

Therefore, we began our studies by initially examining whether SGE from *Lu. longipalpis* was capable of enhancing TOSV infection in our mouse model. We found that the quantity of viral RNA was significantly higher in the SGE group than the virus alone group in the skin inoculation site and spleen at 72hpi (Figure 4.17A, B).

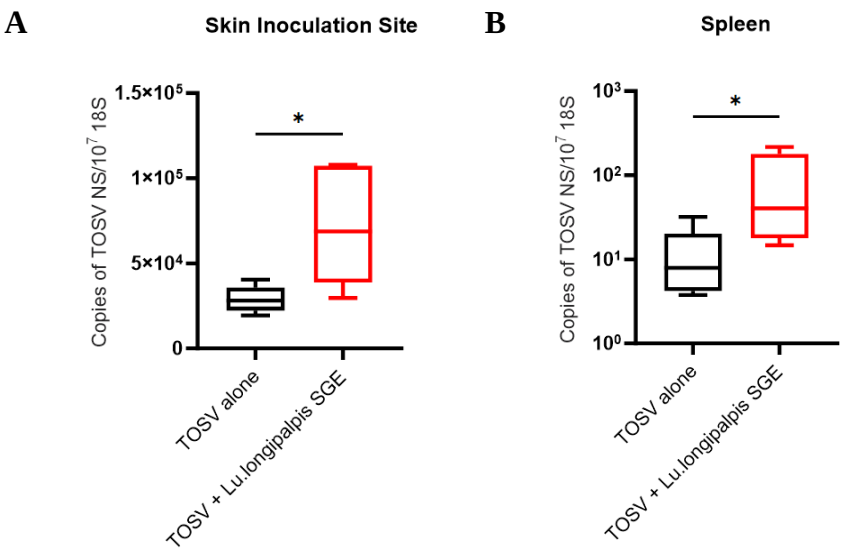


Figure 4.17: *Lutzomyia Longipalpis* Sandfly Salivary Gland Extract Augment TOSV Infection in a Mammalian Host

Mice were inoculated subcutaneously in the left upper foot skin with either 100,000 PFU of TOSV alone or mixed *Lutzomyia Longipalpis* sandfly salivary gland extract (SGE) (1 pair gland extract/µl). **(A)** Skin and **(B)** spleen samples were taken from infected *ifnar1-/-* mice at 72hpi and TOSV RNA (Ns gene) copy number determined by qPCR (n=6 mice). Blood was also collected to assess quantity of infectious units by plaque assay, although no plaques were found. Plots show the median value ± interquartile range. Significant *P < 0.05.

4.5 SANDFLY BITE ENHANCES TOSV INFECTION IN A MAMMALIAN HOST

We have already shown that sandfly SGE enhances susceptibility to TOSV infection. However, saliva/SGE injection via needle missed some aspects of natural biting. Therefore, we next determined whether the presence of a sandfly bite at the inoculation site modulated host susceptibility to TOSV (Figure 4.18) as described in Section 2.4.1.2 of Chapter 2.

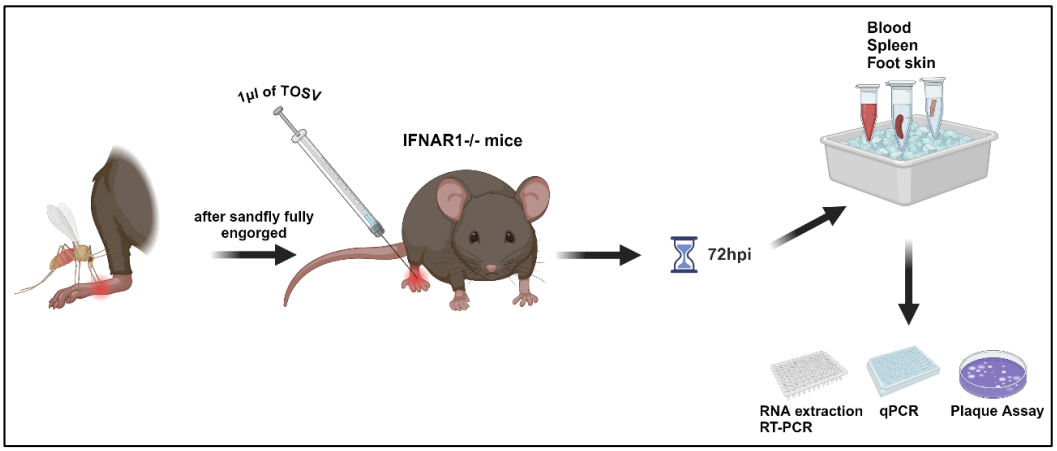


Figure 4.18: *In Vivo* Model for TOSV Infection with Sandfly Bite

A drawing to summarise the infection process of *ifnar1* null mice with TOSV, with or without prior exposure to sandfly bites.

Here, we investigated whether sandfly bites could enhance TOSV infection with the same efficacy as sandfly SGE. Mice were bitten with sandflies (*Lutzomyia longipalpis*) and then immediately 1µl of TOSV inoculated into the same site (upper side of the left foot). Interestingly, those mice receiving sandfly bites not only induced enhanced infection compared to TOSV alone inoculated mice, but also exhibited an additional enhancement effect compared to mice that received SGE with virus, as assessed quantifying virus RNA in skin and spleen samples at 72hpi (Figure 4.19A). Here, the quantity of TOSV RNA in the skin and spleen of the bite group was significantly higher than in mice inoculated with TOSV plus SGE or the TOSV-alone. Crucially, we also detected infectious virus at significantly higher quantities in the mice receiving sandfly bites as determined by plaque analysis (Figure 4.19B). Indeed, all mice became viremic if inoculated at sandfly bites, as compared to mice inoculated in resting skin.

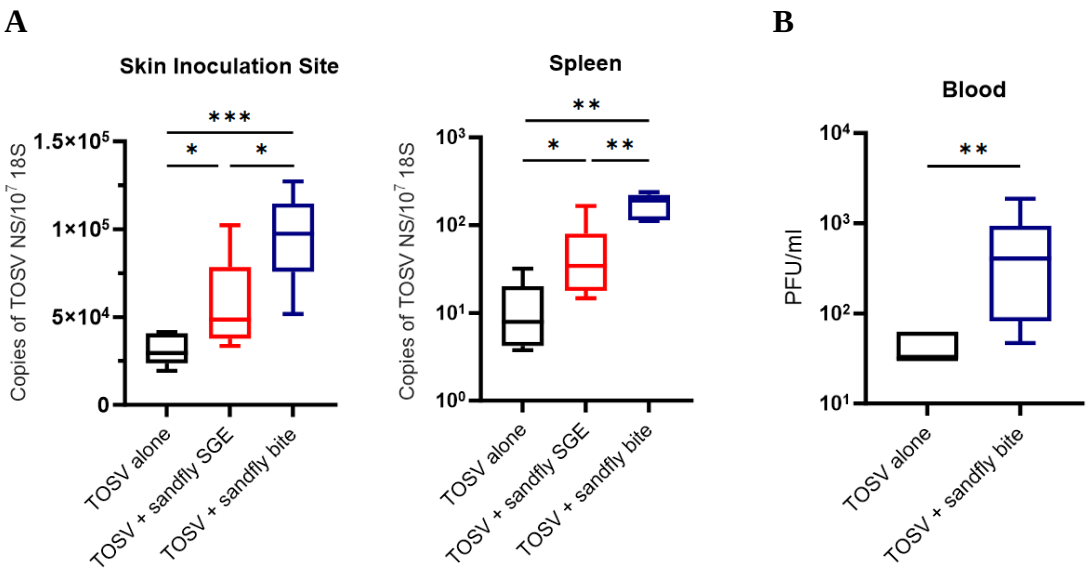


Figure 4.19: Sandfly Bite Enhance Severity of TOSV Infection in a Mammalian Host

Mice were exposed to the bites of two to four *Lutzomyia longipalpis* species sandflies, on the upper side of the left foot. Sandflies were left to feed until fully engorged. Immediately afterwards, 1µl of virus was injected (100,000 PFU of TOSV) subcutaneously directly at the bite site using a Hamilton needle. **(A)** Skin and spleen samples were taken from infected *ifnar1-/-* mice at 72hpi and TOSV RNA (Ns gene) copy number determined by qPCR (n=6 mice), **(B)** Virus titers in the blood serum at 72hpi were also quantified by plaque assay. Plots show the median value ± interquartile range. Significant *P < 0.05, **p < 0.01.

To help understand the mechanism by which sandfly bites enhance TOSV infection, we next defined the impact of the presence of bites on cutaneous innate immune responses. The expression of key innate immune genes was assayed in the skin of mice that were either bitten by sandflies and then infected with the virus or infected with the virus

without bites. At bite sites, *il-6* and *isg15* levels were significantly elevated compared to the groups exposed to sandfly SGE+virus or to virus alone. *Ccl2*, monocytic chemoattractive chemokine, was also upregulated significantly in the presence of bites. Interestingly, a neutrophil-attracting chemokine, *cxcl2*, did not show significant differences between the experiment groups (Figure 4.20). However, sandfly bites have been shown to cause rapid and prolonged neutrophilic infiltration at the localised bite sites, although this may be independent of CXCL2-CXCR2 axis signalling (Peters et al., 2008; Guimaraes et al., 2017). Together, this suggests that there is increased inflammatory gene expression with TOSV in bitten skin as compared to TOSV infection alone. This may be a consequence of either biting directly, or because there is enhanced quantities of TOSV.

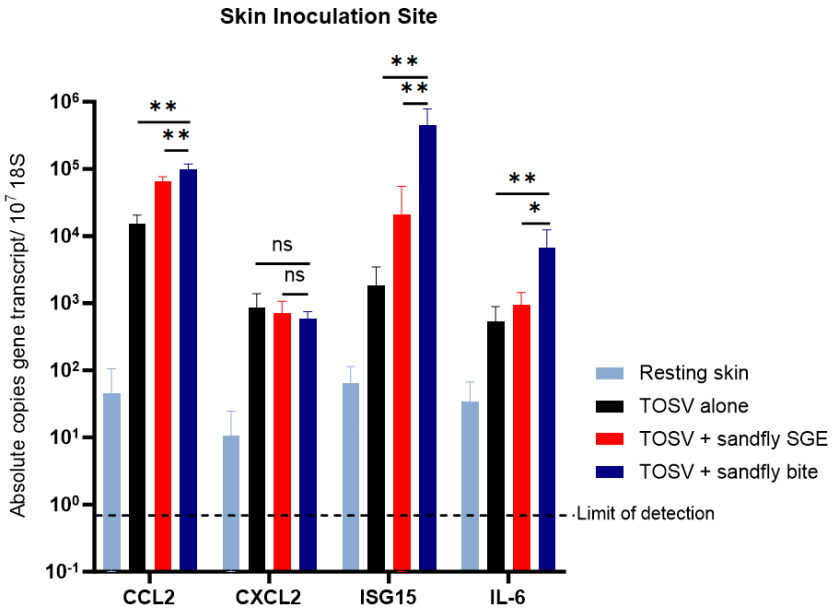


Figure 4.20: Sandfly Bite Causes Upregulation of Key Innate Immune Genes in The Skin

The quantities of immune gene expression either in skin at rest or following TOSV infection alone or in presence of sandfly SGE or with sandfly bites. The skin samples were taken from *ifnar1-/-* mice at 72hpi. Gene expression was measured in these samples by qPCR (n=6 mice). Plots show the median value ± interquartile range. Dotted line represents the assay's limits of detection based on qPCR values. ns=not significant, significant *P < 0.05, **p < 0.01.

4.6 DEFINING THE POTENTIAL FOR MICROBIOTA TO EXPLAIN SALIVA-INDUCED TOSV INFECTION

Although there is no clear information about whether sandfly saliva contains microorganisms alongside pharmacologically active compounds that aid feeding, the gut microbiota of sandflies has been studied several times to investigate the relationship

between microorganisms in the gut and *Leishmania* parasite transmission. *Leishmania* parasites develop solely within the gut lumen of the insect before being transmitted to a suitable host during the insect's subsequent blood meal. Notably, although the midgut microbiome is one of three or four major factors affecting *Leishmania* transmission, there is no specific or stable midgut microbiome. It differs between sandfly species, even colonies; most bacteria in the midgut are associated with contamination from the environment (e.g., plants, animals) (Volf et al., 2002; Sant'Anna et al., 2012; Telleria et al., 2018).

Other factors affecting *Leishmania* transmission are sandfly saliva, promastigote secretory gel (PSG) and other compounds synthesised by *Leishmania* in sandfly midgut. It has been demonstrated that when sandfly gut microbes and saliva are egested into the skin of a host, together they trigger the activation of the host's inflammasome. The inflammasome, a multi-protein signalling platform of the innate immune system comprising various sensors and receptors (Broz and Dixit, 2016; Chen et al., 2017), subsequently promotes the expression of proinflammatory cytokines, such as *il-1β*. This cascade of events increases the recruitment of neutrophils to the bite site, which become infected, and facilitates greater dissemination of the parasite within the host. These findings indicate that the immune response initiated by the microbes unintentionally supports the spread of the parasite (Dey et al., 2018).

Interestingly, the microbiome is regurgitated along with *Leishmania* parasites, as the parasites damage the sandfly's stomodeal valve. Positioned at the junction between the foregut and midgut, the stomodeal valve primarily ensures a 'one-way' flow of blood or sugar meal during feeding and prevents the regurgitation of gut contents (Volf et al., 2004). Thus, in non-*Leishmania* infected flies, such as ours, with properly working stomodaeal valves, the regurgitation of the gut microbiome is unlikely. Although there is a knowledge gap between sandfly salivary gland or saliva and microbiome presence in them, a study of mosquito salivary glands revealed a diverse microbiota in glands, likely injected into the skin during blood feeding (Sharma et al., 2014). Abiotic mosquito saliva is also less inflammatory in skin, than microbiota sufficient mosquito saliva (Lefteri et al., 2022).

In this section, we wanted to assess whether potential microbial components in sandfly saliva effect on saliva-induced TOSV infection. There are no studies in the literature investigating the influence of these components on the host's susceptibility to TOSV.

4.6.1 LPS and PAM3CSK4 Enhance TOSV Infection in Mice

Here, pro-inflammatory bacterial agents and mimics were used to test whether microbiota could play a role in the phenotype of enhancing TOSV infection. These were LPS (lipopolysaccharide) and Pam3CSK4 (a synthetic lipopeptide that activates TLR2), both of which induce cutaneous *cxcl2* and *il-1β* expression in a mouse model (Pingen et al., 2016). Here, 1µl/µg of Pam3CSK4 and 1µl/µg of LPS were injected subcutaneously into the left upper foot skin of mice. After 2 hours post-inoculation, 1µl of 100,000 PFU TOSV was injected subcutaneously into the same inoculation area. The skin, foot joint, spleen and serum were collected at 72hpi. Both of these bacterial compounds substantially enhanced TOSV replication in the tissues of infected mice. Quantifying viral RNA at the inoculation site, foot joint, and spleen revealed higher levels of viral RNA in mice co-injected with the virus, LPS, and Pam3CSK4 compared to those infected with the virus alone (Figure 4.21A, B, C). However, the viral titer in blood serum did not show a significant increase in the LPS and Pam3CSK4 group compared to the virus-only group (Figure 4.21D). Overall, this suggested that the infection-promoting ability is not specific to sandfly bites/SGE and that it is possible to induce a similar phenotype using solely microbial products.

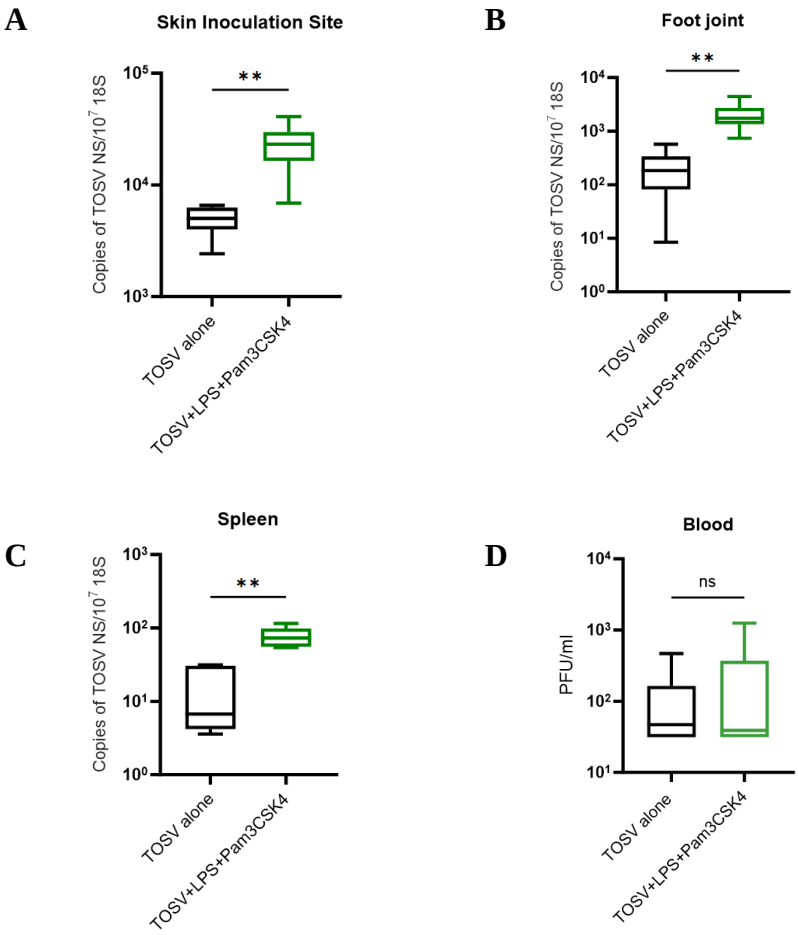


Figure 4.21: Pro-Inflammatory Agents Enhance TOSV Infection In Vivo

Ifnar1-/- mice were infected with TOSV on the dorsal side of their left foot, either with or without the addition of Pam3CSK4 and LPS. The expression of the viral TOSV NS gene was measured using qPCR in the skin **(A)**, foot joint **(B)**, and spleen **(C)** at 72hpi, **(D)** Infectious units in serum were determined by plaque assay (n=6 mice). Plots show the median value ± interquartile range. ns=not significant, significant **p < 0.01.

4.6.1.1 Quantification of LPS in Sandfly Salivary Gland Extract

In the previous experiment (see Section 4.6.1), we found that LPS and Pam3CSK4 at high concentrations could copy the enhancement phenotype. To determine whether this could explain how sandfly SGE enhances TOSV infection, we wanted to assess the endotoxin concentration in the SGE preps that had been used in our *in vivo* experiments. Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria that is known for stimulating immune responses and is recognised by the Toll-like receptor 4 (TLR4) complex (Rosadini and Kagan, 2017).

Endotoxin concentration of sandfly salivary gland extract (SGE) was determined using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, USA). The assay was performed as per the manufacturer's instructions (Section 2.13 in Chapter 2). A standard curve of endotoxin concentrations between 0.6 EU/mL, 0.3 EU/mL and 0 EU/mL was generated to calculate final endotoxin concentration using the subsequent equation of the line. The result of this indicating that the SGE contained a total of 0.2 EU/mL (Figure 4.22). One EU (Endotoxin Unit) is defined as approximately 0.1 to 0.2 ng endotoxin/mL of solution. Therefore, this suggests that a concentration of 0.2 EU/mL equates to an endotoxin concentration of 0.02 - 0.04 ng/ml of endotoxin. Therefore, the quantity of endotoxin was substantially lower (1000/0.02 = 50 000-fold) than we used in the previous experiment (Figure 4.21). Therefore, this suggested that microbiota in sandfly saliva is not likely responsible for the enhancement of TOSV infection.

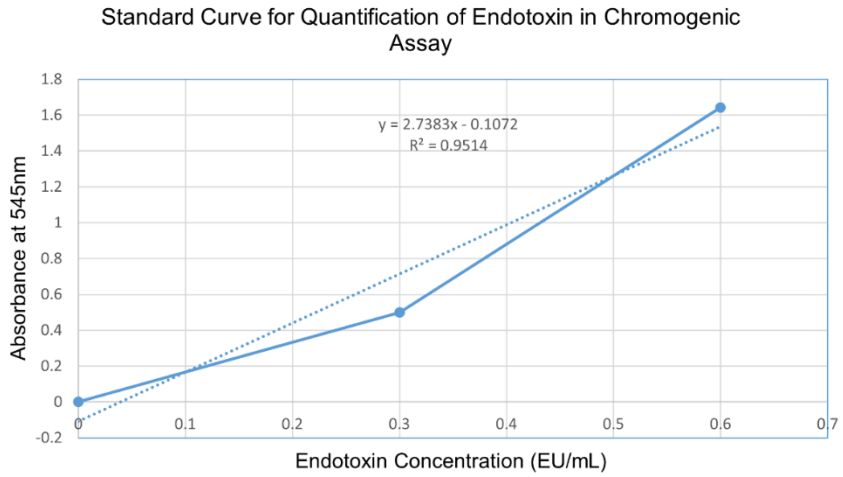


Figure 4.22: Standard Curve for Quantification of LPS in Sandfly Salivary Gland Extract

The y-axis shows the absorbance output readout from the samples mixed with the colorimetric reagent, and the x-axis demonstrates the endotoxin concentration. The sample's EU concentration was calculated by using the equation of the line (y=2.7383x-0.1072), which was generated through the line of best fit.

4.6.2 Pre-antibiotic-treated Sandflies Has Similar TOSV Enhancing Properties as Bacteria Sufficient Flies

We discovered that LPS and Pam3CSK4 in high concentration can enhance TOSV infection similarly to sandfly SGE, but their presence in SGE are unlikely to drive a similar phenotype, being at such low concentration. Nevertheless, we decided to generate abiotic sandflies, which were previously published as lacking microbiota (Kelly et al., 2017) to investigate further whether salivary microbiota modulates TOSV

infection levels *in vivo*. For this purpose, we used a similar antibiotic cocktail, Penicillin 500 U/ml, Streptomycin 500 µg/ml, Gentamicin sulphate 100 µg/ml, was incorporated into the sugar meal (25% sucrose w/v), which was fed to sandflies in soaked cotton wool pads and changed daily. Then, mice were anesthetised and exposed to 2 to 4 sandfly bites of either untreated or antibiotic treated *Lu. longipalpis* sandfly in the dorsal side of their left foot, before being injected with 100,000 PFU of TOSV. Skin, foot joint, spleen and blood were collected at 72hpi. The quantity of viral RNA at the bite site, foot joint, and spleen demonstrated no significant difference between mice exposed to antibiotic-treated or untreated sandfly bites (Figure 4.23). Likewise, no difference in viral titers was observed in the serum between antibiotic-treated and untreated sandfly bites (Figure 4.23D). This suggested that sandfly expressed salivary factors, rather than bacteria, is the primary factor enhancing virus infection. This aligns with findings from mosquito-borne viral diseases, in which mosquito microbiota does not modulate susceptibility to arbovirus infection in mice (Lefteri et al., 2022).

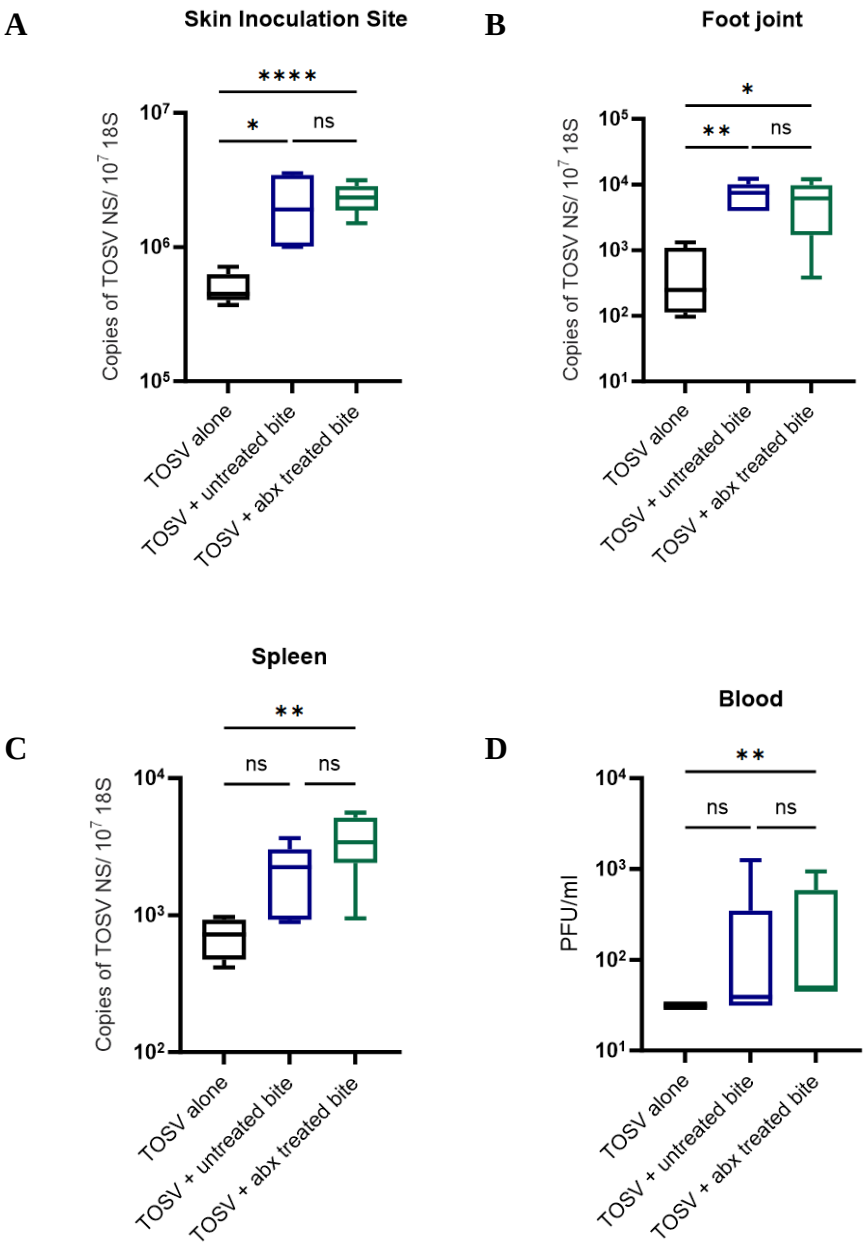


Figure 4.23: Salivary Microbiota Does Not Influence the Ability of Sandfly Bites to Enhance Infection

To determine if salivary microbiota affects the modulation of TOSV infection in a mammalian host, mice were subcutaneously inoculated on the upper side of their left foot with 100,000 PFU of TOSV, either alone or in combination with 2-4 sandfly bites, which were either untreated or pre-antibiotic treated. Antibiotics included a cocktail of penicillin, streptomycin and gentamicin sulphate. The expression of the viral TOSV NS gene was measured using qPCR in the skin **(A)**, foot joint **(B)**, and spleen **(C)** at 72hpi (n=6 mice). Infectious units in serum were determined by plaque assay **(D)**. Plots show the median value ± interquartile range. ns=not significant, significant *P < 0.05, **p < 0.01, ****p < 0.0001.

Furthermore, we wanted to examine whether depleting salivary microbiota affects the upregulation of inflammatory genes in the skin and in the foot joint, which is an important tissue for TOSV pathogenesis (section 4.2.2.2). The expression of selected genes previously identified as upregulated by sandfly SGE (see Figure 4.3) was compared to that in unbitten resting skin and the virus-only skin group using qPCR.

Data showed that bites from both untreated and antibiotic-treated sandflies significantly upregulated the expression of *il-6* and *isg15* compared to resting skin and the virus-only group. We found that there was no significant difference in expression levels of cytokines when comparing mice receiving bites from Abx-treated and untreated sandflies. However, whilst bites from untreated sandflies resulted in a significant upregulation of *ccl2*, bites from antibiotic-treated sandflies did not significantly induce *ccl2* or *cxcl2* (Figure 4.24A).

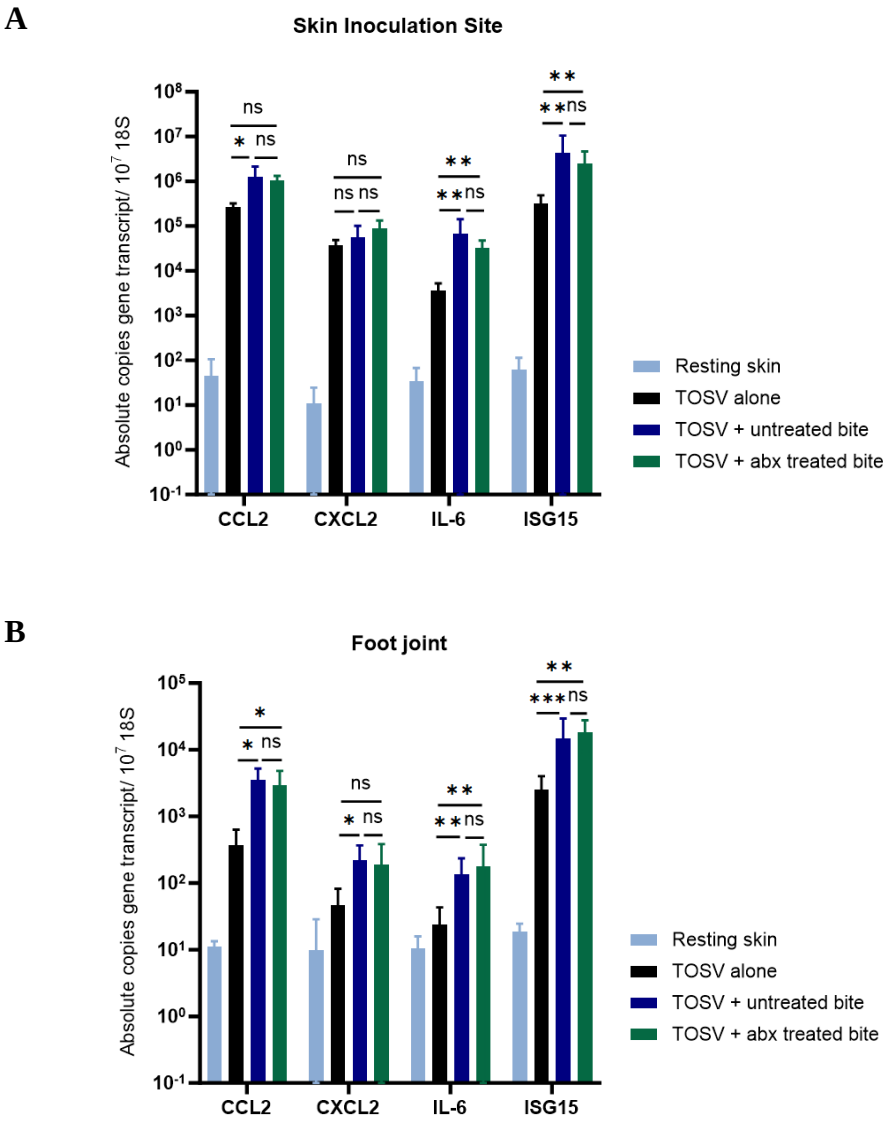


Figure 4.24: Microbiota-Independent Upregulation of Inflammatory Genes in the Skin and Foot Joint in Response to Sandfly Bites

The levels of *ccl2*, *cxcl2*, *il-6*, and *isg15* genes expression either in resting skin or TOSV alone or with untreated sandfly bites or with antibiotic (Abx) treated sandfly bites. The skin **(A)** and foot joint **(B)** samples were taken from *ifnar1-/-* mice at 72hpi. Gene expression was measured in these samples by qPCR (n=6 mice). Plots show the median value ± interquartile range. ns=not significant, significant *P < 0.05, **p < 0.01, ***p<0.001.

Similarly in the foot joint, there was no significant difference when comparing mice that had received bites from Abx-treated and untreated bites. However, bites from sandflies with intact microbiota significantly upregulated more *cxcl2* in foot joints compared to mice receiving virus alone, whereas joints in mice that received bites from antibiotic-treated sandflies did not, despite the similar gene quantities in both bite groups (Figure 4.24B). However, in summary, the upregulation of inflammatory genes in response to sandfly bite/SGE was independent of microbiota.

4.7 SUMMARY AND CONCLUSIONS

In this chapter, we developed our mouse model for TOSV infection adding sandfly SGE in the inoculation. We aimed to investigate whether sandfly SGE enhance TOSV infection in a mammalian host. As a result, we showed that sandfly SGE had ability to increase host susceptibility to the infection at 24hpi and 72hpi. However, the results suggested that the replication of TOSV in host tissues was higher and consistent at later times. Therefore, we decided to examine the 72-hour post-infection time point for future experiments. We also investigated whether sandfly SGE modulates the gene expression in the cutaneous immune response to TOSV. Two key chemokines (*ccl2*, *cxcl2*), a pro-inflammatory cytokine (*il-6*), and an interferon-stimulated gene (*isg15*) were significantly upregulated by the presence of sandfly SGE in the inoculum compared to resting skin of mice at 24hpi and at 72hpi. Although there was no significant increase for the *ccl2* and *cxcl2* levels in the sandfly SGE group compared to mice that received only the TOSV group, the plots showed an upward trend in these genes in the SGE group than the virus-only group. In addition, we showed that the enhancement effect of sandfly SGE was dose-dependent, resulting in a ten-fold higher increase in viral load in the inoculation site and peripheral tissue once TOSV was inoculated with three times more SGE. It is noteworthy that while TOSV was detectable in tissues distant from the initial infection site, such as the spleen, plaque assays revealed only minimal levels of infectious virus in the blood. This likely highlights the superior sensitivity of the qPCR assay, which is more effective at detecting viral RNA than the plaque assay is at identifying infectious units.

In the previous chapter, we suggested that *Phlebotomus perniciosus* sandfly SGE has a general pro-viral effect because it augments SFV infection, a genetically unrelated virus to TOSV. Here, we wanted to determine whether the phenomenon of TOSV infection enhancement by sandfly SGE is specific to the sandfly vector that naturally transmits

the virus. To do this, we used *Lu. longipalpis* sandfly SGE, a potent vector of *Leishmania*. The results suggested that TOSV infection could be enhanced by this distinct blood-feeding sandfly, even if it is not a natural vector.

Next, we wanted to determine the effect of sandfly SGE on TOSV dissemination and pathogenesis in our mouse model. The experiment concluded that inclusion of sandfly SGE in the TOSV inoculum causes more severe outcomes of TOSV infection; mice receiving sandfly SGE within the inoculum exhibited more inflamed foot joints and atypical neurological signs at 15 days post-infection (dpi) and at 21dpi. As a neurotropic virus in humans, we showed that TOSV could also cause CNS infection in animals. Moreover, the mice infected with TOSV and sandfly SGE had a higher viral load in the brain than those that received TOSV only. The foot joint inflammation by TOSV and TOSV with sandfly SGE was also first described. Accordingly, it can be characterised by a condition that begins on the 6dpi, worsens on the 7-8dpi, and then resolves on its own. In foot joint tissues, the chemokines (*ccl2*, *cxcl2*, *cxcl10*) and the inflammatory cytokines (*tnf-α*, *il-6*) were significantly upregulated by co-injected sandfly SGE. In addition, despite the host generating a protective virus-neutralizing response to TOSV infection with sandfly SGE in the serum, the presence of sandfly SGE nevertheless exacerbated the disease. Histological evaluation of H&E-stained samples from inflamed skin and inflamed foot joints revealed significant infiltration of inflammatory cells in the dermis of TOSV co-infected with sandfly SGE, compared to the resting and TOSV-alone groups. In inflamed foot joints, TOSV notably caused gaps to appear in muscle tissue, suggestive of myofiber degeneration and/or oedema. Moreover, adding sandfly SGE led to a greater aggregation of inflammatory cells in the subcutaneous tissue, extending into the muscular layer, than TOSV alone in foot joint samples.

Following the discovery that inoculation sandfly SGE via needle enhances susceptibility to TOSV infection *in vivo*, we wanted to assess the sandfly bites capacity on the virus enhancement. The upper foot skin of mice was exposed to up to 4 sandfly bites and then immediately infected with TOSV. Significant increases in TOSV RNA were observed in the skin and spleen, as well as in its titer in the serum. This suggested that sandfly bites enhance TOSV replication in a mammalian host. In addition, we showed increased inflammatory responses to bites.

Lastly, we aimed to evaluate whether potential microbial components in sandfly saliva influence saliva-induced TOSV infection. Firstly, we illustrated that pro-inflammatory agent like LPS and Pam3CSK4 in high concentration could enhance TOSV infection *in*

vivo. However, within a subsequent endotoxin analysis, the amount of LPS in the sandfly SGE was determined to be 0.02-0.04 ng/ml, which is well below the amount that we used LPS and Pam3CSK4 experiment. Although the high concentrations of LPS and Pam3CSK4 copy the phenotype of sandfly saliva, they are unlikely to be the agents responsible for enhancing infection in sandfly saliva. Next, based on the hypothesis that microbiota in sandfly SGE could modulate TOSV infection *in vivo* by triggering inappropriate immune responses that inadvertently enhance infection, we aimed to determine if the salivary microbiota in sandfly saliva is responsible for the observed virus-enhancing effect. The sandflies became abiotic using an antibiotic cocktail before they were allowed to bite. As a result, we demonstrated for the first time that microbiota in sandfly saliva does not influence virus infection *in vivo*. Both microbiota-depleted and untreated sandfly bites enhanced infection to a similar extent.

CHAPTER 5: DEFINING CUTANEOUS CELLULAR TARGETS FOR TOSV AND WHETHER THIS IS MODULATED BY SANDFLY SALIVARY GLAND EXTRACT OR SANDFLY BITES

5.1 INTRODUCTION

The focus of the Chapter is on the initial infection of a mammalian host by TOSV from a cellular perspective. In addition, we examined the influence of sandfly SGE on cell infection in mammalian skin, where infected arthropods introduce saliva and TOSV during feeding.

Firstly, the resident and migratory cells in the epidermis and dermis encounter arboviruses by infected arthropod bites. Therefore, the infection of the skin by arboviruses is a critical phase of the disease progression. This is supported by a study showing that 99% of WNV was deposited into the extracellular spaces in the cutaneous compartment, rather than entering the bloodstream (Styer et al., 2007). Furthermore, surgical removal of the inoculation site has successfully protected mice from infections with the St. Louis encephalitis (SLE) (Turell et al., 1995) and the Rift Valley fever virus (Spielman and Turell, 1992), underscoring the role of skin-derived viruses in the subsequent development of the disease.

Various studies on mosquito-borne viruses have demonstrated that fibroblasts, dendritic cells (DCs) and macrophages can be infected by virus in mice e.g., WNV, CHIKV and DENV (Pingen et al., 2017). Collectively, this indicates that the infection of hematopoietic cells, alongside cutaneous fibroblasts, plays a significant role in several arbovirus infections. However, our knowledge about the cellular targets of TOSV, and indeed most bunyavirales, is limited by few studies. An *in vitro* study showed that TOSV could infect immature DCs derived from human PBMCs (Lozach et al., 2011). In addition, HUVECs and moDCs were also susceptible to infection with TOSV *in vitro* (Cusi et al., 2016). The same study detected no virus in circulating PBMCs generated from mice infected with TOSV. However, TOSV-infected mouse endothelial cells were defined by an immunofluorescence assay (Cusi et al., 2016). Here, in this thesis, we showed that skin derived fibroblasts, M-CSF Macrophages, and Flt3-Ligand Dendritic cells were susceptible to TOSV infection at a range of MOIs *in vitro* (Section 3.4 of Chapter 3).

Therefore, to better define early stage of TOSV infection in skin, **the aim of this chapter is to interrogate the cellular tropism of TOSV at skin inoculation sites.**

We hypothesise that inflammatory responses to sandfly enhance infection with TOSV, by more frequent infection of inflammatory cells that are recruited to the skin by sandfly saliva. We have shown that sandfly SGE and bite increase host

susceptibility to TOSV infection and worsen clinical outcomes. For mosquito-borne disease – infection of leukocytes recruited by saliva helps explain the phenotype. Our previous group member's data suggests that the host's inflammatory response to mosquito saliva increases susceptibility to the virus by recruiting monocytic cells that become infected and replicate the virus. Thus, this influx of monocytes provides new cellular targets for infection (Pingen et al., 2016). Here, we suggested that a similar early inflammatory response in the bite site to sandfly SGE/bite may boost replication of TOSV. Sandfly bites induce a strong local inflammatory response using various saliva components (Lestinova et al., 2017), in which neutrophils infiltrate rapidly and localise to the bite site, followed by monocytes/macrophages recruitment (Teixeira et al., 2005; Peters et al., 2008).

Thus, by defining cellular targets of TOSV infection we **aim to define the mechanism by which sandfly saliva enhances TOSV infection susceptibility.**

These aims and objectives were investigated using the Fluorescence-Activated Cell Sorting (FACS) to sort live infected cells from mouse skin. This technique allowed us to separate cells based on their surface marker expression and determine whether stromal cells or leukocytes were being infected *in vivo*. Novel antibody panels and genetically modified TOSV-mCherry were utilised.

5.2 ANTIBODY PANELS FOR FACS EXPERIMENT

5.2.1 Leukocytes

This thesis used two panels to dissect neutrophils, macrophages, and dendritic cells, monocytes in skin, respectively.

PANEL 1

Antibody	Clone	Fluorophore	Marker for
CD45	30-F11	FITC	common leukocyte antigen
CD11b	M1/70	APC	monocytes, macrophages
MERTK (Mer)	2B10C42	APC-Cy7	macrophages
Ly-6G	1A8	Brilliant Violet 421	neutrophils
TOSV	n/a	mCherry	infected TOSV cells
Live/Dead dye	n/a	Ultraviolet	cell viability

Table 5.1: List of Antibodies, Antibody Clones, Fluorophores for Panel 1.

Flow cytometry analysis is founded on the principle of gating. To investigate and quantify these specific populations, gates and regions are established around cell populations that share common characteristics, such as forward scatter (FSC), side scatter (SSC), and marker expression.

Therefore, the gating strategy of Panel 1 was as follows:

- FSC-H vs FSC-A plot to determine single cells
- Exclude Live/Dead dye + cells (these cells are debris)
- CD45+ (all leukocytes)
- CD45+Ly-6G+ or CD45+Ly-6G+CD11b+ (refers to neutrophils positive cells)
- CD45+Ly-6G- (non-neutrophil leukocytes)
- CD45+Ly-6G-CD11b+MerTK+ (refers to macrophages positive cells)
- Defining mCherry+ refers to TOSV-infected cell populations

PANEL 2

Antibody	Clone	Fluorophore	Marker for
CD45	30-F11	FITC	common leukocyte antigen
Ly-6C	HK1.4	PE	monocytes
MHC-II	M5/114.15.2	APC	antigen-presenting cells (APCs)
CD11c	N418	PE-Cy7	dendritic cells (DCs)
TOSV	n/a	mCherry	infected TOSV cells
Live/Dead dye	n/a	Ultraviolet	cell viability

Table 5.2: List of Antibodies, Antibody Clones, Fluorophores for Panel 2.

Gating strategy was as follows for Panel 2:

- FSC-H vs FSC-A plot to determine single cells
- Exclude Live/Dead dye + cells (these cells are debris)
- CD45+ (all leukocytes)
- CD45+Ly-6C-CD11c+MHC II+ (refers to activated DCs)
- CD45+Ly-6C^{hi}CD11c-MHC II- (refers to monocytes positive cells)
- Defining mCherry+ refers to TOSV-infected cell populations

5.2.2 Stromal Cells and Fibroblasts Subpopulations

Here, we wanted to investigate the susceptibility of non-leukocyte cells to TOSV and, later, the effect of sandfly SGE on these cells *in vivo* systems.

Therefore, two distinct panels were established to define skin-resident stromal cells and fibroblast subpopulations.

STROMAL CELLS PANEL

Antibody	Clone	Fluorophore	Marker for
CD45	30-F11	FITC	common leukocyte antigen
Vimentin	280618	APC	mesenchymal cells, including fibroblasts
CD31	390	PE	pan-endothelial cells
CD326 (Ep-CAM)	G8.8	APC-Cy7	epithelial cells, including keratinocytes
TOSV	n/a	mCherry	infected TOSV cells
Live/Dead dye	n/a	Ultraviolet	cell viability

Table 5.3: List of Antibodies, Antibody Clones, Fluorophores for Stromal Cells.

Gating strategy was as follows for stromal cells panel:

- FSC-H vs FSC-A plot to determine single cells
- Exclude Live/Dead dye + cells (these cells are debris)
- CD45- (leukocyte-specific CD45 that is not expressed on epithelial or stromal cells)
- CD45-Vimentin+ (refers to positive fibroblasts)
- CD45-CD31+ (refers to positive endothelial cells)
- CD45-EpCAM+ (refers to positive epithelial cells)

FIBROBLAST SUBPOPULATIONS PANEL

Antibody	Clone	Fluorophore	Marker for
CD45	30-F11	FITC	common leukocyte antigen
CD31	390	FITC	endothelial cells
CD326 (Ep-CAM)	G8.8	FITC	epithelial cells
CD90.2 (Thy1.2)	30-H12	Pacific Blue	fibroblasts
Podoplanin	PMab-1	Alexa Fluor 647	activated fibroblasts
CD140a (PDGFR-α)	APA5	PE-Cy7	activated fibroblasts
Ly-6A/E (Sca-1)	W18174A	PE	pluripotent mesenchymal
TOSV	n/a	mCherry	infected TOSV cells
Live/Dead dye	n/a	Ultraviolet	cell viability

Table 5.4: List of Antibodies, Antibody Clones, and Fluorophores for Fibroblast Subpopulations.

Because there are a number of ways for defining activated fibroblasts, we used the following gating strategy to define fibroblast subpopulations:

- FSC-H vs FSC-A plot to determine single cells
- Exclude Live/Dead dye + cells (these cells are debris)
- CD45-CD31-EpCAM- (exclude leukocytes, endothelial and epithelial cells)
- CD45-CD31-EpCAM-CD90.2+ (refers to fibroblasts)
- CD45-CD31-EpCAM-Podoplanin+ (refers to activated fibroblasts)
- CD45-CD31-EpCAM-CD140a+ (refers to activated fibroblasts)
- CD45-CD31-EpCAM-Sca-1+ (refers to more primitive, pluripotent mesenchymal cells, including those that differentiate into fibroblasts)

5.3 *IN VIVO* MODEL FOR TOSV EXPRESSING MCHERRY FLUORESCENT PROTEIN

There is limited knowledge about which cells in the skin, especially those bitten by hematophagous arthropods, are susceptible to infection by bunyavirales. Indeed, TOSV infection on specific cells in the skin has not been studied. For this purpose, we wanted to develop an *in vivo* murine model that would allow us to track live infected TOSV cells from mouse skin. Prof. Alain Kohl kindly provided genetically modified TOSV that express mCherry fluorescent protein in infected cells. The TOSV-mCherry strain (1500590) and the TOSV strain (Pt SI-1812) are distinct, yet they belong to the same lineage (Lineage A). However, even if they were not from the same lineage, this would be likely inconsequential, as there is no evidence suggesting that genetic differences between lineages (A, B, C) influence virulence or clinical symptoms (Ayhan and Charrel, 2020). The recombinant TOSV was produced using reverse genetic systems, which allowed the deletion of the NSs gene and replacement of it with a reporter gene, mCherry (Alexander et al., 2020). Although the non-structural NSs protein of TOSV is best known as a type I IFN antagonist, the absence does not affect cellular transcription (Kalveram and Ikegami, 2013; Wuerth and Weber, 2016). Because we are using *ifnar1* -/- mice, the lack of NSs is likely to not have much impact on TOSV fitness *in vivo*. It has been suggested that the NSs protein could have a role in the tropism or propagation of TOSV, due to an increase in neuroinvasiveness in mice infected with the recombinant RVFV MP-12 strain encoding TOSV NSs (rMP12-TOSNSs) (Indran et al., 2013). However, these studies were done in IFN sufficient wild type mice, and the most likely reason for these observations is the ability of NSs to modulate IFN function.

Firstly, we wanted to define whether TOSV-mCherry exhibits similar ability replicate more efficiently in mice following infection with SGE, as compared to the wild type TOSV strain used in Chapters 3 and 4. Here, we infected *ifnar1-/-* mice with 100,000 PFU of TOSV-mCherry in 1µl inoculum with or without sandfly SGE. The skin samples were collected at 72hpi, and the quantity of TOSV N gene in the inoculation site was investigated using qPCR (Figure 5.1).

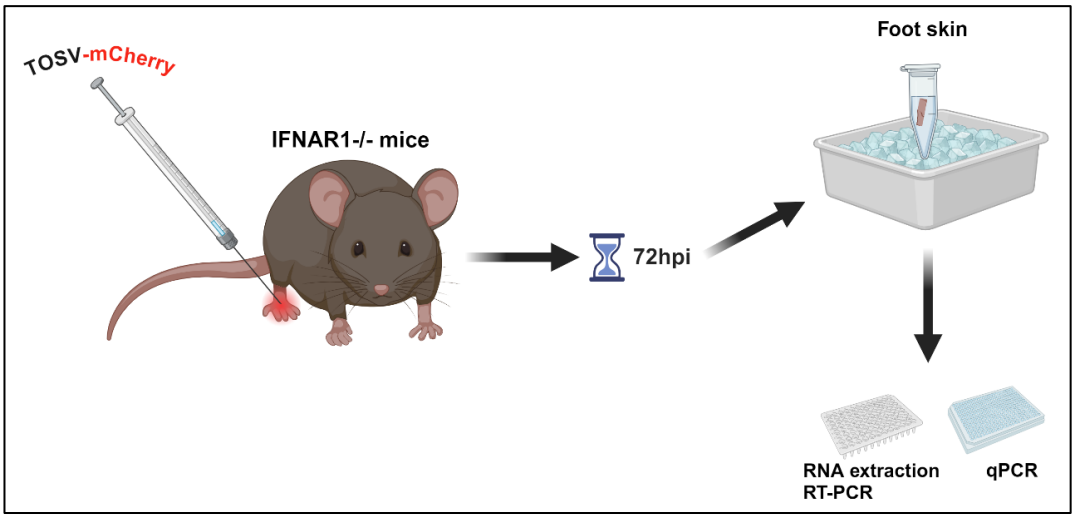


Figure 5.1: *In Vivo* Model for TOSV-mCherry Infection in the Skin Inoculation Site
A cartoon to summarise the infection of *ifnar1* null mice with TOSV-mCherry.

As a result, we showed that TOSV-mCherry could infect the mice when inoculated into the upper foot skin with low volume inoculum. In addition, the quantity of virus RNA was higher in sandfly SGE group than the virus alone group at 72hpi (Figure 5.2). This suggested that the recombinant TOSV acts as wild-type TOSV in our mouse model. However, likely due to the fitness cost of carrying extra protein, the rTOSV caused around less 10-fold replication in the tissue compared to infection with wild-type TOSV (Figure 4.2 in Chapter 4).

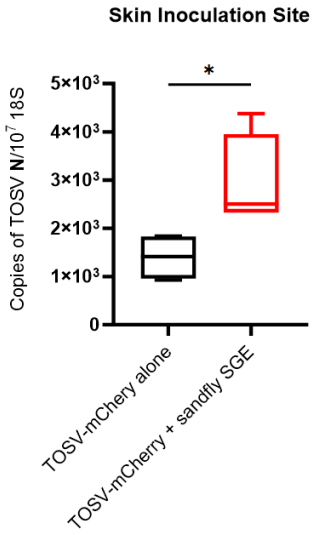


Figure 5.2: Sandfly Salivary Gland Extract Enhances TOSV-mCherry Infection in a Mammalian Host

Ifnar1-/- mice were infected with 100,000 PFU TOSV-mCherry with or without sandfly salivary gland extract (SGE) (1 pair gland extract/µl). Skin samples were taken from infected *ifnar1-/-* mice at 72hpi and TOSV RNA (N gene) copy number determined by qPCR (n=4 mice). Plots show the median value ± interquartile range. Significant, *P < 0.05.

5.4 DEFINING CELLULAR TROPISM OF TOSV INFECTION

5.4.1 Detecting TOSV-mCherry in Infected BHK-21 Cells Using FACS

We aimed to determine if TOSV-mCherry-infected cells could be detected using flow cytometry. To achieve this, BHK-21 cells were infected with 0.1 and 1 MOI of TOSV-mCherry. Following an overnight infection, the cells were collected and subjected to a FACS protocol. This included washing with FACS buffer to remove residual tissue culture reagents and washing with PBS to create a protein-free environment before staining the cells with a live/dead stain and fixing them with 4% PFA. Infected BHK-21 cells were identified by excluding live/dead stain-positive cells and gating on mCherry-positive cells.

We demonstrated that TOSV-mCherry-infected BHK-21 cells can be identified using FACS analysis (Figure 5.3), with a higher number of infected cells detected as the viral titer increases (Figure 5.3C). Therefore, we chose to use BHK-21 cells infected with TOSV-mCherry at an MOI of 1 as a marker for mCherry (required for single-stain compensation when analysing multiple fluorophores).

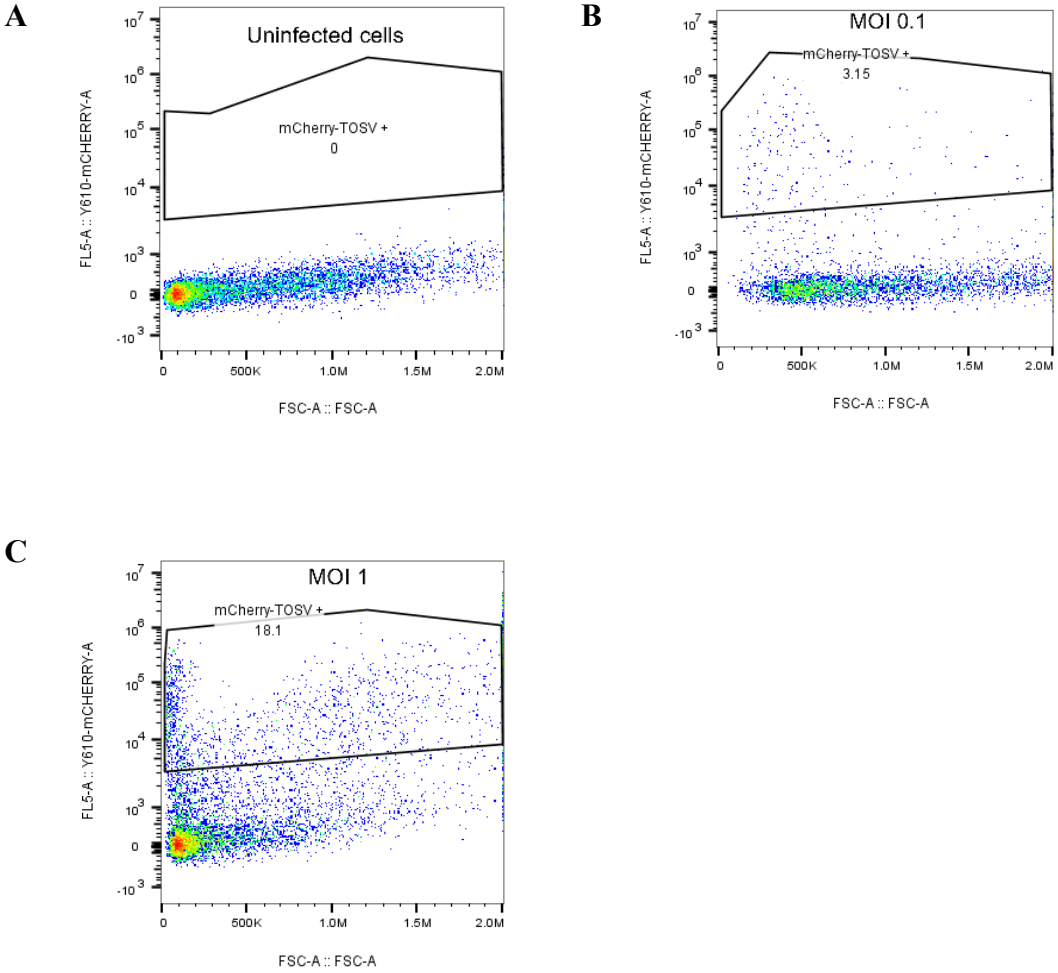


Figure 5.3: Detecting Infected BHK-21 Cells with TOSV-mCherry via FACS

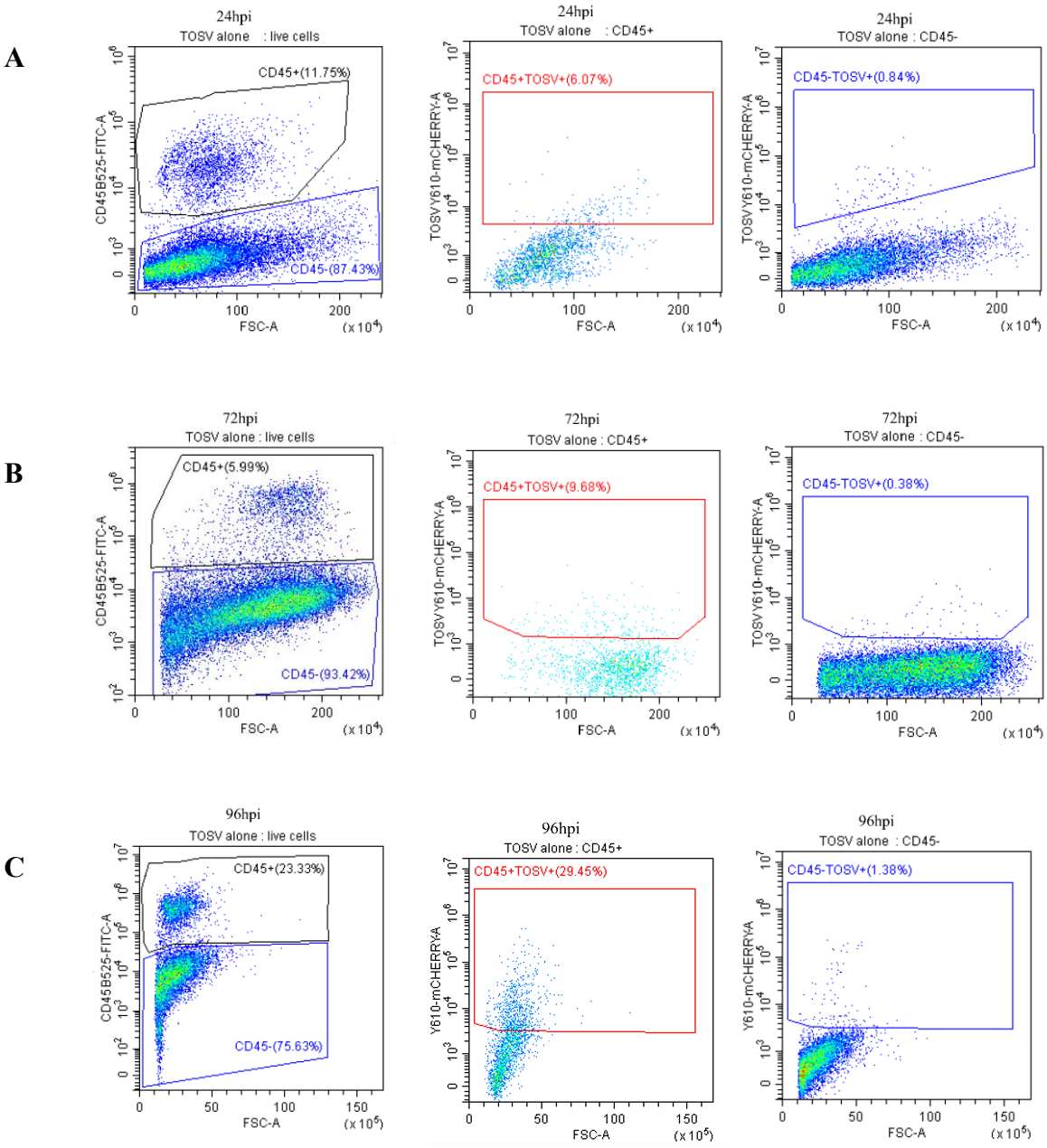
BHK-21 cells were infected with TOSV-mCherry at MOIs of 0.1 and 1 and analysed at 24hpi. The cells were collected via trypsinization, centrifuged, and washed twice with FACS buffer and PBS. They were then stained with a viability stain to exclude dead cells during FACS analysis. Infected cells were identified by gating on the mCherry channel, with mCherry+ cells appearing between ~10^4 and 10^6 on the y-axis of the FACS graphs. Panel **(A)** shows uninfected cells, Panel **(B)** shows 3.15% mCherry+ cells at an MOI of 0.1, and Panel **(C)** shows 18.1% mCherry+ cells at an MOI of 1. The y-axis represents the mCherry channel, and the x-axis represents FSC-A. Data shows 1 of repeat experiment.

5.4.2 TOSV Infects Leukocytes and Non-leukocyte Cells *In Vivo*

After optimizing the detection of TOSV-mCherry-infected cells, we undertook a preliminary experiment with a small number of mice to define whether leukocytes at skin inoculation sites could be infected with TOSV *in vivo.* Mice were infected with 100,000 PFU of TOSV-mCherry. At 24-, 72-, and 96-hours post-infection (hpi), the skin was digested and stained for CD45, a leukocyte surface marker. The location of gates in FACS scatter plots was determined based on stained uninfected skin samples. The parent percentage of TOSV+ leukocytes was lower at 24hpi compared to 72hpi (Figures 5.4A, B). Additionally, the parent rate was lower at 72hpi than at 96hpi (Figures 5.4B,

C). The number of CD45+TOSV+ cells per live cell was higher at 96hpi compared to 24hpi and 72hpi (Figure 5.4D). The number of infected stromal cells was also higher at 96hpi compared to earlier time points (Figure 5.4E). These findings suggested that TOSV can infect both leukocytes and non-leukocyte cells in the skin, with a higher number of infections occurring at later time points.

In terms of CD45+ cells, their numbers were lower at 72hpi than at 24hpi, with a trend for highest number at 96hpi (Figure 5.4F). Note these trends are not statistically different, as the study was not powered to demonstrate differences in leukocyte number, but rather show qualitatively which cell types are infected.



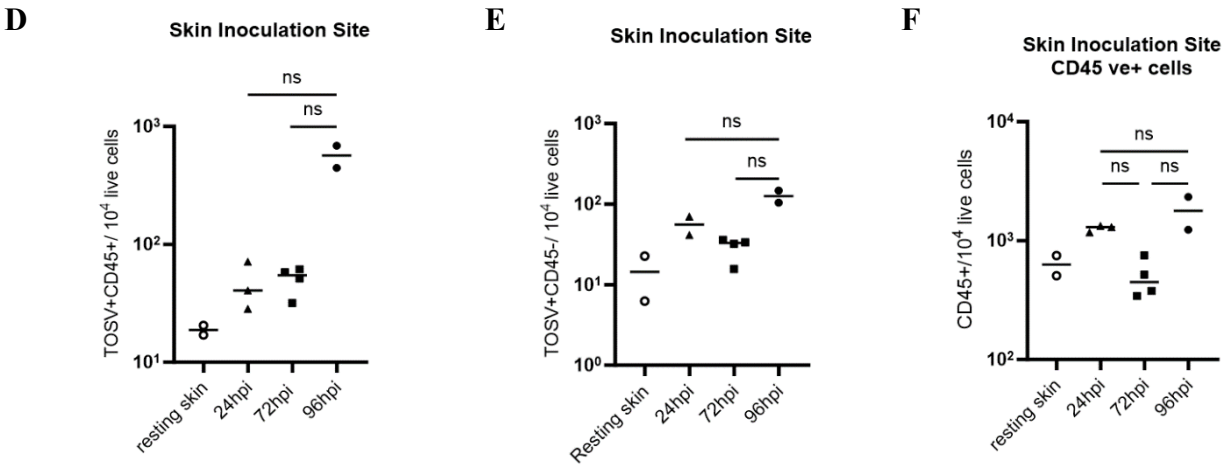


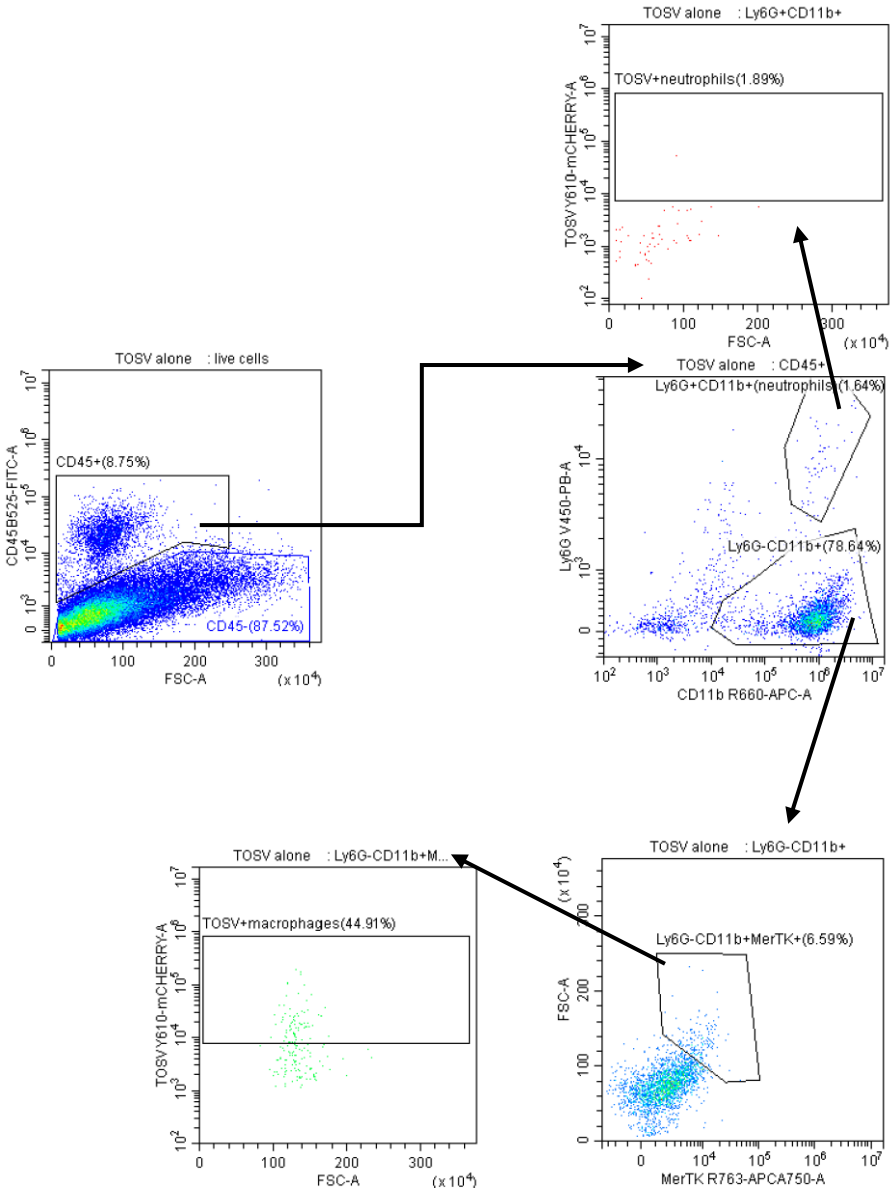
Figure 5.4: TOSV Infects Leukocytes and Stromal Cells *In Vivo*

The skin of *ifnar1-/-* mice was either left as resting control or infected with 100,000 PFU TOSV-mCherry. The skin samples were taken at 24hpi, 72hpi and 96hpi and stained with a live/dead dye and common leukocyte marker, CD45, via applying FACS protocol. **(A-B-C)** demonstrates gating strategies. The percentages on the gate in the graphs refer to the proportion of cells within a specific gate relative to the number of cells in the parent gate (the gate immediately preceding the current gate) **(D-E)** displays numbers of TOSV+CD45+ and TOSV+CD45- cells in the skin as % of all live cells. **(F)** shows number of CD45+ cells in the skin as % of all live cells. Presented as dot plots, representing a separate biological sample with a line at the population median. ns=not significant.

5.4.2.1 Defining TOSV-Infected Leukocytes

Next, we wanted to define which leukocytes can be infected with TOSV-mCherry. 96 hours post-infection was selected for subsequent experiments, as it represented the time point with the highest level of infection, unless otherwise specified. At 96hpi, the infected skin of mice was digested to release single cells and divided in equal amounts into two antibody panels for staining. In the first panel, the cells were stained for CD45, CD11b, MerTK, and Ly-6G; in the second panel, they were stained for CD45, Ly-6C, MHC II, and CD11c. Figure 5.5A illustrates the gating strategy used to identify specific leukocyte populations, including neutrophils and macrophages. This analysis revealed that neutrophils were not infected during TOSV infection, whereas macrophages were susceptible to infection, as defined by their positivity for mCherry. Figure 5.5B details the representative gating strategy for identifying infected dendritic cells (DCs) and monocytes at the skin inoculation site. We found that both DCs and monocytes could be infected, though at a lower frequency compared to macrophages.

A


TOSV alone : live cells
CD45+(8.75%)
CD45-(87.52%)
CD45B525-FITC-A
FSC-A
(x 10^4)
TOSV alone : CD45+
Ly6G+CD11b+(neutrophils)(1.64%)
Ly6G-CD11b+(78.64%)
Ly6G V450-PB-A
CD11b R660-APC-A
TOSV alone : Ly6G+CD11b+
TOSV+neutrophils(1.89%)
TOSV Y610-mCHERRY-A
TOSV alone : Ly6G-CD11b+
Ly6G-CD11b+MerTK+(6.59%)
MerTK R763-APCA750-A
TOSV alone : Ly6G-CD11b+M...
TOSV+macrophages(44.91%)

B

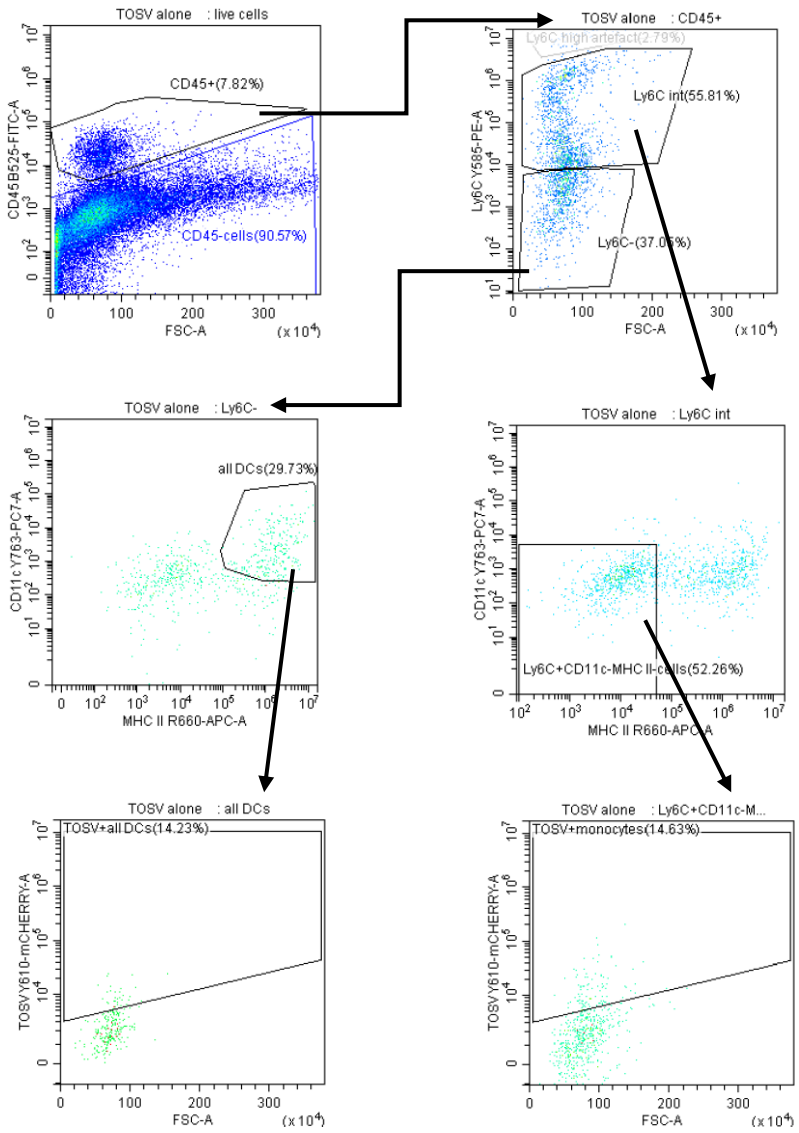


Figure 5.5: TOSV Infects Macrophages, Monocytes and Dendritic Cells but not Neutrophils in the Skin

The skin of *ifnar1-/-* mice was infected with 100,000 PFU TOSV-mCherry subcutaneously. The skin samples were taken at 96hpi, and the FACS protocol was used to analyse the infected cells. **(A)** representing the gating strategy for neutrophils and macrophages, **(B)** demonstrating the gating strategy for DCs and monocytes. The percentages on the gate in the graphs refer to the proportion of cells within a specific gate relative to the number of cells in the parent gate (the gate immediately preceding the current gate). This was representative of six individual experiments.

5.4.2.2 Defining TOSV-Infected Stromal Cells

We have already shown that skin fibroblasts are highly susceptible to TOSV *in vitro* (Section 3.4 of Chapter 3). In addition, a previous study suggested that endothelial cells could be infected with TOSV (Cusi et al., 2016). Therefore, we wanted to investigate whether these stromal cells can be infected in the skin inoculation site. We designed a primary FACS experiment that included staining for CD45, Vimentin, CD31, and

EpCAM to define non-leukocytes, fibroblasts, endothelial cells, and epithelial cells, respectively. In conclusion, while TOSV did not infect keratinocytes or endothelial cells, it successfully infected fibroblasts in the skin (Figure 5.6).

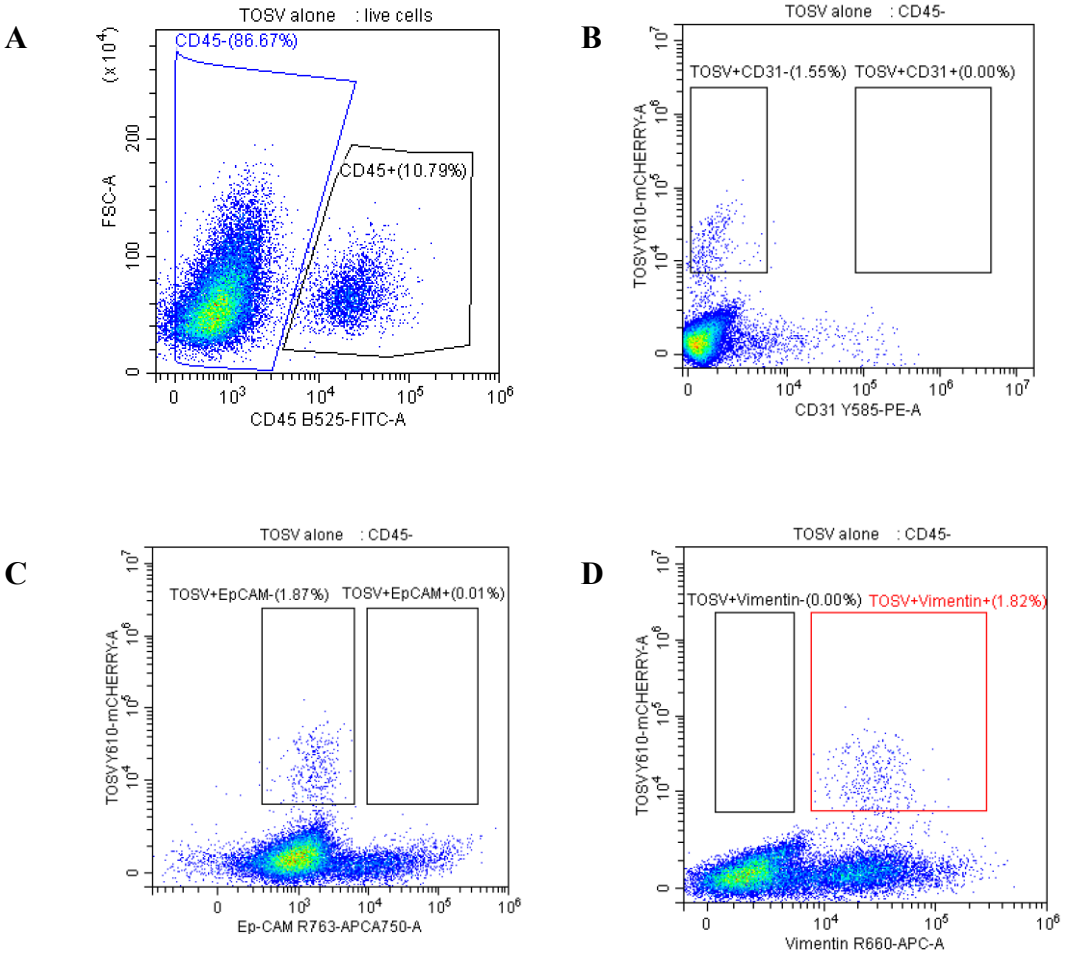


Figure 5.6: TOSV Infects Fibroblasts *In Vivo*

Ifnar1-/- mice were infected with 100,000 PFU of TOSV-mCherry. Skin samples were collected at 96hpi and stained for certain non-leukocyte markers. The infected cells were analysed using the FACS protocol. **(A)** illustrates gating of CD45+ (leukocytes) and CD45- cells. The CD45- cells were then interrogated in B-D. **(B)** displays TOSV positivity in CD31+ (endothelial) and CD31- cells, **(C)** shows TOSV+EpCAM+ cells (epithelial e.g., keratinocytes) and TOSV+EpCAM-, and **(D)** depicts TOSV+Vimentin- and TOSV+Vimentin+ cells (fibroblasts). The percentages on the gates in the graphs represent the proportion of cells within each specific gate relative to the parent gate (the gate immediately preceding the current gate).

5.5 DEFINING CELLULAR TROPISM OF TOSV INFECTION IN SANDFLY SALIVARY GLAND EXTRACT (SGE) INOCULATED SKIN

In previous chapters of this thesis, we found that sandfly SGE enhances TOSV infection in a mammalian host either by increasing viral load in tissues or worsening clinical outcomes. Although our knowledge about the underlying mechanism of this phenotype is limited, **we hypothesise that inflammatory responses to sandfly bites/SGE enhances infection with TOSV, supported by more frequent infection of inflammatory cells recruited to the skin.**

Here, the mice's skin was either left resting or infected subcutaneously with 100,000 PFU of TOSV-mCherry or 100,000 PFU of TOSV-mCherry with sandfly SGE. At 96hpi, the samples were processed the same as previously. Cell numbers for each of the populations in each condition for one representative experiment are shown in Table 5.5 and Figure 5.7. Together, this shows that inclusion of SGE in the inoculum caused an increase in the number of leukocytes (CD45+ cells) and infected leukocytes (TOSV+ CD45+), with approximately 809 events per live cell, compared to the skin infected with TOSV alone, which had about 417 events per live cell. The same phenomenon was also valid in stromal cell infection showing with TOSV+CD45-; there were around 104 events per live cell in the TOSV alone, whereas approximately 322 events per cell in the sandfly SGE.

		CD45+	CD45-	TOSV+CD45+	TOSV+CD45-
Mouse (1)	TOSV alone	1234.37	8693.54	417.83	104.74
Mouse (2)	TOSV+sandfly SGE	2620.03	7328.86	809.62	322.43

Table 5.5: Cell counts of FACS sort.

Ifnar1-/- mice were either (1) injected with 100,000 PFU of TOSV-mCherry alone, (2) injected with 100,000 PFU of TOSV-mCherry + sandfly SGE (1 pair gland extract/µl). At 96hpi, skin was digested and stained for CD45. Table displays cell counts per live cells for 1 repeat of experiment.

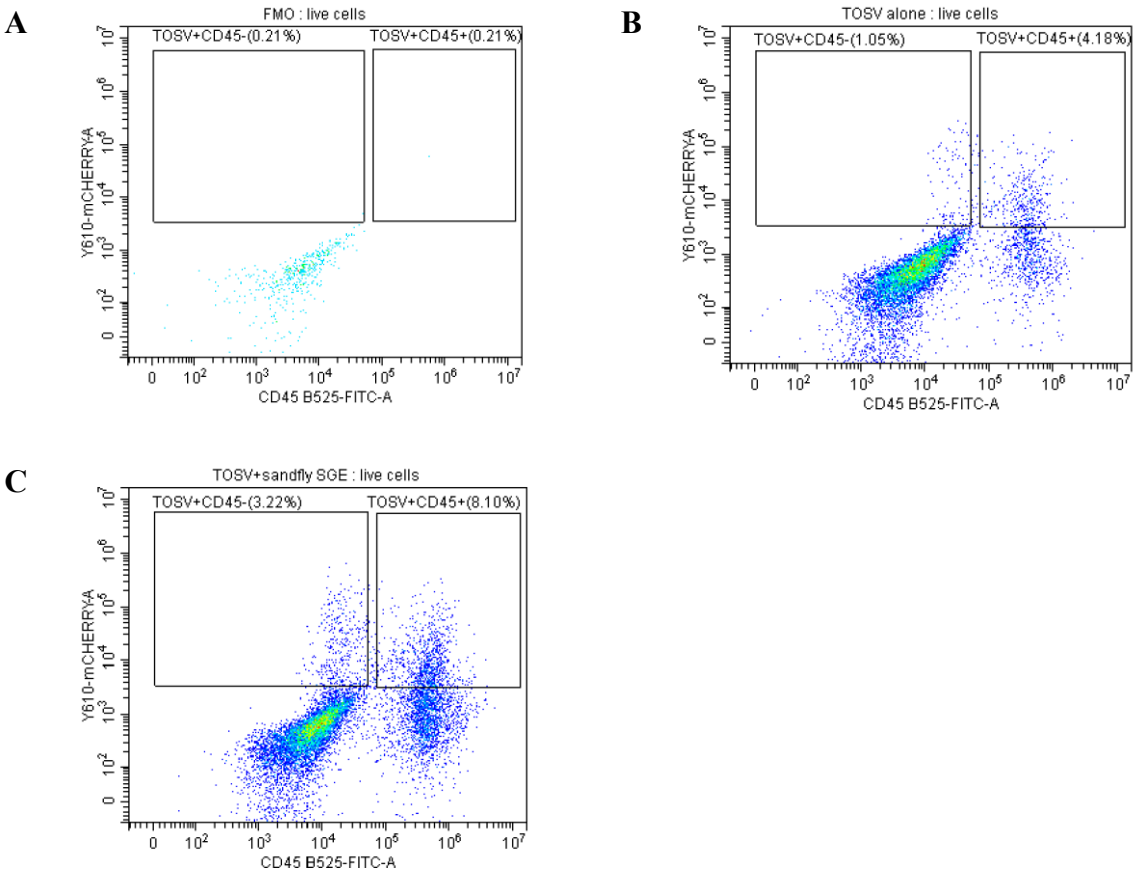


Figure 5.7: Sandfly SGE Supports TOSV Infection Infecting Leukocytes and Stromal Cells *In Vivo*

Ifnar1-/- mice were either (1) injected with 100,000 PFU of TOSV-mCherry alone, or (2) injected with 100,000 PFU of TOSV-mCherry along with sandfly SGE (1 pair gland extract/µl). At 96hpi, the skin was digested and stained for CD45. **(A)** represents the Fluorescence Minus One (FMO) control that lacked infection with TOSV-mCherry, **(B)** shows the gating strategy for CD45+TOSV+ and CD45-TOSV+ cells in the skin infected with TOSV only, and **(C)** illustrates the gating strategy for CD45+TOSV+ and CD45-TOSV+ cells in the skin infected with both TOSV and sandfly SGE. The data shown were generated from one optimisation experiment that primarily aimed to validate the staining panel (n=1 mice).

5.5.1 Defining Stromal Cells Tropism of TOSV Infection in Sandfly Salivary SGE Inoculated Skin

Next, we wanted to investigate which stromal cell types are getting infected with TOSV and whether sandfly SGE has affected the virus tropism on them. We have already found that TOSV-infected cells were negative for CD31 (endothelial cells) and EpCAM (epithelial cell) markers while positive for Vimentin (fibroblasts). Therefore, we chose to investigate a range of fibroblast markers, including Sca-1, CD90.2, CD140a, and podoplanin, to explore the hypothesis that TOSV may replicate more efficiently in immature/primitive fibroblast cells compared to fully differentiated ones. Sca-1 and CD90.2 are particularly notable as markers of primitive fibroblasts, which are involved

in tissue repair and wound healing. These markers suggest a less differentiated, more regenerative state of the cells. While CD140a and podoplanin are associated with more differentiated fibroblasts that also play key roles in the synthesis and remodelling of the extracellular matrix.

Ifnar1-/- mice were infected with 5000 PFU of TOSV-mCherry with or without sandfly SGE (1 pair gland extract/µl). The skin samples were collected at 72hpi and digested to single cells. This time point was selected because the viral RNA load was higher in infected tissues *in vivo*. The cells were stained for CD45, CD31, EpCAM, CD90.2, Podoplanin, CD140a (PDGRF-α) and Sca-1 (Ly-6A/E). We gated out the leukocytes, epithelial and endothelial cells (which we labelled lineage positive), enabling more targeted assessment of fibroblast cell population (which we labelled lineage negative).

Firstly, before assessing number of infected cells, we wanted to assess whether total number of each cell type was altered by inclusion of SGE. The number of CD90.2-, CD140a-, and Podoplanin- cells was higher than those positive for these markers. However, there were more Sca1+ cells than Sca1- cells (Figure 5.8). Importantly, the frequency of these cell types did not change with the addition of sandfly SGE.

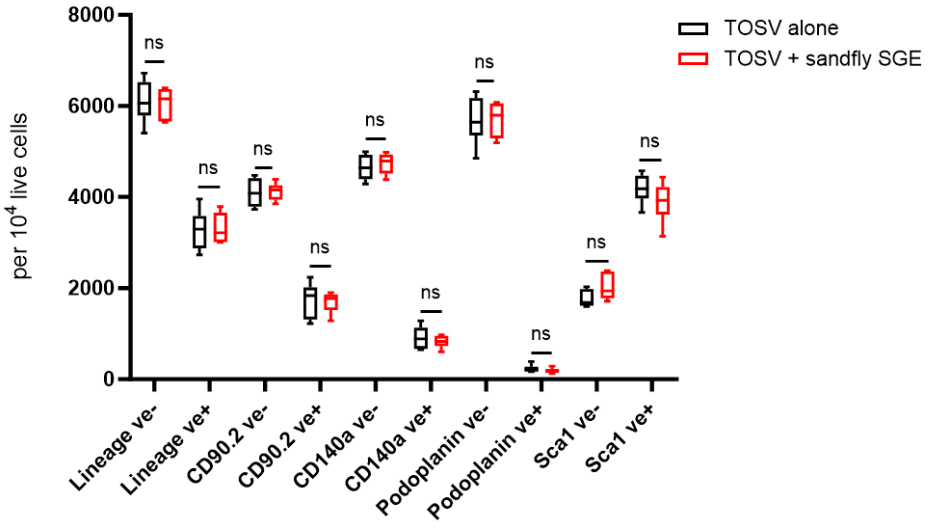


Figure 5.8: The Number of Cells for Each Marker Does Not Change Significantly in the Presence of Sandfly SGE

Showing the numbers of fibroblast subpopulations for each marker in the skin per all live cells (n=6 mice). Plot shows the median value ± interquartile range. ns=not significant

To define whether these cell types exhibited different frequencies of mCherry positivity, we undertook the following gating strategy. Here, we firstly removed TOSV+CD45+CD31+EpCAM+ cells that are a heterogeneous population of leukocytes (CD45+), endothelial cells (CD31+), and epithelial cells (EpCAM+), which are referred to as lineage positive (Figure 5.9A). The remaining cell populations were then interrogated for mCherry expression in gates that included several types of stromal and mesenchymal cells; TOSV+CD90.2+, TOSV+CD140a+, TOSV+Podoplanin+, and TOSV+Sca1+ cells within the lineage-negative population gate (Figure 5.9B). The placement of the gates was determined based on assessing fluorescence of uninfected samples and FMO controls.

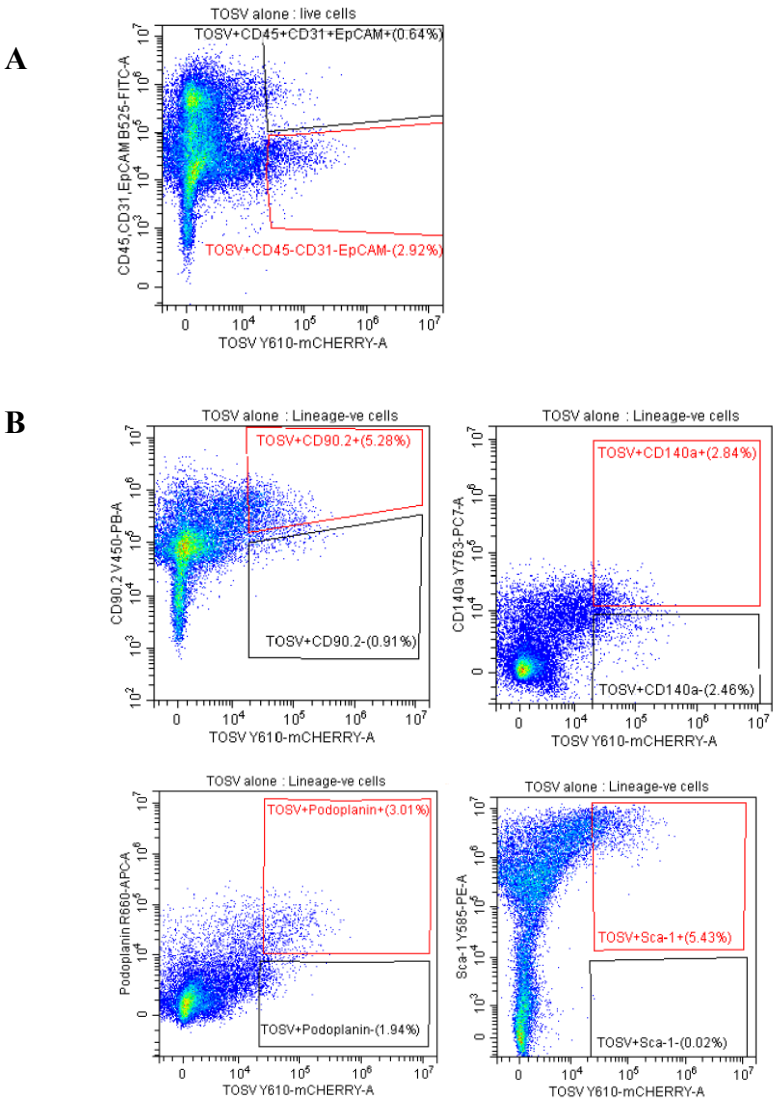


Figure 5.9: Further Characterization of TOSV-Infected Mouse Dermal Fibroblast Subpopulations

Displays an example of a gating strategy for detecting infected fibroblast subpopulations. **(A)** Single-cell suspension from mouse skin was initially gated for TOSV+CD45-CD31-EpCAM- cells, indicating the exclusion of immune, endothelial, and epithelial cells, **(B)** From this gate, subpopulations were further identified as TOSV+CD90.2+, TOSV+CD140a+, TOSV+Podoplanin+, and TOSV+Sca1+ cells (n=6 mice). The data shown are derived from one independent experimental run.

Using this gating strategy, we found that significantly more lineage-negative cells (fibroblasts) were positive for TOSV, compared to lineage positive cells (leukocytes); in both the TOSV alone and TOSV with sandfly SGE groups (Figure 5.10A). This shows that fibroblasts were the most commonly infected cell type in skin, and was somewhat unexpected as was somewhat different to the data in Figure 5.7. When we interrogated the fibroblast specific markers, we found that TOSV only infected CD90.2+ and Sca1+ fibroblasts irrespective of SGE inclusion in inoculum (Figure 5.10B, C). The virus was

present in both Podoplanin+ and Podoplanin- fibroblasts (Figure 5.10D). Finally, TOSV infected both CD140a+ and CD140a- cells, with a significantly higher infection rate in CD140a+ cells (Figure 5.10E). Together shows that TOSV preferentially infects these cell types compared to the marker negative cells.

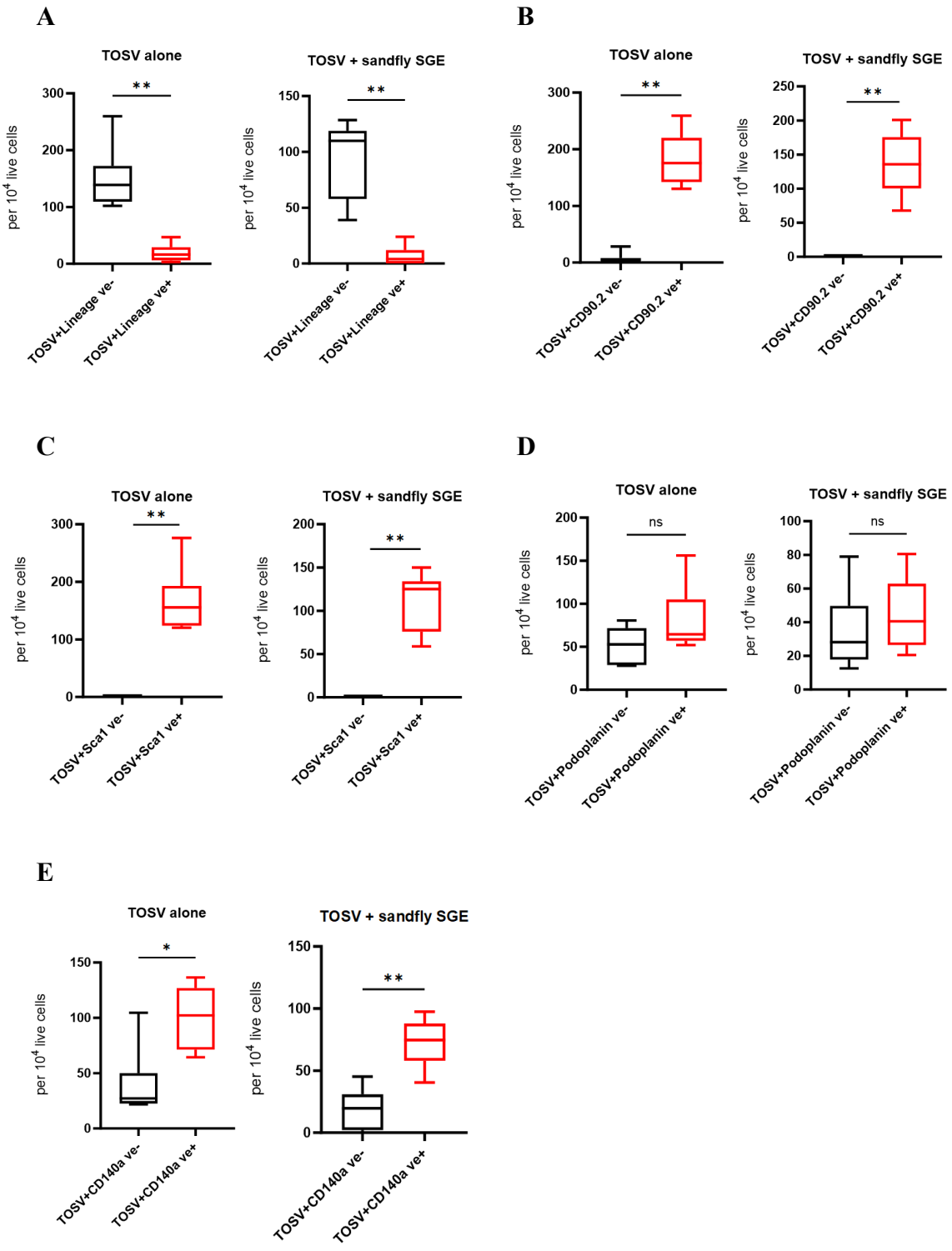


Figure 5.10: Fibroblast Subpopulations Are Getting Infected by TOSV In Different Frequencies

5000 PFU of TOSV-mCherry, either with or without sandfly SGE (1 pair gland extract/µl), was injected into the foot skin of *ifnar1-/-* mice. Skin samples were collected 72 hours post-infection (hpi) and digested into single cells. The cells were then stained for CD45, CD31, EpCAM, CD90.2, Podoplanin, CD140a (PDGFR-α), and Sca-1 (Ly-6A/E). **(A)** shows TOSV infected linege ve+ and lineage ve- cells **(B)** TOSV+CD90.2 ve+ and TOSV+CD90.2 ve- cells, **(C)** TOSV+Sca1 ve+ and TOSV+Sca1 ve- cells, **(D)** TOSV+Podoplanin ve+ and TOSV+Podoplanin ve- cells, **(E)** TOSV+CD140a ve+ and TOSV+CD140a ve- cells in TOSV alone and TOSV with sandfly SGE group (n=6 mice). Plots show the median value ± interquartile range. ns=not significant, significant *P < 0.05, **p < 0.01.

The above analyses define an infected cells using a binary +ve or -ve characterisation of TOSV infection. To further help identify which fibroblast subtypes are permissive to most efficient TOSV infection, and perhaps therefore support higher levels of viral replication, we defined numbers of cells that had “high” mCherry expression, using flow cytometry. High mCherry expression may indicate a higher viral load or more active viral replication within these cells. Therefore, we identified mCherry^{hi} cells for each marker (Figure 5.11A). The number of mCherry^{hi} cells was similar among fibroblast markers, but TOSV+ lineage+ cells (CD45+CD31+EpCAM+) exhibited lower numbers of cells in the high expression gate, compared to fibroblasts. This suggests that although leukocytes can become positive for mCherry, there are few of these cells that exhibit high expression of mCherry. Additionally, we also assessed the geometric mean fluorescence intensity (geomean FI) of each cell type. This analysis for mCherry revealed that TOSV-positive cells have similar levels of mCherry intensity (Figure 5.11B).

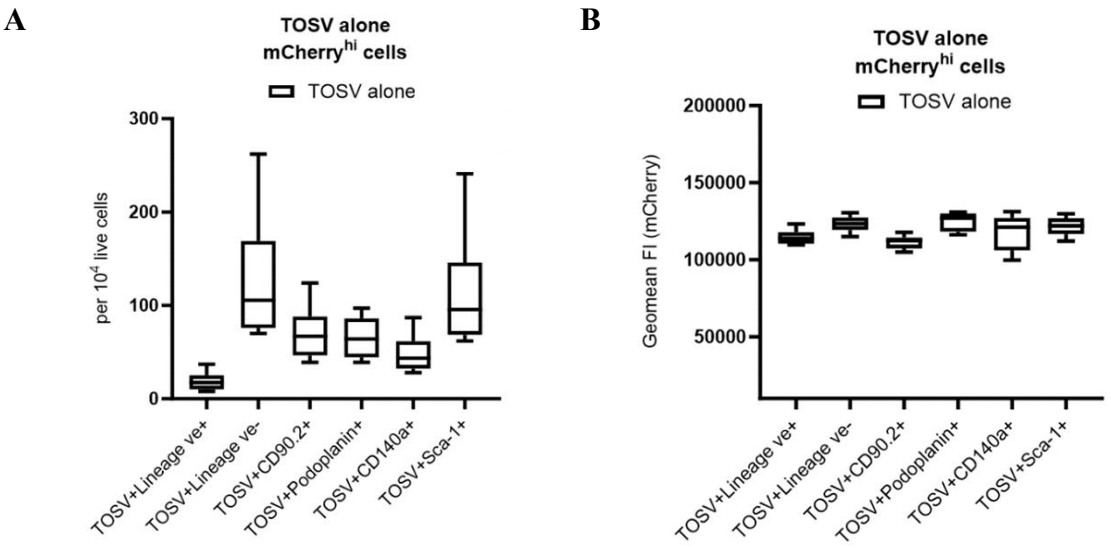


Figure 5.11: TOSV-Positive Cells Exhibited Similar Levels of mCherry Intensity
(A) Graph illustrates which cell types have highest number of mCHerry^{hi} cells, **(B)** Plot shows which cells have highest MFI in the mCherry^{hi} gates (n=6 mice). Lineage ve+ (CD45+CD31+EpCAM+), Lineage ve- (CD45-CD31-EpCAM-).

Finally, when we then assessed what impact SGE might have on mCherry geomean FI for all gates, we saw no significant effect, except a minor if statistically significant difference in lineage positive cells (CD45+CD31+EpCAM+) (Figure 5.12).

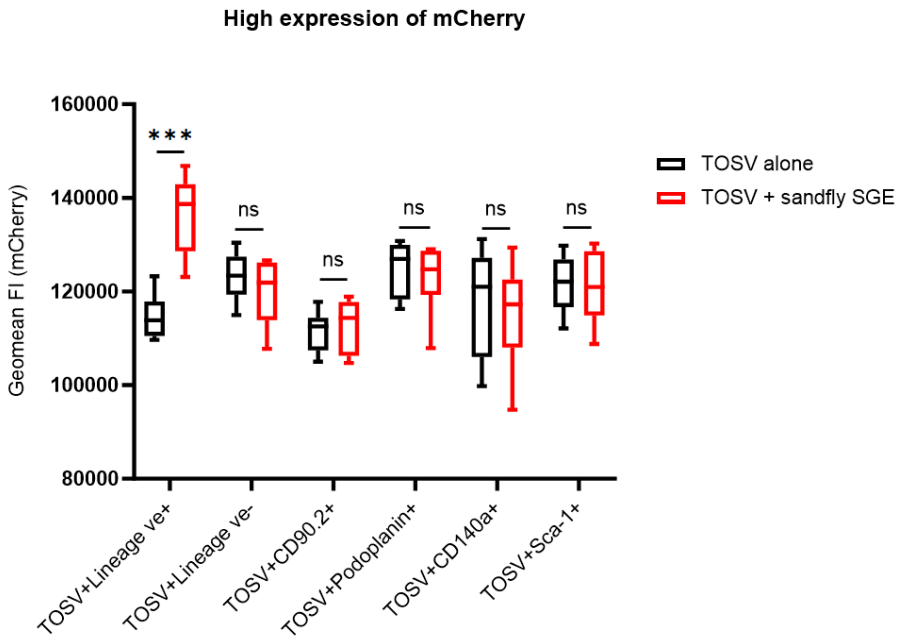


Figure 5.12: High Expression of mCherry in TOSV-Positive Cells
Plot shows which cell types have highest intensity of mCHerry^{hi} cells in TOSV alone and co-infected with sandfly SGE (n=6 mice). Lineage ve+ (CD45+CD31+EpCAM+), Lineage ve- (CD45-CD31-EpCAM-).

5.5.2 Determining the Capacity of TOSV-Infected Cells to Produce New Infectious Virus

The above data indicate that both macrophages and fibroblasts can become infected with TOSV, as defined by mCherry expression. Importantly, the mCherry only shows if a cell is positive for this virus-encoded gene product. The macrophages could be positive because they have phagocytosed virus-infected cells or debris. Thus, we wanted to determine whether infection of cells at skin inoculation sites results in the release of new infectious virus. To explore this, we isolated macrophages at 96hpi, magnetically sorted cells based on the macrophage marker F4/80 on columns and quantified the amount of infectious virus released after 24h and 48h in cell culture by plaque assay (Figure 5.13A, B). In addition, the quantity of TOSV RNA at 48h post-isolation in the fractions was also assessed (Figure 5.13C). We chose macrophages, as they exhibited the highest frequency of TOSV infection in the skin among other leukocytes (Figure 5.5). Importantly we also controlled for cell number, placing exactly the same number of cells for each cell type, into each well.

Interestingly, our findings indicate that the infected cells in the F4/80- fraction, which our FACS data above shows are primarily composed of dermal fibroblasts, shows a significantly higher propensity for replicating and releasing new infectious virus,

compared to the F4/80+ enriched fraction (Figure 5.13A, B). Moreover, the viral RNA quantity was higher in negative fraction than positive fraction cells (Figure 5.13C). This suggested that, despite previous FACS data showing positivity for virus-encoded mCherry in macrophages, they are inefficient at generating infectious virus. In addition, despite the stromal cells having similar geometric mean mCherry fluorescence (Figure 5.11B), here we show that these cells, likely fibroblasts, have higher capacity to generate more infectious virus than leukocytes on a per cell basis. Therefore, the findings suggest that infected fibroblasts might contribute a key cellular target for TOSV in the skin, enabling efficient replication that drives pathogenesis in the mammalian host.

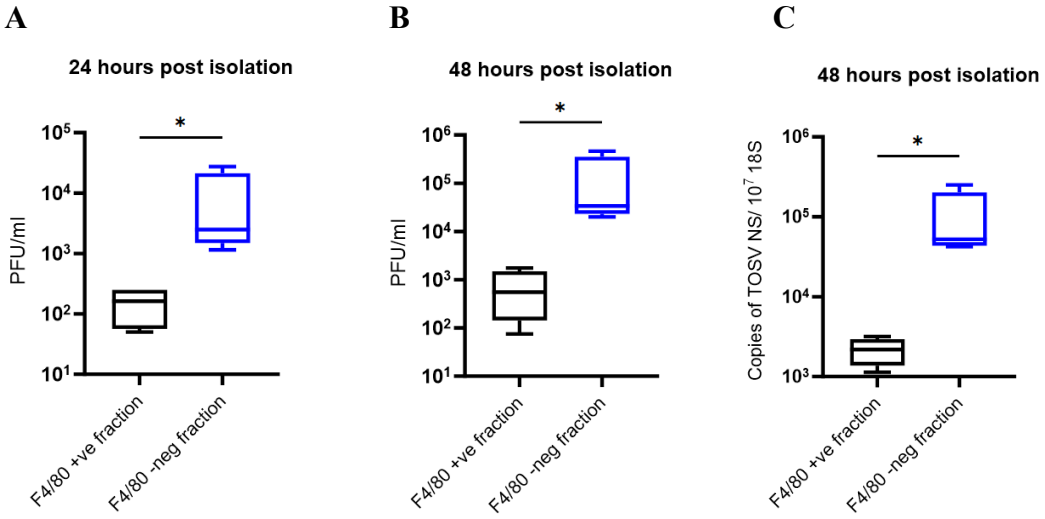


Figure 5.13: Macrophages and Dermal Fibroblasts Support Productive Infection by TOSV

100,000 PFU TOSV with sandfly SGE (1 pair gland extract/µl) were injected into the skin of *ifnar1-/-* mice. At 96hpi, the skin samples were digested to release cells, and F4/80+ cells (macrophages) were sorted on magnetic cell separation columns, applying the MACS protocol in Section 2.10 of Chapter 2. This generated a F4/80+ enriched fraction and a F4/80- fraction (all other cells, that our above FACS analysis demonstrated were fibroblasts). **(A-B)** Infectious virus released into tissue culture media by each cell fraction after 24 and 48 hours post isolation in culture was quantified by plaque assay (n=4). **(C)** Both cell fractions were collected at 48h and the TOSV NS gene copy numbers were determined by qPCR assay (n=4 mice). Plots show the median value ± interquartile range. Significant *$p < 0.05$.

5.6 DEFINING CELLULAR TROPISM OF TOSV INFECTION IN SANDFLY BITTEN SKIN

In this thesis, it has been shown that in addition to SGE, sandfly bites also enhance subsequent TOSV infection severity (Section 4.5 of Chapter 4). Here, we aimed to investigate the cutaneous cellular tropism of TOSV infection at the skin-biting site. For this purpose, mice were either infected with TOSV-mCherry alone or exposed to

Lutzomyia longipalpis sandfly bites and then infected with 100,000 PFU of TOSV-mCherry at the same site. At 96hpi, skin samples were digested and stained with specific antibody panels, which allowed us to dissect the innate immune and stromal cell populations in the skin. Detailed information on panels was explained in Section 5.2.

We showed that the innate immune response in the skin to sandfly bite and TOSV infection was characterised by increased number of leukocytes (Figure 5.14). The number of leukocytes at the bitten skin site was higher when infected with TOSV (Figure 5.14B) than resting skin at 96hpi (Figure 5.14A). In addition, we found that the number of leukocytes in the skin inoculation site were remarkably high later at 6dpi (Figure 5.14D) compared to resting tissue (Figure 5.14C) and at 96hpi (Figure 5.14D). When we assessed which cells were positive for mCherry, we found that vimentin positive cells (fibroblasts) were the most frequently infected, although macrophages were also positive for mCherry (Table 5.6).

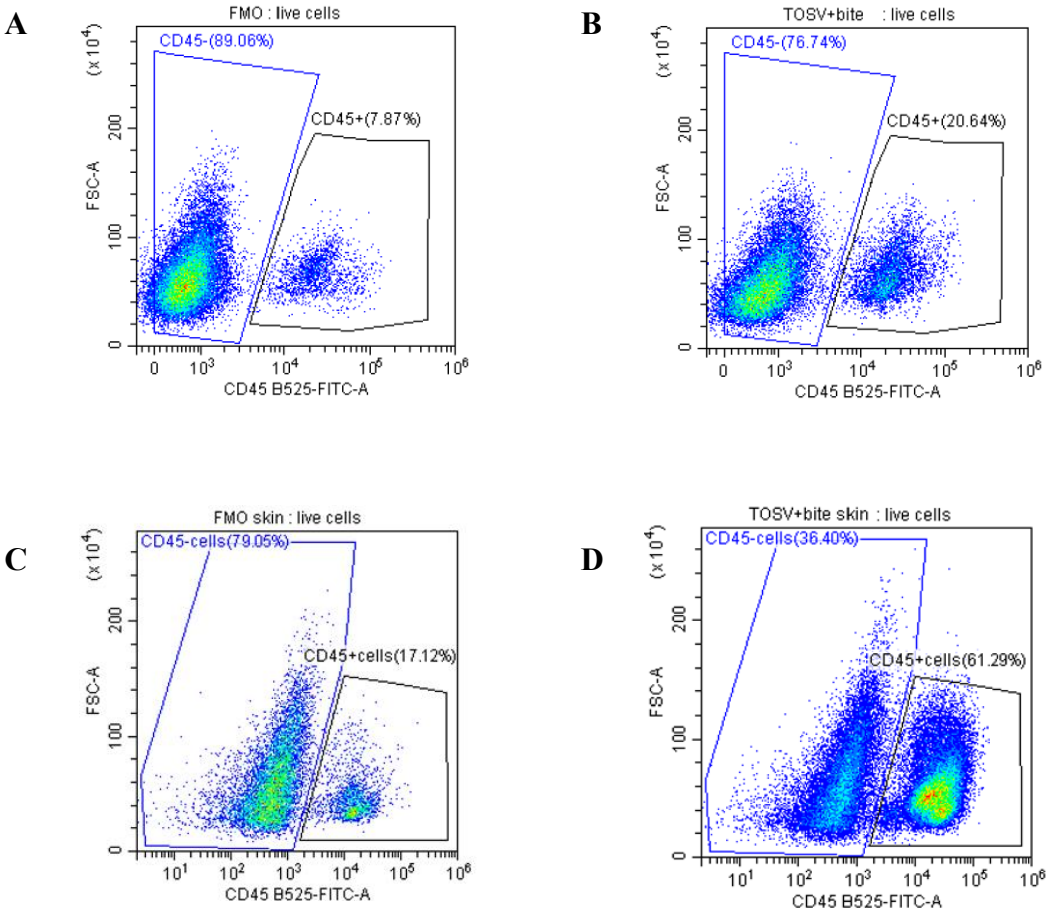


Figure 5.14: Sandfly Bite Causes the Influx of Leukocytes into the Skin Inoculation Site

Ifnar1-/- mice were either left uninfected or exposed to *Lutzomyia longipalpis* sandfly bites and then immediately infected with 100,000 PFU of TOSV-mCherry. Skin was digested at 96hpi **(A-B)** and at 6dpi **(C-D)** and stained with CD45. **(A-B-C-D)** Gating strategy defining the population of leukocyte cells (CD45+) and stromal cells (CD45-) in uninfected control **(A-C)** and TOSV-infected bitten skin **(B-D)**.

	TOSV alone 96hpi	TOSV alone 96hpi	TOSV +bite 96hpi	TOSV +bite 96hpi	TOSV +bite 6dpi	TOSV +bite 6dpi
TOSV+CD45+	634.30	549.56	795.05	416.63	231.75	153.12
TOSV+CD45-	159.42	167.00	226.20	143.30	81.68	80.32
TOSV+Macrophages+	9.66	20.35	74.42	38.37	114.24	59.78
TOSV+Neutrophils+	0	0	0	0	0	0
TOSV+Monocytes+	1	1	9.71	91.63	0	0
TOSV+DCs+	12.26	5.82	11.17	6.63	0	0
TOSV+EpCAM+	0	0	0	0	4	6
TOSV+CD31+	0	0	0	0	0	0
TOSV+Vimentin+	105.23	157.55	255.90	109.45	37.56	42.50

Table 5.6: FACS Cell Counts for TOSV-Only and Sandfly-Bitten Skin.

Ifnar1-/- mice were either infected with 100,000 PFU of TOSV-mCherry alone or exposed to biting *Lutzomyia longipalpis* sandflies prior to infection with 100,000 PFU of TOSV-mCherry. At 96hpi and 6dpi, skin was digested and stained for macrophages, neutrophils, monocytes, DCs and stromal cells. Table displays average cell counts for 1 repeat of experiment.

Although these experiments had an n=2 mice for each condition, there was trend for increased frequency of TOSV+macrophages+ (CD45+ Ly6G-CD11b+MerTK+) at 96hpi and 6dpi and TOSV+monocytes+ (CD45+Ly-6ChiCD11c-MHC II-) at 96hpi after exposure to sandfly biting. However, the frequency of DCs showed a similarity between TOSV alone and within sandfly-bitten skin samples at 96hpi, but there were no infected DCs at 6dpi. Similar to our findings with mice receiving SGE above, we found that neutrophils (CD45+ Ly6G+CD11b+), epithelial cells (CD45-EpCAM+) and endothelial cells (CD45-CD31+) did not get infected with TOSV in the skin, irrespective of whether they also received a sandfly bite. Interestingly, the frequencies of TOSV+Vimentin+ cells were higher than those of any TOSV-positive leukocytes in TOSV alone skin and the virus-infected bitten skin at 96hpi. In contrast, the number of mCherry-positive macrophages was higher than that of the infected vimentin cells at 6dpi (Table 5.6).

5.7 INVESTIGATING TOSV CELL TROPISM IN INFECTED INFLAMED FOOT

In chapter 4, we identified that the presence of SGE in the inoculation caused frequent development of foot limb inflammation (in Section 4.2.2.2). Here, we asked whether a sandfly bite led to the same phenotype in the foot joints of TOSV-mCherry infected mice. *Ifnar1-/-* mice skin were exposed two up to 4 sandflies bite then 100,000 PFU of TOSV-mCherry was injected into the bitten part of the skin. At 6 days post-infection

(dpi), inflamed foot joints were observed (Figure 5.15); the skin and foot joint samples were then processed into single cells for FACS analysis.

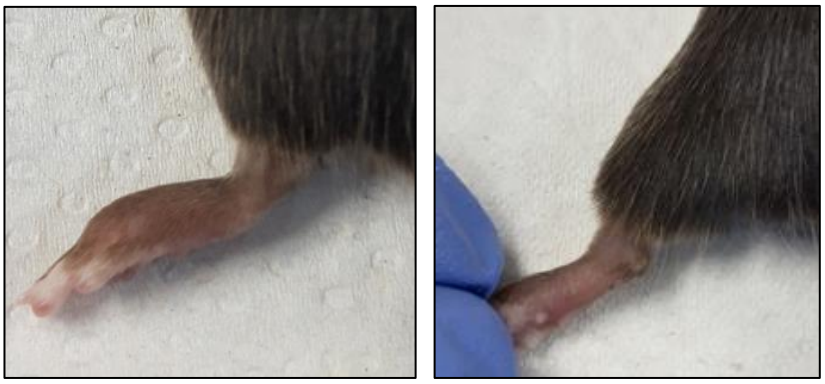


Figure 5.15: Sandfly Bites Co-inoculated with Genetically Modified TOSV Cause Foot Joint Inflammation by 6 days post infection

At least two to four *Lutzomyia longipalpis* sandflies were allowed to bite the upper left foot skin until fully engorged. Subsequently, 100,000 PFU of TOSV-mCherry was injected subcutaneously directly at the bite site. Skin and foot joint samples were collected from infected *ifnar1-/-* mice six days post-infection.

In this section, we also wanted to investigate the infected cells of the foot joint caused by TOSV with sandfly bites at 6dpi. In foot joint samples, the number of leukocytes changed moderately in mice receiving a bite (Figure 5.16B) compared to resting foot joint tissue (Figure 5.16A).

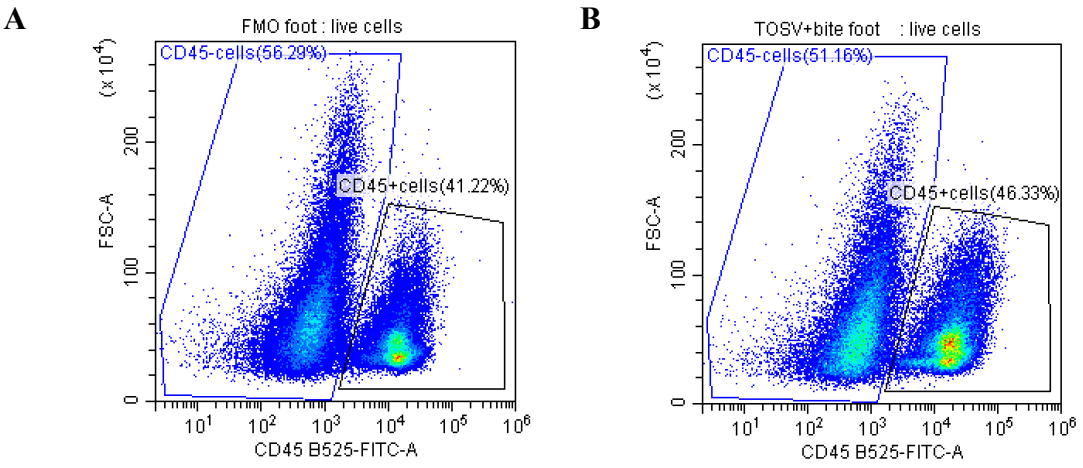


Figure 5.16: Sandfly Bite Causes the Influx of Leukocytes into the Foot Joint Tissue

Ifnar1-/- mice were either left uninfected or exposed to *Lutzomyia longipalpis* sandfly bites and then immediately infected with 100,000 PFU of TOSV-mCherry. Foot joint tissues were digested at 6dpi after observing foot joint inflammation and stained with CD45. **(A-B)** Gating strategy defining the population of leukocyte cells (CD45+) and stromal cells (CD45-) in uninfected control and TOSV-infected bitten skin, respectively.

Like our findings above in skin, we found that inflamed foot joints did not contain TOSV-infected neutrophils (Figure 5.17A). However, our analyses indicated that a large frequency of macrophages were infected at 6dpi, when foot joint inflammation was the most severe (Figure 5.17B). TOSV-infected fibroblasts and DC were present, (Figure 5.17C), but they were quite rare.

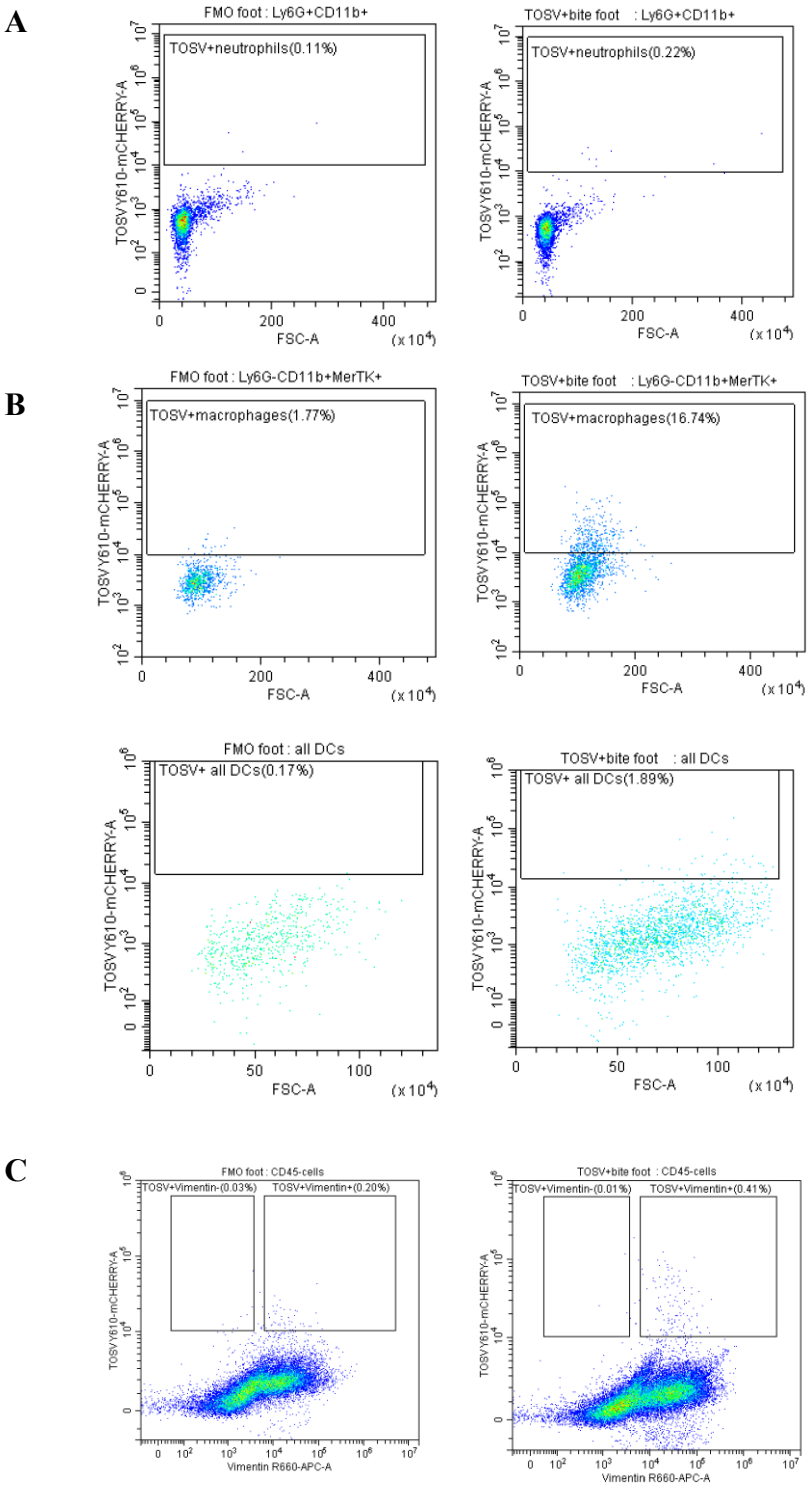


Figure 5.17: Changes in Leukocytes and Stromal Cells Frequencies in Inflamed Foot Joint at 6dpi

(A) Gating strategy used to define the population of TOSV+ neutrophils in foot joint compared to the uninfected Fluorescence Minus One (FMO) control that lacked infection with TOSV-mCherry, **(B)** illustrates TOSV+ macrophages/DCs in foot joint compared to the uninfected FMO control, and **(C)** shows TOSV+ vimentin+ cells in foot joint compared to the uninfected FMO control. Results belonged to 1 repeat of experiment (n=2 mice).

5.8 SUMMARY AND CONCLUSIONS

In this chapter, we explored the cellular targets of TOSV in the skin and examined how sandfly salivary gland extract or sandfly bites influence the cellular tropism of the virus. Many arboviruses have the capacity to infect keratinocytes, fibroblasts, and immune cells (mostly defined as by *in vitro* studies). Replication in these cells to high quantities in the skin facilitates virus spread throughout the host for a number of mosquito-borne viruses. However, we have limited knowledge as to whether TOSV shares a similar cellular tropism in the skin.

Initially, based on studies of mosquito-borne viruses, we hypothesised that inflammatory responses to sandfly saliva or their bites enhanced TOSV infection by promoting infection of inflammatory cells recruited to the skin. To investigate this hypothesis, we developed novel antibody panels for flow cytometry analysis. Furthermore, we successfully infected *ifnar1-/-* mice with genetically modified TOSV expressing mCherry, allowing us to identify TOSV-infected cells in multiple FACS experiments following optimization of our methods.

Our findings indicated that TOSV infects both leukocytes and stromal cells in the skin. Interestingly, we observed no infection of neutrophils with TOSV alone or co-infected with sandfly SGE or bites. While monocytes and dendritic cells (DCs) showed varying levels of infection with TOSV alone or with sandfly SGE/bites, macrophages were the primary leukocyte population infected by TOSV. Additionally, we found no infection of endothelial or epithelial cells. However, TOSV was capable of infecting dermal fibroblasts, as demonstrated by infection of vimentin+ cells, a marker classically associated with fibroblasts (Rinkevich et al., 2015). Next, we wanted to investigate which fibroblast subpopulations are permissive to TOSV infection and whether more primitive fibroblasts exhibit differing susceptibilities. Notably, we found that TOSV preferentially infected fibroblasts that were positive for CD90.2 and Sca1, fibroblast progenitor markers. In addition, CD140a ve+ cells were getting infected significantly with TOSV. The typical markers of proliferating stromal cells (CD140 and Sca1) indicate those cells that have the potential to differentiate and tend to be present more frequently during tissue repair. TOSV also infected Podoplanin ve+ and ve- cells. Important to note that podoplanin is also on lymphatic endothelial cells, but we have excluded these cells by using the lineage negative gate (CD45-CD31-EpCAM-), so we are confident that podoplanin in these cells is indicative of activated fibroblasts. Importantly, when we only assessed mCherry hi cells, the frequency of infected

leukocytes was low, compared to fibroblasts. This suggested that fibroblasts more efficiently replicate TOSV than leukocytes.

This inference was further supported by the MACS separation study, which examined the quantity of infectious virus generated by the most frequently TOSV-infected leukocyte (macrophages) and the non-macrophage fraction. This indicated that infected macrophages have limited ability to produce new infectious virus compared to non-macrophage fractions, which our flow data indicated were almost exclusively fibroblasts. Therefore, we concluded fibroblasts generate more infectious virus. This finding suggests that assessing intensity of fluorophore signal in flow cytometry, may not be the best method for investigating TOSV cell tropism in mCherry-positive cells.

Regarding the effect of SGE on these cell types, we observed no difference in frequency or geomean FI across all marker gates except for lineage-positive cells. However, these cells represent a minority of the TOSV-infected cells present, suggesting minimal impact on overall TOSV titers in the skin. Important to note that, some results suggested that sandfly SGE increased frequency of TOSV infection in leukocytes and stromal cells compared to infection with TOSV alone. However, subsequent experiments did not replicate this finding, possibly due to selected incorrect time point and variations in TOSV-mCherry replication kinetics.

We have two hypotheses to explain why the enhancement of TOSV infection is not apparent in our flow data. The first is that each infected cell generates more virus in the presence of sandfly SGE. Second, we also suggest that sandfly bites/or SGE trigger a wound healing response, resulting in higher frequency of more primitive cells (e.g., Sca-1 +ve cells), which are more susceptible to TOSV infection. Certainly, our data indicates TOSV preferentially infects those fibroblasts with more primitive/activated markers.

Based on these new results **we now hypothesise that SGE may cause a change in fibroblast biology that inadvertently increases susceptibility to virus infection.** Furthermore, despite no significant difference between TOSV alone and TOSV with the sandfly SGE group in FACS experiments that investigated mCherry positivity, **we suggest that each infected cell may generate more virus if also stimulated with SGE.** Further experimental evidence is required to justify this new hypothesis.

We also demonstrated that sandfly bites induce inflammation and enhance leukocyte influx into the skin at inoculation sites compared to resting skin.

Interestingly, a recently published study demonstrated that the mosquito-borne bunyavirus La Crosse virus (LACV) targets muscle cells of the panniculus carnosus (PC) in the skin (Schneider et al., 2024). In our model of foot skin, lacking a muscle layer, TOSV infection was primarily observed in fibroblasts and macrophages. Although we did not completely rule out a role for muscle cells in our FACS panels, this discrepancy underscores the importance of understanding the specific cellular targets and pathogenesis of different bunyaviruses in diverse tissue contexts. Additionally, it should be noted that human skin lacks a muscle layer, making our findings more pertinent for human disease models.

CHAPTER 6: OVERALL DISCUSSION

6.1 INTRODUCTION

The present thesis aimed to elucidate the interactions between the Toscana virus (TOSV), its sandfly vector, and the mammalian host, focusing on the role of sandfly salivary components in modulating viral infection and pathogenesis. Despite TOSV's significance as a neuroinvasive pathogen causing meningitis and encephalitis, research on its transmission dynamics and host-pathogen interactions has been limited. This knowledge gap is particularly evident in the context of skin infection sites, where arthropod-derived factors may play a crucial role.

Arboviruses, including TOSV, are almost exclusively transmitted through the bites of hematophagous arthropods such as mosquitoes, ticks, and sandflies. These vectors introduce not only the virus but also a complex mixture of salivary components that can significantly influence host immune responses. Previous studies have shown that mosquito-derived factors can enhance viral replication and dissemination by inducing local inflammation and recruiting susceptible immune cells to the bite site (Pingen et al., 2016). Similar mechanisms have been observed with tick-borne viruses, where tick saliva exacerbates disease severity (Hermance and Thangamani, 2015).

Sandflies, as vectors of various pathogens, have been extensively studied in the context of *Leishmania* parasites. Sandfly bites elicit strong local inflammatory responses, characterised by the rapid infiltration of neutrophils followed by macrophages, creating a favourable environment for parasite establishment (Peters et al., 2008). However, the impact of sandfly saliva on viral transmission, particularly at the skin inoculation site, remains unstudied.

This thesis aimed to increase our understanding of the relationship between TOSV and its vector components. More specifically we were interested in investigating how sandfly salivary components are responsible for the pathogenesis of TOSV on a mammalian host. In addition, we tested whether these sandfly salivary factors play a pan-proviral enhancement effect on genetically different arboviruses. It is important to understand whether this is the case if we are to use this knowledge to develop a treatment targeting multiple arbovirus infections. Finally, we looked at cellular tropism of TOSV in the skin inoculation site. The early virus replication and dissemination in the bite area are strongly related to outcome of the infection.

Therefore, the overall aims of this thesis were:

1. **To establish a unique TOSV infection model that incorporate sandfly bite and/or salivary components.**

2. **To investigate whether the ability of sandfly bite and salivary compounds can modulate host susceptibility to arbovirus infection, primarily TOSV.**

3. **To define the cellular tropism of TOSV in the skin and whether sandfly-derived factors affect these phenomena.**

6.2 A MOUSE MODEL FOR TOSV INFECTION WAS ESTABLISHED

Chapter 3 focused on optimizing several techniques to investigate TOSV infection at the vector-host interface. We evaluated whether *in vitro* infection models could reliably test our hypothesis. In addition, we tested whether sandfly salivary gland extract (SGE) enhances arbovirus infection using a well-established mouse model (SFV). Importantly, we also undertook crucial steps to establish a tractable animal model for TOSV infection. Therefore, our main aims of this chapter were:

1. **To optimise plaque and qPCR assays for determining infectious TOSV titres and measuring TOSV RNA levels in infected cells and mouse tissues**

2. **To develop an *in vitro* system for TOSV infection of primary skin cell cultures**

3. **To determine whether sandfly SGE modulates arbovirus infection *in vivo***

4. **To establish an *in vivo* model for TOSV infection**

Initially, we optimised TOSV primers and standards for qPCR analysis, enabling precise measurement of TOSV RNA in mouse tissues and infected cells. Additionally, we advanced plaque assays to determine the titers of both wild-type and genetically modified TOSV. These foundational optimizations were essential in ensuring the accuracy and reliability of our experimental results.

We developed an *in vitro* model system to study the potential enhancement of TOSV infection by sandfly SGE. Utilizing murine primary cell cultures for dermal fibroblasts, macrophages, and dendritic cells, we demonstrated that, *in vitro*, sandfly SGE did not consistently enhance TOSV infection in those cells. Additionally, the *ifn-β*, *ccl2*, and *cxcl2* expression during TOSV infection with or without sandfly SGE were variable and inconsistent across experiments. These findings suggested that if sandfly saliva can enhance arbovirus infection, it likely requires an *in vivo* system. This is similar to how mosquito saliva only enhances arbovirus in *in vivo* models of arbovirus infection (Lefteri et al., 2022).

To test whether sandfly SGE could enhance arbovirus infection, we firstly used Semliki Forest virus (SFV) infection, as a well-established mouse model, in which infection is enhanced in a mammalian host by mosquito saliva. The findings showed that sandfly SGE could indeed enhance SFV infection in the mammalian host, and this enhancement occurred independently of the host's type I IFN response. Furthermore, we showed sandfly SGE modulates the induction of innate immune genes. Encouraged by these results, we proceeded to investigate whether sandfly SGE could similarly enhance TOSV infection in mice.

Initial optimization experiments showed low but reproducible levels of TOSV RNA in the skin, lymph nodes, and spleen. We hypothesised that the limitation of TOSV replication in wild type mice might be due to an efficient activation of the type I IFN response. Indeed, when IFN signalling was temporarily repressed through administration of a blocking antibody, TOSV RNA expression increased in the lymph nodes and skin compared to IFN-sufficient mice. Interestingly, mosquito bites did not enhance TOSV infection in wild type mice. Together this suggests that inefficient IFN signalling antagonism by TOSV in mouse cells might be limiting TOSV replication.

Following an extensive search of the literature, we found only one published study describing TOSV infection in mice. This had used TOSV derived from Vero cells for mouse infection (Grazia Cusi et al., 2005), which suggested that the cell line from which the virus is derived could influence its infectivity in mice. Therefore, we infected mice with TOSV derived from Vero cells and assessed viral levels at 48- and 72-hours post-infection. Although virus RNA was detectable in the skin, lymph nodes, and spleen, the quantities were low, and none of the mice became viremic. These findings indicated that IFN-sufficient mice do not support efficient TOSV replication.

To overcome this challenge, we developed a TOSV mouse model using *ifnar1-/-* mice, which lack a functional type I interferon receptor. These genetically deficient mice exhibited a pronounced and permanent lack of IFN signalling, allowing for higher levels of TOSV replication. We infected the mice by subcutaneously injecting TOSV into skin on the upper side of the foot, resembling natural arbovirus transmission (not the foot pad). At 72 hours post-infection, high levels of TOSV RNA were detected in the skin and spleen tissues, but not in the lymph nodes, suggesting that TOSV may not utilise lymph node infection for dissemination. Although viral replication in lymph nodes is commonly observed in Flaviviruses, a recent study has also indicated that La Crosse Virus (LACV), a bunyavirus, does not primarily proliferate in lymph nodes (Schneider et al., 2024). Additionally, TOSV modulated the host inflammatory response by upregulating the expression of innate immune genes.

In conclusion, in Chapter 3, we successfully established an *in vivo* mouse model for TOSV infection using *ifnar1-/-* mice, which is essential for studying TOSV pathogenesis and the role of sandfly-derived factors in modulating viral infection. The optimization of plaque assays, qPCR techniques provided a solid foundation for these investigations. Our findings highlight the complexity of TOSV interactions with the host immune system and underscore the importance of using appropriate animal models to study viral pathogenesis. The establishment of this novel *in vivo* model represents a significant advancement in TOSV research and provides a valuable tool for future studies on arbovirus-vector-host interactions.

6.3 INCLUSION OF SANDFLY SALIVARY GLAND EXTRACT (SGE) OR SANDFLY BITES WORSEN TOSV INFECTION IN A MAMMALIAN HOST

In this thesis, we sought to define whether mammalian host susceptibility to TOSV is altered by host response to sandfly salivary factors. Chapter 4 of this thesis aimed to elucidate the role of sandfly SGE and sandfly bites in enhancing TOSV infection, define the pathogenesis of TOSV in a mammalian host, and explore the potential involvement of microbiota in modulating saliva-induced TOSV infection. Therefore, our main aims of this chapter were:

1. **To examine whether sandfly SGE and bites enhance TOSV infection *in vivo.***

2. **To investigate the pathogenesis of TOSV infection in a mouse model.**

3. **To explore the potential role of microbiota of saliva in contributing to saliva-induced TOSV infection *in vivo.***

We developed a mouse model for TOSV infection incorporating sandfly SGE in the inoculum to investigate its impact on viral infection. Our findings revealed that sandfly SGE from *Phlebotomus pernicious* and *Lutzomyia longipalpis* significantly increased host susceptibility to TOSV infection. This enhancement effect was dose-dependent, with higher amounts of SGE leading to a substantial increase in viral load at both the inoculation site and peripheral tissues, with more viral RNA detected at later time points. The same phenotype was observed with sandfly bites, even though the sandfly species, *Lu. longipalpis,* used is not known as a natural vector of TOSV, with each endemic area being geographically separate. This highlights the broad pro-viral effect of sandfly saliva components and that they are not vector species specific. Also, sandfly SGE injections or bites caused the upregulation of key inflammatory genes, including *cxcl2*, *ccl2*, and *il-6*, at different levels in skin and foot joint samples.

We further investigated the impact of sandfly SGE on the dissemination and pathogenesis of TOSV in our mouse model. We concluded that TOSV replicated and disseminated to the foot joints and brain, with sandfly SGE exacerbating the infection and leading to more severe outcomes. These included increased inflammation in the foot joints and brain, and higher viral loads in the brain, indicating that TOSV can cause CNS infection in mice, mirroring its neurotropic nature in humans. The inflammatory response in the foot joints, characterised by significant upregulation of chemokines and cytokines, was more pronounced with the inclusion of sandfly SGE. The histopathological evaluation also demonstrated this enhanced inflammatory response, showing accumulation of leukocytes in the dermis and among muscle fibres, along with associated tissue damage.

The specific factors within sandfly SGE or bites that enhance viral infection remain unknown. However, we do know that sandfly salivary components are numerous and highly diverse, primarily aimed at facilitating blood meals from vertebrate hosts. While it is possible that a single sandfly salivary factor, akin to sialokinin from *Aedes* mosquitoes, could significantly enhance viral infection, our focus in this thesis was on understanding the general nature of sandfly salivary components involved in this enhancement. Previous research has shown that mosquito saliva can enhance viral infection through sialokinin-dependent vascular leakage (Lefteri et al., 2022).

Lastly, we assessed whether microbiota in sandfly saliva influenced TOSV infection. High concentrations of the pro-inflammatory agents LPS and Pam3CSK4 enhanced TOSV infection, but the low levels of LPS found in SGE were insufficient to enhance infection. By using an antibiotic cocktail to render sandflies abiotic, we demonstrated that the microbiota in sandfly saliva did not significantly influence virus infection. Both microbiota-depleted and untreated sandfly bites similarly enhanced TOSV infection.

Chapter 4 provided comprehensive insights into sandfly SGE, and bites enhance TOSV infection in a mammalian host. The findings highlighted the critical role of sandfly-derived factors in modulating viral infection and the host immune response, thereby exacerbating disease outcomes. The broad pro-viral effect of sandfly saliva, irrespective of the natural vector, and the absence of impact of salivary microbiota underscore the complexity of vector-host-pathogen interactions.

6.4 TOSV ENCODED MCHERRY WAS DETECTED IN BOTH MACROPHAGES AND PRIMITIVE/ACTIVATED FIBROBLASTS

Chapter 5 focused on identifying the cutaneous cellular targets of TOSV and examining how sandfly SGE or sandfly bites might modulate these interactions. Our investigation **aimed to fill significant gaps in our understanding of the cellular tropism of TOSV and the influence of vector-derived factors on viral infection dynamics.**

Initially, we developed an animal model that incorporated infection with the genetically modified TOSV expressing mCherry reporter gene. Thus, it allowed us to collect infected skin cells. Next, we optimised FACS antibody panels, including various cell surface and intracellular markers, to dissect TOSV-infected cell populations precisely.

Our research demonstrated that TOSV infects leukocytes and stromal cells in the skin inoculation site. Notably, we found that neutrophils were not susceptible to TOSV infection, regardless of the presence of sandfly SGE or bites. In contrast, macrophages emerged as the primary leukocyte population infected by TOSV, with varying levels of infection observed in monocytes and dendritic cells. Importantly, our data indicated that endothelial and epithelial cells were not infected by TOSV; instead, dermal fibroblasts were susceptible. Importantly, we discovered that infected fibroblasts produce new infectious virus more efficiently than infected macrophages. This indicates that fibroblasts play a role in TOSV replication and dissemination within the host.

Due to the inflammatory features of sandfly bites and sandfly SGE, we observed an influx of leukocytes into the skin inoculation site compared to resting skin. Therefore,

we found that some of them were also infected with TOSV. However, except for one experiment result, we did not see a clear rise in the frequency of TOSV-infected leukocytes or stromal cells in those mice receiving SGE, compared to TOSV alone injection.

Our investigation into fibroblast subpopulations showed that TOSV almost exclusively infects fibroblasts that are positive for CD90.2 and Sca1, markers associated with fibroblast progenitors. Cells that express these markers are known for their heightened capacity for aiding tissue repair, wound healing, and tissue remodelling. We found that the mCherry-positive cells were also positive for CD140a and podoplanin markers, which are found in differentiated cells and have roles in the synthesis and remodelling of the extracellular matrix. These findings suggest that TOSV targets primitive/activated fibroblasts, which are more prevalent during tissue repair.

Indeed, these findings suggest that TOSV-associated clinical signs (swollen feet and muscle pathology) match the observed viral cell tropism. Something similar is observed with CHIKV infection of ifnar-1 null mice. Here, CHIKV antigens were observed in fibroblasts of the deep dermis in the human and mouse skin, as well as in fibroblasts located beneath the synovial wall of the joint connective tissue. This study suggested that CHIKV primarily targets fibroblasts, which may explain its preference for joints and skin connective tissues (Couderc et al., 2008). TOSV also disseminated to the brain, resulting in expression of inflammatory cytokines. Defining which cells TOSV infects in neural tissue will aid our understanding of TOSV infection.

The main conclusion from Chapter 5 emphasises the critical role of fibroblasts in TOSV replication and dissemination within vertebrate hosts.

6.5 CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis, we have demonstrated for the first time that sandfly SGE and sandfly bites enhance arbovirus infection, including SFV, an alphavirus, and TOSV, a bunyavirale. Our research has significantly advanced the understanding of the complex interactions between TOSV, its sandfly vector, and the mammalian host, with a particular focus on the role of sandfly salivary components in modulating viral infection and pathogenesis.

By establishing robust *in vivo* models and optimizing key experimental techniques, we have shown that sandfly SGE/bites exacerbates TOSV infection. This leads to more severe disease outcomes, including increased inflammation and higher viral loads in the skin inoculation site and the brain and foot joints. Additionally, our findings suggest that

the enhancement effect of sandfly saliva is not specific to a particular vector species but rather a general pro-viral phenomenon.

We have shown a critical role of dermal fibroblasts in TOSV replication that is key for its dissemination. Regarding the sandfly SGE effect on the cutaneous cellular targets of TOSV, our flow cytometry analysis gave some important insights. Together this data suggests that SGE and sandfly biting could re-model fibroblast biology so that there are more primitive, a necessary step for wound healing to commence, but thereby also putatively make them more efficient at replicating virus. However, further work is required to determine exactly how sandfly SGE enhances susceptibility to TOSV infection in these cells. One such future approach could include experiments in which once isolates cells from mice infected with TOSV alone and co-infected with sandfly SGE; then after normalising the cell numbers, we could culture them and determine whether the cells infected with TOSV and sandfly SGE release more infectious virus on a per-cell basis using plaque assay technique.

To conclude, I would argue that this research underscores the importance of considering vector-derived factors in the study of arbovirus pathogenesis and highlights the potential for targeting these interactions in developing novel therapeutic and vaccine strategies.

REFERENCES

Abbink, P., Stephenson, K.E. and Barouch, D.H. 2018. Zika virus vaccines. *Nature Reviews Microbiology*. **16**(10), pp.594–600.

Abdeladhim, M., V. Coutinho-Abreu, I., Townsend, S., Pasos-Pinto, S., Sanchez, L., Rasouli, M., B. Guimaraes-Costa, A., Aslan, H., Francischetti, I.M.B., Oliveira, F., Becker, I., Kamhawi, S., Ribeiro, J.M.C., Jochim, R.C. and Valenzuela, J.G. 2016. Molecular Diversity between Salivary Proteins from New World and Old World Sand Flies with Emphasis on Bichromomyia olmeca, the Sand Fly Vector of Leishmania mexicana in Mesoamerica E. Ghedin, ed. *PLOS Neglected Tropical Diseases*. **10**(7), p.e0004771.

Abdeladhim, M., Jochim, R.C., Ben Ahmed, M., Zhioua, E., Chelbi, I., Cherni, S., Louzir, H., Ribeiro, J.M.C. and Valenzuela, J.G. 2012. Updating the salivary gland transcriptome of Phlebotomus papatasi (Tunisian strain): the search for sand fly-secreted immunogenic proteins for humans. *PloS one*. **7**(11), pp.1–16.

Abdeladhim, M., Kamhawi, S. and Valenzuela, J.G. 2014. What's behind a sand fly bite? The profound effect of sand fly saliva on host hemostasis, inflammation and immunity. *Infection, Genetics and Evolution*. **28**, pp.691–703.

Acosta-Ampudia, Y., Monsalve, D.M., Castillo-Medina, L.F., Rodríguez, Y., Pacheco, Y., Halstead, S., Willison, H.J., Anaya, J.M. and Ramírez-Santana, C. 2018. Autoimmune neurological conditions associated with zika virus infection. *Frontiers in Molecular Neuroscience*. **11**(April).

Adler, S. and Theodor, O. 1926. The Mouth Parts, Alimentary Tract, and Salivary Apparatus of the Female in Phlebotomus Papatasii. *Annals of Tropical Medicine & Parasitology*. **20**(1), pp.109–142.

Afonso, M.M.D.S., Duarte, R., Miranda, J.C., Caranha, L. and Rangel, E.F. 2012. Studies on the feeding habits of Lutzomyia (Lutzomyia) longipalpis (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) populations from endemic areas of American Visceral Leishmaniasis in Northeastern Brazil. *Journal of Tropical Medicine*. **2012**.

Agarwal, A., Joshi, G., Nagar, D.P., Sharma, A.K., Sukumaran, D., Pant, S.C., Parida, M.M. and Dash, P.K. 2016. Mosquito saliva induced cutaneous events augment Chikungunya virus replication and disease progression. *Infection, Genetics and Evolution*. **40**, pp.126–135.

Ahata, B. and Akçapınar, G.B. 2023. CCHFV vaccine development, current challenges, limitations, and future directions. *Frontiers in Immunology*. **14**(September), pp.1–18.

Akhoundi, M., Kuhls, K., Cannet, A., Votýpka, J., Marty, P., Delaunay, P. and Sereno, D. 2016. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies A.-L. Bañuls, ed. *PLOS Neglected Tropical Diseases*. **10**(3), p.e0004349.

Akkaya, M., Kwak, K. and Pierce, S.K. 2020. B cell memory: building two walls of protection against pathogens. *Nature Reviews Immunology*. **20**(4), pp.229–238.

Albornoz, A., Hoffmann, A.B., Lozach, P.Y. and Tischler, N.D. 2016. Early bunyavirus-host cell interactions. *Viruses*. **8**(5).

Alexander, A.J.T., Confort, M.P., Desloire, S., Dunlop, J.I., Kuchi, S., Sreenu, V.B., Mair, D., Wilkie, G.S., da Silva Filipe, A., Brennan, B., Ratinier, M., Arnaud, F. and Kohl, A. 2020. Development of a reverse genetics system for Toscana virus (Lineage A). *Viruses*. **12**(4), pp.1–15.

Alho, A.M., Pita, J., Amaro, A., Amaro, F., Schnyder, M., Grimm, F., Custódio, A.C., Cardoso, L., Deplazes, P. and De Carvalho, L.M. 2016. Seroprevalence of vector-borne pathogens and molecular detection of Borrelia afzelii in military dogs from Portugal. *Parasites and Vectors*. **9**(1), pp.1–6.

Alkan, C., Allal-Ikhlef, A.B., Alwassouf, S., Baklouti, A., Piorkowski, G., de Lamballerie, X., Izri, A. and Charrel, R.N. 2015. Virus isolation, genetic characterization and seroprevalence of Toscana virus in Algeria. *Clinical Microbiology and Infection*. **21**(11), 1040.e1-1040.e9.

Alkan, C., Bichaud, L., De Lamballerie, X., Alten, B., Gould, E.A. and Charrel, R.N. 2013. Sandfly-borne phleboviruses of Eurasia and Africa: Epidemiology, genetic diversity, geographic range, control measures. *Antiviral Research*. **100**(1), pp.54–74.

Alkan, C., Moin Vaziri, V., Ayhan, N., Badakhshan, M., Bichaud, L., Rahbarian, N., Javadian, E.A., Alten, B., de Lamballerie, X. and Charrel, R.N. 2017. Isolation and sequencing of Dashli virus, a novel Sicilian-like virus in sandflies from Iran; genetic and phylogenetic evidence for the creation of one novel species within the Phlebovirus genus in the Bunyaviridae family. *PLoS Neglected Tropical Diseases*. **11**(12), pp.1–22.

Al-numaani, S.A., Al-Nemari, A.T., El-Kafrawy, S.A., Hassan, A.M., Tolah, A.M., Alghanmi, M., Zawawi, A., Masri, B.E., Hindawi, S.I., Alandijany, T.A., Bajrai, L.H., Bukhari, A., Mahmoud, A.B., Al Salem, W.S., Algaissi, A., Charrel, R.N., Azhar, E.I. and Hashem, A.M. 2023. Seroprevalence of Toscana and sandfly fever Sicilian viruses in humans and livestock animals from western Saudi Arabia. *One Health*. **17**(July), p.100601.

Alspach, E., Lussier, D.M. and Schreiber, R.D. 2019. Interferon γ and its important roles in promoting and inhibiting spontaneous and therapeutic cancer immunity. *Cold Spring Harbor Perspectives in Biology*. **11**(3), pp.1–20.

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J. and de Boer, M. 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE*. **7**(5).

Alwassouf, S., Christodoulou, V., Bichaud, L., Ntais, P., Mazeris, A., Antoniou, M. and Charrel, R.N. 2016. Seroprevalence of Sandfly-Borne Phleboviruses Belonging to Three Serocomplexes (Sandfly fever Naples, Sandfly fever Sicilian and Salehabad) in Dogs from Greece and Cyprus Using Neutralization Test. *PLoS Neglected Tropical Diseases*. **10**(10), pp.1–13.

Alwassouf, S., Maia, C., Ayhan, N., Coimbra, M., Cristovao, J.M., Richet, H., Bichaud, L., Campino, L. and Charrel, R.N. 2016. Neutralization-based seroprevalence of toscana virus and sandfly fever sicilian virus in dogs and cats from Portugal. *Journal of General Virology*. **97**(11), pp.2816–2823.

Amarante-Mendes, G.P., Adjemian, S., Branco, L.M., Zanetti, L.C., Weinlich, R. and Bortoluci, K.R. 2018. Pattern recognition receptors and the host cell death molecular machinery. *Frontiers in Immunology*. **9**(OCT), pp.1–19.

AMARO, F., LUZ, T., PARREIRA, P., MARCHI, A., CIUFOLINI, M.G. and ALVES, M.J. 2012. Serological evidence of Toscana virus infection in Portuguese patients. *Epidemiology and Infection*. **140**(6), pp.1147–1150.

Amaro, F., Zé-Zé, L., Luz, M.T. and Alves, M.J. 2021. Toscana Virus: Ten Years of Diagnostics in Portugal. *Acta Médica Portuguesa*. **34**(10), pp.677–681.

Amodio, E., Cusi, M.G., Valenti, R.M., Valentini, M., Mammina, C., Gori-Savellini, G., Vitale, F., Romano, N., Goedert, J.J. and Calamusa, G. 2012. Immunoglobulin M seropositivity

for Toscana virus in a random population sample in Sicily. *International Journal of Infectious Diseases*. **16**(8), pp.e633–e635.

Amor, S., Scallan, M.F., Morris, M.M., Dyson, H. and Fazakerley, J.K. 1996. Role of immune responses in protection and pathogenesis during Semliki Forest virus encephalitis. *Journal of General Virology*. **77**(2), pp.281–291.

Amroun, A., Priet, S., de Lamballerie, X. and Quérat, G. 2017. Bunyaviridae RdRps: structure, motifs, and RNA synthesis machinery. *Critical Reviews in Microbiology*. **43**(6), pp.753–778.

Anagnostou, V. and Papa, A. 2013. Prevalence of antibodies to phleboviruses within the sand fly fever Naples virus species in humans, northern Greece. *Clinical Microbiology and Infection*. **19**(6), pp.566–570.

Anagnostou, Vassiliki and Papa, A. 2013. Seroprevalence of Toscana virus among residents of Aegean Sea islands, Greece. *Travel Medicine and Infectious Disease*. **11**(2), pp.98–102.

Anaguano, D.F., Ponce, P., Baldeón, M.E., Santander, S. and Cevallos, V. 2015. Blood-meal identification in phlebotomine sand flies (Diptera: Psychodidae) from Valle Hermoso, a high prevalence zone for cutaneous leishmaniasis in Ecuador. *Acta Tropica*. **152**, pp.116–120.

Andayi, F., Charrel, R.N., Kieffer, A., Richet, H., Pastorino, B., Leparc-Goffart, I., Ahmed, A.A., Carrat, F., Flahault, A. and de Lamballerie, X. 2014. A Sero-epidemiological Study of Arboviral Fevers in Djibouti, Horn of Africa. *PLoS Neglected Tropical Diseases*. **8**(12).

Anderson, J.F. 2002. The natural history of ticks. *Medical Clinics*. **86**(2), pp.205–218.

Andrews, J.M., Quinby, G.E. and Langmuir, A.D. 1950. Malaria eradication in the United States. *American journal of public health*. **40**(11), pp.1405–1411.

Aronson, N., Herwaldt, B.L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., Carvalho, E., Ephros, M., Jeronimo, S. and Magill, A. 2017. Diagnosis and treatment of leishmaniasis: Clinical practice guidelines by the infectious diseases society of America (IDSA) and the American Society of tropical medicine and hygiene (ASTMH). *American Journal of Tropical Medicine and Hygiene*. **96**(1), pp.24–45.

Ashley, E.A., Pyae Phyo, A. and Woodrow, C.J. 2018. Malaria. *The Lancet*. **391**(10130), pp.1608–1621.

Aspöck, H., Gerersdorfer, T., Formayer, H. and Walochnik, J. 2008. Sandflies and sandfly-borne infections of humans in Central Europe in the light of climate change. *Wiener Klinische Wochenschrift*. **120**(SUPPL. 4), pp.24–29.

Assumpção, T.C.F., Ma, D., Schwarz, A., Reiter, K., Santana, J.M., Andersen, J.F., Ribeiro, J.M.C., Nardone, G., Yu, L.L. and Francischetti, I.M.B. 2013. Salivary Antigen-5/CAP Family Members Are Cu2+-dependent Antioxidant Enzymes That Scavenge O2⁻· and Inhibit Collagen-induced Platelet Aggregation and Neutrophil Oxidative Burst. *Journal of Biological Chemistry*. **288**(20), pp.14341–14361.

Atkins, G.J., Sheahan, B.J. and Dimmock, N.J. 1985. Semliki Forest virus infection of mice: a model for genetic and molecular analysis of viral pathogenicity. *The Journal of general virology*. **66 (Pt 3)**, pp.395–408.

Atkins, G.J., Sheahan, B.J. and Liljeström, P. 1999. The molecular pathogenesis of Semliki Forest virus: A model virus made useful? *Journal of General Virology*. **80**(9), pp.2287–2297.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G. and Geissmann, F. 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. **317**(5838), pp.666–670.

Avirutnan, P., Fuchs, A., Hauhart, R.E., Somnuke, P., Youn, S., Diamond, M.S. and Atkinson, J.P. 2010. Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. *Journal of Experimental Medicine*. **207**(4), pp.793–806.

Ayhan, N., Baklouti, A., Prudhomme, J., Walder, G., Amaro, F., Alten, B., Moutailler, S., Ergunay, K., Charrel, R.N. and Huemer, H. 2017. Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part I: Important Points to Consider Ante Field Work. *Vector borne and zoonotic diseases (Larchmont, N.Y.)*. **17**(1), pp.73–80.

Ayhan, N. and Charrel, R.N. 2020. An update on Toscana virus distribution, genetics, medical and diagnostic aspects. *Clinical Microbiology and Infection*. **26**(8), pp.1017–1023.

Ayhan, N. and Charrel, R.N. 2018. Emergent Sand Fly–Borne Phleboviruses in the Balkan Region. *Emerging Infectious Diseases*. **24**(12), pp.2324–2330.

Ayhan, N. and Charrel, R.N. 2017. Of phlebotomines (sandflies) and viruses: a comprehensive perspective on a complex situation. *Current Opinion in Insect Science*. **22**, pp.117–124.

Ayhan, N., López-Roig, M., Monastiri, A., Charrel, R.N. and Serra-Cobo, J. 2021. Seroprevalence of toscana virus and sandfly fever sicilian virus in european bat colonies measured using a neutralization test. *Viruses*. **13**(1), pp.0–7.

Ayhan, N. and N. Charrel, R. 2019. Sandfly-Borne Viruses of Demonstrated/Relevant Medical Importance *In*: *Vectors and Vector-Borne Zoonotic Diseases* [Online]. IntechOpen, p.13. Available from: http://dx.doi.org/10.1039/C7RA00172J%0Ahttps://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics%0Ahttp://dx.doi.org/10.1016/j.colsurfa.2011.12.014.

Ayhan, N., Prudhomme, J., Laroche, L., Bañuls, A. and Charrel, R.N. 2020. Broader Geographical Distribution of Toscana Virus in the Mediterranean Region Suggests the Existence of Larger Varieties of Sand Fly Vectors. *Microorganisms*. **8**(1), p.114.

Ayhan, N., Rodríguez-Teijeiro, J.D., López-Roig, M., Vinyoles, D., Ferreres, J.A., Monastiri, A., Charrel, R. and Serra-Cobo, J. 2023. High rates of antibodies against Toscana and Sicilian phleboviruses in common quail Coturnix coturnix birds. *Frontiers in Microbiology*. **13**(January), pp.1–9.

Ayhan, N., Sherifi, K., Taraku, A., Bërxholi, K. and Charrel, R.N. 2017. High rates of neutralizing antibodies to toscana and sandfly fever sicilian viruses in livestock, Kosovo. *Emerging Infectious Diseases*. **23**(6), pp.989–992.

Azar, S.R., Campos, R.K., Bergren, N.A., Camargos, V.N. and Rossi, S.L. 2020. Epidemic alphaviruses: Ecology, emergence and outbreaks. *Microorganisms*. **8**(8), pp.1–35.

Bahri, O., Fazaa, O., Ben Alaya-Bouafif, N., Bouloy, M., Triki, H. and Bouattour, A. 2010. Rôle du virus Toscana dans les infections neuroméningées en Tunisie. *Pathologie Biologie*. **59**(6), pp.2010–2012.

Balaska, S., Fotakis, E.A., Chaskopoulou, A. and Vontas, J. 2021. Chemical control and insecticide resistance status of sand fly vectors worldwide. *PLoS Neglected Tropical Diseases*. **15**(8), pp.1–23.

Baldelli, F., Ciufolini, M.G., Francisci, D., Marchi, A., Venturi, G., Fiorentini, C., Luchetta, M.L., Bruto, L. and Pauluzzi, S. 2004. Unusual Presentation of Life-Threatening Toscana Virus Meningoencephalitis. *Clinical Infectious Diseases*. **38**(4), pp.515–520.

Bañuls, A.L., Bastien, P., Pomares, C., Arevalo, J., Fisa, R. and Hide, M. 2011. Clinical pleiomorphism in human leishmaniases, with special mention of asymptomatic infection. *Clinical Microbiology and Infection*. **17**(10), pp.1451–1461.

Bardina, S. V., Bunduc, P., Tripathi, S., Duehr, J., Frere, J.J., Brown, J.A., Nachbagauer, R., Foster, G.A., Krysztof, D., Tortorella, D., Stramer, S.L., García-Sastre, A., Krammer, F. and Lim, J.K. 2017. Enhancement of Zika virus pathogenesis by preexisting antiflavivirus immunity. *Science*. **356**(6334), pp.175–180.

Barras, V. and Greub, G. 2014. History of biological warfare and bioterrorism. *Clinical Microbiology and Infection*. **20**(6), pp.497–502.

Barrett, A.D.T. 2017. Yellow fever live attenuated vaccine : A very successful live attenuated vaccine but still we have problems controlling the disease. *Vaccine*. **35**(44), pp.5951–5955.

Bartels, S., Boni, L., Kretzschmar, H.A. and Heckmann, J.G. 2012. Lethal encephalitis caused by the Toscana virus in an elderly patient. *Journal of Neurology*. **259**(1), pp.175–177.

Barzon, L., Pacenti, M., Franchin, E., Pagni, S., Martello, T., Cattai, M., Cusinato, R. and Palù, G. 2013. Excretion of west nile virus in urine during acute infection. *Journal of Infectious Diseases*. **208**(7), pp.1086–1092.

Bautista-Hernández, L.A., Gómez-Olivares, J.L., Buentello-Volante, B. and Bautista-de Lucio, V.M. 2017. Fibroblasts: the unknown sentinels eliciting immune responses against microorganisms. *European Journal of Microbiology and Immunology*. **7**(3), pp.151–157.

Beard, J. 2006. DDT and human health. *Science of the Total Environment*. **355**(1–3), pp.78–89.

Beersma, M.F., Grimbergen, Y.A., Kroon, F.P. and Veldkamp, P.J. 2004. Meningitis caused by Toscana virus during a summer stay in Italy. *Nederlands Tijdschrift Voor Geneeskunde*. **148**(6), pp.286–288.

Belkaid, Y., Kamhawi, S., Modi, G., Valenzuela, J., Noben-Trauth, N., Rowton, E., Ribeiro, J. and Sacks, D.L. 1998. Development of a Natural Model of Cutaneous Leishmaniasis: Powerful Effects of Vector Saliva and Saliva Preexposure on the Long-Term Outcome of Leishmania major Infection in the Mouse Ear Dermis. *Journal of Experimental Medicine*. **188**(10), pp.1941–1953.

Bell-Sakyi, L. and Attoui, H. 2013. Endogenous tick viruses and modulation of tick-borne pathogen growth. *Frontiers in Cellular and Infection Microbiology*. **4**(JUL), pp.1–10.

Benbetka, C., Hachid, A., Benallal, K.E., Khardine, F.A., Ayhan, N., Bouredjoul, N., Boulehbal, W.M., Bellila, D., Khaldi, A. and Charrel, R. 2023. Epidemiology, Isolation, and Genetic Characterization of Toscana Virus in Algerian Patients Displaying Neurological Infection, 2016–2018. *IJID Regions*. **7**(March), pp.193–198.

Bendall, L. 2005. Chemokines and their receptors in disease. *Histology and Histopathology*. **20**(3), pp.907–926.

Benelli, G., Jeffries, C.L. and Walker, T. 2016. Biological control of mosquito vectors: Past, present, and future. *Insects*. **7**(4), pp.1–18.

Bente, D.A., Forrester, N.L., Watts, D.M., McAuley, A.J., Whitehouse, C.A. and Bray, M. 2013. Crimean-Congo hemorrhagic fever: History, epidemiology, pathogenesis, clinical syndrome and genetic diversity. *Antiviral Research*. **100**(1), pp.159–189.

Berdjane-Brouk, Z., Koné, A.K., Djimdé, A.A., Charrel, R.N., Ravel, C., Delaunay, P., del Giudice, P., Diarra, A.Z., Doumbo, S., Goita, S., Thera, M.A., Depaquit, J., Marty, P., Doumbo, O.K. and Izri, A. 2012. First detection of Leishmania major DNA in Sergentomyia (Spelaeomyia) darlingi from cutaneous leishmaniasis foci in Mali. *PLoS ONE*. **7**(1), pp.1–5.

Bergren, N. and Kading, R. 2018. The Ecological Significance and Implications of Transovarial Transmission among the Vector-Borne Bunyaviruses: A Review. *Insects*. **9**(4), p.173.

Bernareggi, D., Pouyanfard, S. and Kaufman, D.S. 2019. Development of innate immune cells from human pluripotent stem cells. *Experimental Hematology*. **71**, pp.13–23.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., Myers, M.F., George, D.B., Jaenisch, T., Wint, G.R.W., Simmons, C.P., Scott, T.W., Farrar, J.J. and Hay, S.I. 2013. The global distribution and burden of dengue. *Nature*. **496**(7446), pp.504–507.

Bichaud, L., Izri, A., de Lamballerie, X., Moureau, G. and Charrel, R.N. 2014. First detection of Toscana virus in Corsica, France. *Clinical Microbiology and Infection*. **20**(2), pp.O101–O104.

Bichaud, L., Souris, M., Mary, C., Ninove, L., Thirion, L., Piarroux, R.P., Piarroux, R., de Lamballerie, X. and Charrel, R.N. 2011. Epidemiologic relationship between Toscana virus infection and Leishmania infantum due to common exposure to Phlebotomus perniciosus sandfly vector. *PLoS Neglected Tropical Diseases*. **5**(9), pp.1–8.

Bird, J.A., Sánchez-Borges, M., Ansotegui, I.J., Ebisawa, M. and Ortega Martell, J.A. 2018. Skin as an immune organ and clinical applications of skin-based immunotherapy. *World Allergy Organization Journal*. **11**(1), p.38.

Boehm, T., Hess, I. and Swann, J.B. 2012. Evolution of lymphoid tissues. *Trends in immunology*. **33**(6), pp.315–321.

Bonilla, F.A. and Oettgen, H.C. 2010. Adaptive immunity. *Journal of Allergy and Clinical Immunology*. **125**(2 SUPPL. 2), pp.S33–S40.

Bonnet, S.I., Bertagnoli, S., Falchi, A., Figoni, J., Fite, J., Hoch, T., Quillery, E., Moutailler, S., Raffetin, A., René-Martellet, M., Vourc'h, G. and Vial, L. 2023. An Update of Evidence for Pathogen Transmission by Ticks of the Genus Hyalomma. *Pathogens*. **12**(4), pp.1–19.

Borregaard, N. 2010. Neutrophils, from Marrow to Microbes. *Immunity*. **33**(5), pp.657–670.

Van Bortel, W., Dorleans, F., Rosine, J., Blateau, A., Rousseau, D., Matheus, S., Leparc-Goffart, I., Flusin, O., Prat, C.M., Césaire, R., Najioullah, F., Ardillon, V., Balleydier, E., Carvalho, L., Lemaître, A., Noël, H., Servas, V., Six, C., Zurbaran, M., Léon, L., Guinard, A., van den Kerkhof, J., Henry, M., Fanoy, E., Braks, M., Reimerink, J., Swaan, C., Georges, R., Brooks, L., Freedman, J., Sudre, B. and Zeller, H. 2014. Chikungunya outbreak in the Caribbean region, December 2013 to March 2014, and the significance for Europe. *Eurosurveillance*. **19**(13), p.20759.

Borucki, M.K., Chandler, L.J., Parker, B.M., Blair, C.D. and Beaty, B.J. 1999. Bunyavirus superinfection and segment reassortment in transovarially infected mosquitoes. *Journal of General Virology*. **80**(12), pp.3173–3179.

Bowles, D., Britch, S., Linthicum, K. and Johnson, R. 2015. Sand Flies (Diptera: Psychodidae: Phlebotominae): Significance, Surveillance, and Control in Contingency Operations. *Armed Forces Pest Management Board*. (49), p.72.

Boylston, A. 2012. The origins of inoculation. *Journal of the Royal Society of Medicine*. **105**(7), pp.309–313.

Bradish, C.J., Allner, K. and Maber, H.B. 1971. The virulence of original and derived strains of Semliki forest virus for mice, guinea-pigs and rabbits. *The Journal of general virology*. **12**(2), pp.141–160.

Braito, Assunta, Ciufolini, M.G., Pippi, L., Corbisiero, R., Fiorentini, C., Gistri, A. and Toscano, L. 1998. Phlebotomus-transmitted Toscana virus infections of the central nervous system: A seven-year experience in Tuscany. *Scandinavian Journal of Infectious Diseases*. **30**(5), pp.505–508.

Braito, A., Corbisiero, R., Corradini, S., Fiorentini, C. and Ciufolini, M.G. 1998. Toscana virus infections of the central nervous system in children: A report of 14 cases. *Journal of Pediatrics*. **132**(1), pp.144–148.

Braito, A., Corbisiero, R., Corradini, S., Marchi, B., Sancasciani, N., Fiorentini, C. and Ciufolini, M.G. 1997. Evidence of Toscana virus infections without central nervous system involvement: A serological study. *European Journal of Epidemiology*. **13**(7), pp.761–764.

Brasil, P. and Gabaglia, C.R. 2019. The story of chikungunya virus. *The Lancet Infectious Diseases*. **19**(7), p.702.

Briant, L., Desprès, P., Choumet, V. and Missé, D. 2014. Role of skin immune cells on the host susceptibility to mosquito-borne viruses. *Virology*. **464–465**(1), pp.26–32.

Brisbarre, N., Plumet, S., Cotteaux-Lautard, C., Emonet, S.F., Pages, F. and Leparc-Goffart, I. 2015. A rapid and specific real time RT-PCR assay for diagnosis of Toscana virus infection. *Journal of Clinical Virology*. **66**, pp.107–111.

Broz, P. and Dixit, V.M. 2016. Inflammasomes: Mechanism of assembly, regulation and signalling. *Nature Reviews Immunology*. **16**(7), pp.407–420.

Bruce-Chwatt, L.J. and De Zulueta, J. 1980. *The rise and fall of malaria in Europe: a historico-epidemiological study.*

Bryden, S.R., Pingen, M., Lefteri, D.A., Miltenburg, J., Delang, L., Jacobs, S., Abdelnabi, R., Neyts, J., Pondeville, E., Major, J., Müller, M., Khalid, H., Tuplin, A., Varjak, M., Merits, A., Edgar, J., Graham, G.J., Shams, K. and McKimmie, C.S. 2020. Pan-viral protection against arboviruses by activating skin macrophages at the inoculation site. *Science Translational Medicine*. **12**(527), p.eaax2421.

Buchmann, K. 2014. Evolution of innate immunity: Clues from invertebrates via fish to mammals. *Frontiers in Immunology*. **5**(SEP), pp.1–8.

Bustos-Arriaga, J., García-Machorro, J., León-Juárez, M., García-Cordero, J., Santos-Argumedo, L., Flores-Romo, L., Méndez-Cruz, A.R., Juárez-Delgado, F.J. and Cedillo-Barrón, L. 2011. Activation of the Innate Immune Response against DENV in Normal Non-Transformed Human Fibroblasts B. A. Lopes da Fonseca, ed. *PLoS Neglected Tropical Diseases*. **5**(12), p.e1420.

Calamusa, G., Valenti, R.M., Vitale, F., Mammina, C., Romano, N., Goedert, J.J., Gori-Savellini, G., Cusi, M.G. and Amodio, E. 2012. Seroprevalence of and risk factors for Toscana and Sicilian virus infection in a sample population of Sicily (Italy). *Journal of Infection*. **64**(2), pp.212–217.

Calisher, C.H. and Calzolari, M. 2021. Taxonomy of phleboviruses, emphasizing those that are sandfly-borne†. *Viruses*. **13**(5), pp.1–7.

Calisher, CharlesH., Weinberg, ArnoldN., Muth, DavidJ. and Lazuick, JohnS. 1987. TOSCANA VIRUS INFECTION IN UNITED STATES CITIZEN RETURNING FROM ITALY. *The Lancet*. **329**(8525), pp.165–166.

Caminade, C., McIntyre, K.M. and Jones, A.E. 2019. Impact of recent and future climate change on vector-borne diseases. *Annals of the New York Academy of Sciences*. **1436**(1), pp.157–173.

Campino, L., Cortes, S., Dionísio, L., Neto, L., Afonso, M.O. and Maia, C. 2013. The first detection of Leishmania major in naturally infected Sergentomyia minuta in Portugal. *Memorias do Instituto Oswaldo Cruz*. **108**(4), pp.516–518.

Cardeñosa, N., Kaptoul, D., Fernández-Viladrich, P., Aranda, C., de Ory, F., Niubó, J., Plans, P., Domínguez, A., Fedele, G., Tenorio, A. and Sánchez-Seco, M.P. 2013. Toscana Virus Infection In Catalonia (Spain). *Vector-Borne and Zoonotic Diseases*. **13**(4), pp.273–275.

Carlton-Smith, C. and Elliott, R.M. 2012. Viperin, MTAP44, and Protein Kinase R Contribute to the Interferon-Induced Inhibition of Bunyamwera Orthobunyavirus Replication. *Journal of Virology*. **86**(21), pp.11548–11557.

Carregaro, V., Valenzuela, J.G., Cunha, T.M., Verri, W.A., Grespan, R., Matsumura, G., Ribeiro, J.M.C., Elnaiem, D.-E., Silva, J.S. and Cunha, F.Q. 2008. Phlebotomine salivas inhibit immune inflammation-induced neutrophil migration via an autocrine DC-derived PGE2/IL-10 sequential pathway. *Journal of Leukocyte Biology*. **84**(1), pp.104–114.

De Carvalho, M.S., De Lara Pinto, A.Z., Pinheiro, A., Rodrigues, J.S.V., Melo, F.L., Da Silva, L.A., Ribeiro, B.M. and Dezengrini-Slhessarenko, R. 2018. Viola phlebovirus is a novel Phlebotomus fever serogroup member identified in Lutzomyia (Lutzomyia) longipalpis from Brazilian Pantanal. *Parasites and Vectors*. **11**(1), pp.1–10.

Casals, J. 1969. Antigenic similarity between the virus causing Crimean hemorrhagic fever and Congo virus. *Proceedings of the Society for Experimental Biology and Medicine*. **131**(1), pp.233–236.

Cecílio, P., Cordeiro-da-Silva, A. and Oliveira, F. 2022. Sand flies: Basic information on the vectors of leishmaniasis and their interactions with Leishmania parasites. *Communications Biology*. **5**(1).

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A. and Colonna, M. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nature Medicine*. **5**(8), pp.919–923.

Cerny, D., Haniffa, M., Shin, A., Bigliardi, P., Tan, B.K., Lee, B., Poidinger, M., Tan, E.Y., Ginhoux, F. and Fink, K. 2014. Selective Susceptibility of Human Skin Antigen Presenting Cells to Productive Dengue Virus Infection. *PLoS Pathogens*. **10**(12).

Chalghaf, B., Chemkhi, J., Mayala, B., Harrabi, M., Benie, G.B., Michael, E. and Ben Salah, A. 2018. Ecological niche modeling predicting the potential distribution of Leishmania vectors in the Mediterranean basin: Impact of climate change. *Parasites and Vectors*. **11**(1), pp.1–9.

Champagne, D.E., Smartt, C.T., Ribeiro, J.M.C. and James, A.A. 1995. The salivary gland-specific apyrase of the mosquito Aedes aegypti is a member of the 5′-nucleotidase family. *Proceedings of the National Academy of Sciences of the United States of America*. **92**(3), pp.694–698.

Charrel, R.N. 2012. Emergence of Toscana virus in the mediterranean area. *World Journal of Virology*. **1**(5), p.135.

Charrel, R.N., Gallian, P., Navarro-Marí, J.-M., Nicoletti, L., Papa, A., Sánchez-Seco, M.P., Tenorio, A. and de Lamballerie, X. 2005. Emergence of Toscana Virus in Europe. *Emerging Infectious Diseases*. **11**(12), pp.1657–1663.

Charrel, R.N., Izri, A., Temmam, S., De Lamballerie, X. and Parola, P. 2006. Toscana virus RNA in Sergentomyia minuta flies. *Emerging Infectious Diseases*. **12**(8), pp.1299–1300.

Charrel, R.N., Moureau, G., Temmam, S., Izri, A., Marty, P., Parola, P., Da Rosa, A.T., Tesh, R.B. and De Lamballerie, X. 2009. Massilia virus, a novel phlebovirus (Bunyaviridae) isolated from sandflies in the Mediterranean. *Vector-Borne and Zoonotic Diseases*. **9**(5), pp.519–530.

Chastonay, A.H.M. and Chastonay, O.J. 2022. Housing Risk Factors of Four Tropical Neglected Diseases: A Brief Review of the Recent Literature. *Tropical Medicine and Infectious Disease*. **7**(7).

Chen, C., Chen, A. and Yang, Y. 2022. A diversified role for γδT cells in vector-borne diseases. *Frontiers in Immunology*. **13**(August), pp.1–10.

Chen, W., Foo, S.S., Zaid, A., Teng, T.S., Herrero, L.J., Wolf, S., Tharmarajah, K., Vu, L.D., Van Vreden, C., Taylor, A., Freitas, J.R., Li, R.W., Woodruff, T.M., Gordon, R., Ojcius, D.M., Nakaya, H.I., Kanneganti, T.D., O'Neill, L.A.J., Robertson, A.A.B., King, N.J., Suhrbier, A., Cooper, M.A., Ng, L.F.P. and Mahalingam, S. 2017. Specific inhibition of NLRP3 in chikungunya disease reveals a role for inflammasomes in alphavirus-induced inflammation. *Nature Microbiology*. **2**(10), pp.1435–1445.

Cheung, Y.P., Park, S., Pagtalunan, J. and Maringer, K. 2022. The antiviral role of NF-κB-mediated immune responses and their antagonism by viruses in insects. *Journal of General Virology*. **103**(5).

Chopp, L., Redmond, C., O'Shea, J.J. and Schwartz, D.M. 2023. From thymus to tissues and tumors: A review of T-cell biology. *Journal of Allergy and Clinical Immunology*. **151**(1), pp.81–97.

Chow, K.T., Gale, M. and Loo, Y.M. 2018. RIG-I and Other RNA Sensors in Antiviral Immunity. *Annual Review of Immunology*. **36**, pp.667–694.

Christova, I., Panayotova, E., Trifonova, I., Taseva, E., Gladnishka, T. and Ivanova, V. 2020. Serologic evidence of widespread Toscana virus infection in Bulgaria. *Journal of Infection and Public Health*. **13**(2), pp.164–166.

Chung, Y., Chang, S.H., Martinez, G.J., Yang, X.O., Nurieva, R., Kang, H.S., Ma, L., Watowich, S.S., Jetten, A.M., Tian, Q. and Dong, C. 2009. Critical Regulation of Early Th17 Cell Differentiation by Interleukin-1 Signaling. *Immunity*. **30**(4), pp.576–587.

Cildir, G., Akincilar, S.C. and Tergaonkar, V. 2013. Chronic adipose tissue inflammation: All immune cells on the stage. *Trends in Molecular Medicine*. **19**(8), pp.487–500.

Ciufolini, M.G., Maroli, M., Guandalini, E., Marchi, A. and Verani, P. 1989. Experimental studies on the maintenance of Toscana and Arbia viruses (Bunyaviridae: Phlebovirus). *The American journal of tropical medicine and hygiene*. **40**(6), pp.669–675.

Coccia, E.M., Severa, M., Giacomini, E., Monneron, D., Remoli, M.E., Julkunen, I., Cella, M., Lande, R. and Uzé, G. 2004. Viral infection and toll-like receptor agonists induce a differential expression of type I and λ interferons in humans plasmacytoid and monocyte-derived dendritic cells. *European Journal of Immunology*. **34**(3), pp.796–805.

Coillard, A. and Segura, E. 2019. In vivo differentiation of human monocytes. *Frontiers in Immunology*. **10**(AUG), pp.1–7.

Collin, M. and Milne, P. 2016. Langerhans cell origin and regulation. *Current Opinion in Hematology*. **23**(1), pp.28–35.

Collin, N., Assumpção, T.C.F., Mizurini, D.M., Gilmore, D.C., Dutra-Oliveira, A., Kotsyfakis, M., Sá-Nunes, A., Teixeira, C., Ribeiro, J.M.C., Monteiro, R.Q., Valenzuela, J.G. and Francischetti, I.M.B. 2012. Lufaxin, a novel factor Xa inhibitor from the salivary gland of the sand fly lutzomyia longipalpis blocks protease-activated receptor 2 activation and inhibits inflammation and thrombosis in vivo. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **32**(9), pp.2185–2196.

Collins, N.D. and Barrett, A.D.T. 2017. Live Attenuated Yellow Fever 17D Vaccine: A Legacy Vaccine Still Controlling Outbreaks In Modern Day. *Current Infectious Disease Reports*. **19**(3), p.14.

Colomba, C., Saporito, L., Ciufolini, M.G., Marchi, A., Rotolo, V., De Grazia, S., Titone, L. and Giammanco, G.M. 2012. Prevalence of Toscana sandfly fever virus antibodies in neurological patients and control subjects in Sicily. *New Microbiologica*. **35**(2), pp.161–165.

Coluzzi, M., Sabatinelli, G., Paci, P., Renzi, A., Amaducci, L., Ciufolini, M.G., Caciolli, S., Balducci, M., Bartolozzi, D., Verani, P., Nicoletti, L. and Volpi, G. 1988. Ecology of Viruses Isolated from Sand Flies in Italy and Characterization of a New Phlebovirus (Arbia Virus). *The American Journal of Tropical Medicine and Hygiene*. **38**(2), pp.433–439.

Constant, L.E.C., Rajsfus, B.F., Carneiro, P.H., Sisnande, T., Mohana-Borges, R. and Allonso, D. 2021. Overview on Chikungunya Virus Infection: From Epidemiology to State-of-the-Art Experimental Models. *Frontiers in Microbiology*. **12**(October), pp.1–20.

Conway, Michael J, Colpitts, T.M. and Fikrig, E. 2014. Role of the Vector in Arbovirus Transmission. *Annual Review of Virology*. **1**(1), pp.71–88.

Conway, Michael J., Watson, A.M., Colpitts, T.M., Dragovic, S.M., Li, Z., Wang, P., Feitosa, F., Shepherd, D.T., Ryman, K.D., Klimstra, W.B., Anderson, J.F. and Fikrig, E. 2014. Mosquito Saliva Serine Protease Enhances Dissemination of Dengue Virus into the Mammalian Host. *Journal of Virology*. **88**(1), pp.164–175.

Cordey, S., Bel, M., Petty, T.J., Docquier, M., Sacco, L., Turin, L., Cherpillod, P., Emonet, S., Louis-Simonet, M., Zdobnov, E.M., Ambrosioni, J. and Kaiser, L. 2015. Toscana virus meningitis case in Switzerland: An example of the ezVIR bioinformatics pipeline utility for the identification of emerging viruses. *Clinical Microbiology and Infection*. **21**(4), 387.e1-387.e4.

Costa, D.J., Favali, C., Clarêncio, J., Afonso, L., Conceição, V., Miranda, J.C., Titus, R.G., Valenzuela, J., Barral-Netto, M., Barral, A. and Brodskyn, C.I. 2004. Lutzomyia longipalpis Salivary Gland Homogenate Impairs Cytokine Production and Costimulatory Molecule Expression on Human Monocytes and Dendritic Cells. *Infection and Immunity*. **72**(3), pp.1298–1305.

Costa, J.C.R., Marchi, G.H., Santos, C.S., Andrade, M.C.M., Chaves Junior, S.P., Silva, M.A.N., Melo, M.N. and Andrade, A.J. 2021. First molecular evidence of frogs as a food source for sand flies (Diptera: Phlebotominae) in Brazilian caves. *Parasitology Research*. **120**(5), pp.1571–1582.

Couderc, T., Chrétien, F., Schilte, C., Disson, O., Brigitte, M., Guivel-Benhassine, F., Touret, Y., Barau, G., Cayet, N., Schuffenecker, I., Desprès, P., Arenzana-Seisdedos, F., Michault, A., Albert, M.L. and Lecuit, M. 2008. A Mouse Model for Chikungunya: Young Age and Inefficient Type-I Interferon Signaling Are Risk Factors for Severe Disease M. J. Buchmeier, ed. *PLoS Pathogens*. **4**(2), p.e29.

Le Coupanec, A., Babin, D., Fiette, L., Jouvion, G., Ave, P., Misse, D., Bouloy, M. and Choumet, V. 2013. Aedes Mosquito Saliva Modulates Rift Valley Fever Virus Pathogenicity M. J. Turell, ed. *PLoS Neglected Tropical Diseases*. **7**(6), p.e2237.

Cruickshank, M., Brazzelli, M., Manson, P., Torrance, N. and Grant, A. 2024. What is the impact of long-term COVID-19 on workers in healthcare settings? A rapid systematic review of current evidence. *PLoS ONE*. **19**(3 March), pp.1–21.

Crum-Cianflone, N.F. 2008. Bacterial, fungal, parasitic, and viral myositis. *Clinical Microbiology Reviews*. **21**(3), pp.473–494.

Cusi, M.G., Gandolfo, C., Terrosi, C., Savellini, G.G., Belmonte, G. and Miracco, C. 2016. Toscana virus infects dendritic and endothelial cells opening the way for the central nervous system. *Journal of NeuroVirology*. **22**(3), pp.307–315.

Cusi, M.G. and Savellini, G.G. 2011. Diagnostic tools for Toscana virus infection. *Expert Review of Anti-infective Therapy*. **9**(7), pp.799–805.

Cusi, M.G., Savellini, G.G. and Zanelli, G. 2010. Toscana Virus Epidemiology: From Italy to Beyond. *The Open Virology Journal*. **4**(2), pp.22–28.

Dachraoui, K., Chelbi, I., Labidi, I., Ben Osman, R., Sayadi, A., Ben Said, M., Cherni, S., Abbas, M.A.S., Charrel, R. and Zhioua, E. 2023. The Role of the Leishmania infantum Infected Dogs as a Potential Reservoir Host for Toscana Virus in a Zoonotic Visceral Leishmaniasis Focus of Northern Tunisia. *Viruses*. **15**(4), pp.1–16.

Daffis, S., Szretter, K.J., Schriewer, J., Li, J., Youn, S., Errett, J., Lin, T.-Y., Schneller, S., Zust, R., Dong, H., Thiel, V., Sen, G.C., Fensterl, V., Klimstra, W.B., Pierson, T.C., Buller, R.M., Gale Jr, M., Shi, P.-Y. and Diamond, M.S. 2010. 2′-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature*. **468**(7322), pp.452–456.

Dahmani, M., Alwassouf, S., Grech-Angelini, S., Marié, J. Lou, Davoust, B. and Charrel, R.N. 2016. Seroprevalence of Toscana virus in dogs from Corsica, France. *Parasites and Vectors*. **9**(1), pp.1–4.

Dalskov, L., Gad, H.H. and Hartmann, R. 2023. Viral recognition and the antiviral interferon response. *The EMBO Journal*. **42**(14), pp.1–11.

Dash, P.K., Parida, M.M., Santhosh, S.R., Verma, S.K., Tripathi, N.K., Ambuj, S., Saxena, P., Gupta, N., Chaudhary, M. and Babu, J.P. 2007. East Central South African genotype as the causative agent in reemergence of Chikungunya outbreak in India. *Vector-Borne and Zoonotic Diseases*. **7**(4), pp.519–528.

David, S. and Abraham, A.M. 2016. Epidemiological and clinical aspects on West Nile virus, a globally emerging pathogen. *Infectious Diseases*. **48**(8), pp.571–586.

Defuentes, G., Rapp, C., Imbert, P. and Durand, J. 2005. Acute Meningitis Owing to Phlebotomus Fever Toscana Virus Imported to France. , pp.295–296.

Delrieu, M., Martinet, J.-P., O'Connor, O., Viennet, E., Menkes, C., Burtet-Sarramegna, V., D. Frentiu, F. and Dupont-Rouzeyrol, M. 2023. Temperature and transmission of chikungunya, dengue, and Zika viruses: A systematic review of experimental studies on

Aedes aegypti and Aedes albopictus. *Current Research in Parasitology & Vector-Borne Diseases*. **4**(May), p.100139.

Depaquit, J., Grandadam, M., Fouque, F., Andry, P.E. and Peyrefitte, C. 2010. Arthropod-borne viruses transmitted by Phlebotomine sandflies in Europe: A review. *Eurosurveillance*. **15**(10), pp.40–47.

Dersch, R., Sophocleous, A., Cadar, D., Emmerich, P., Schmidt-Chanasit, J. and Rauer, S. 2021. Toscana virus encephalitis in Southwest Germany: a retrospective study. *BMC Neurology*. **21**(1), p.495.

Déruaz, M., Frauenschuh, A., Alessandri, A.L., Dias, J.M., Coelho, F.M., Russo, R.C., Ferreira, B.R., Graham, G.J., Shaw, J.P., Wells, T.N.C., Teixeira, M.M., Power, C.A. and Proudfoot, A.E.I. 2008. Ticks produce highly selective chemokine binding proteins with antiinflammatory activity. *The Journal of Experimental Medicine*. **205**(9), pp.2019–2031.

Desjardins, P. and Conklin, D. 2010. NanoDrop Microvolume Quantitation of Nucleic Acids. *Journal of Visualized Experiments*. (1), pp.1–4.

Desjeux, P. 2004. Leishmaniasis: Current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases*. **27**(5), pp.305–318.

Dey, R., Joshi, A.B., Oliveira, F., Pereira, L., Guimarães-Costa, A.B., Serafim, T.D., de Castro, W., Coutinho-Abreu, I. V., Bhattacharya, P., Townsend, S., Aslan, H., Perkins, A., Karmakar, S., Ismail, N., Karetnick, M., Meneses, C., Duncan, R., Nakhasi, H.L., Valenzuela, J.G. and Kamhawi, S. 2018. Gut Microbes Egested during Bites of Infected Sand Flies Augment Severity of Leishmaniasis via Inflammasome-Derived IL-1β. *Cell Host & Microbe*. **23**(1), pp.134-143.e6.

Diamond, M.S. and Farzan, M. 2013. The broad-spectrum antiviral functions of IFIT and IFITM proteins. *Nature Reviews Immunology*. **13**(1), pp.46–57.

Dias, F. de O.P., Lorosa, E.S. and Rebêlo, J.M.M. 2003. Blood feeding sources and peridomiciliation of Lutzomyia longipalpis (Lutz & Neiva, 1912) (Psychodidae, Phlebotominae). *Cadernos de saúde pública / Ministério da Saúde, Fundação Oswaldo Cruz, Escola Nacional de Saúde Pública*. **19**(5), pp.1373–1380.

Dick, G.W.A., Kitchen, S.F. and Haddow, A.J. 1952. Zika Virus (I). Isolations and serological specificity. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **46**(5), pp.509–520.

Dincer, E., Karapinar, Z., Oktem, M., Ozbaba, M., Ozkul, A. and Ergunay, K. 2016. Canine Infections and Partial S Segment Sequence Analysis of Toscana Virus in Turkey. *Vector-Borne and Zoonotic Diseases*. **16**(9), pp.611–618.

Dincer, E., Ozkul, A., Gargari, S. and Ergunay, K. 2015. Potential Animal Reservoirs of Toscana Virus and Coinfections with Leishmania infantum in Turkey. *The American Journal of Tropical Medicine and Hygiene*. **92**(4), pp.690–697.

Dionisio, D., Valassina, M., Ciufolini, M.G., Vivarelli, A., Esperti, F., Cusi, M.G., Marchi, A., Mazzoli, F. and Lupi, C. 2001. Encephalitis without Meningitis Due to Sandfly Fever Virus Serotype Toscana. *Clinical Infectious Diseases*. **32**(8), pp.1241–1243.

Dobler, G., Treib, J., Haass, A., Frösner, G., Woesner, R. and Schimrigk, K. 1997. Toscana virus infection in german travellers returning from the mediterranean. *Infection*. **25**(5), pp.325–325.

Dong, S. and Dimopoulos, G. 2021. Antiviral compounds for blocking arboviral transmission in mosquitoes. *Viruses*. **13**(1).

Driskell, R.R., Jahoda, C.A.B., Chuong, C., Watt, F.M. and Horsley, V. 2014. Defining dermal adipose tissue. *Experimental Dermatology*. **23**(9), pp.629–631.

Duffy, M.R., Chen, T.-H., Hancock, W.T., Powers, A.M., Kool, J.L., Lanciotti, R.S., Pretrick, M., Marfel, M., Holzbauer, S., Dubray, C., Guillaumot, L., Griggs, A., Bel, M., Lambert, A.J., Laven, J., Kosoy, O., Panella, A., Biggerstaff, B.J., Fischer, M. and Hayes, E.B. 2009. Zika Virus Outbreak on Yap Island, Federated States of Micronesia. *New England Journal of Medicine*. **360**(24), pp.2536–2543.

Dupuis-Maguiraga, L., Noret, M., Brun, S., Le Grand, R., Gras, G. and Roques, P. 2012. Chikungunya disease: Infection-associated markers from the acute to the chronic phase of arbovirus-induced arthralgia. *PLoS Neglected Tropical Diseases*. **6**(3).

Durbin, A.P., Vargas, M.J., Wanionek, K., Hammond, S.N., Gordon, A., Rocha, C., Balmaseda, A. and Harris, E. 2008. Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever. *Virology*. **376**(2), pp.429–435.

Durden, L.A. and Mullen, G.R. 2018. *Medical and Veterinary Entomology*.

Eash, K.J., Greenbaum, A.M., Gopalan, P.K. and Link, D.C. 2010. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *Journal of Clinical Investigation*. **120**(7), pp.2423–2431.

Eberl, G., Colonna, M., Santo, J.P.D. and McKenzie, A.N.J. 2015. Innate lymphoid cells: A new paradigm in immunology. *Science*. **348**(6237).

Echevarría, J.-M., de Ory, F., Guisasola, M.-E., Sánchez-Seco, M.-P., Tenorio, A., Lozano, Á., Córdoba, J. and Gobernado, M. 2003. Acute meningitis due to Toscana virus infection among patients from both the Spanish Mediterranean region and the region of Madrid. *Journal of Clinical Virology*. **26**(1), pp.79–84.

Eckhoff, P.A. 2011. A malaria transmission-directed model of mosquito life cycle and ecology. *Malaria Journal*. **10**, pp.1–17.

Edwards, J.F., Higgs, S. and Beaty, B.J. 1998. Mosquito Feeding-Induced Enhancement of Cache Valley Virus (Bunyaviridae) Infection in Mice. *Journal of Medical Entomology*. **35**(3), pp.261–265.

Edwards, J.P., Zhang, X., Frauwirth, K.A. and Mosser, D.M. 2006. Biochemical and functional characterization of three activated macrophage populations. *Journal of Leukocyte Biology*. **80**(6), pp.1298–1307.

Egawa, M., Mukai, K., Yoshikawa, S., Iki, M., Mukaida, N., Kawano, Y., Minegishi, Y. and Karasuyama, H. 2013. Inflammatory Monocytes Recruited to Allergic Skin Acquire an Anti-inflammatory M2 Phenotype via Basophil-Derived Interleukin-4. *Immunity*. **38**(3), pp.570–580.

Ehrnst, A., Peters, C.J., Niklasson, B., Svedmyr, A. and Holmgren, B. 1985. Neurovirulent Toscana virus (a sandfly fever virus) in Swedish man after visit to Portugal. *The Lancet*. **325**(8439), pp.1212–1213.

Eitrem, Rickard, Niklasson, B. and Weiland, O. 1991. Sandfly fever among swedish tourists. *Scandinavian Journal of Infectious Diseases*. **23**(4), pp.451–457.

Eitrem, R., Stylianou, M. and Niklasson, B. 1991. High prevalence rates of antibody to three sandfly fever viruses (Sicilian, Naples and Toscana) among Cypriots. *Epidemiology and Infection*. **107**(3), pp.685–691.

Eitrem, R., Vene, S. and Niklasson, B. 1990. Incidence of Sand Fly Fever Among Swedish United Nations Soldiers on Cyprus During 1985. *The American Journal of Tropical Medicine and Hygiene*. **43**(2), pp.207–211.

Elliott, R.M. and Brennan, B. 2014. Emerging phleboviruses. *Current Opinion in Virology*. **5**(1), pp.50–57.

Elong Ngono, A. and Shresta, S. 2018. Immune Response to Dengue and Zika. *Annual Review of Immunology*. **36**(1), pp.279–308.

Epelboin, L., Hausfater, P., Schuffenecker, I., Riou, B., Zeller, H., Bricaire, F. and Bossi, P. 2008. Meningoencephalitis due to Toscana virus in a French traveler returning from central Italy. *Journal of Travel Medicine*. **15**(5), pp.361–363.

Erdem, H., Ergunay, K., Yilmaz, A., Naz, H., Akata, F., Inan, A.S., Ulcay, A., Gunay, F., Ozkul, A., Alten, B., Turhan, V., Oncul, O. and Gorenek, L. 2014. Emergence and co-infections of West Nile virus and Toscana virus in Eastern Thrace, Turkey. *Clinical Microbiology and Infection*. **20**(4), pp.319–325.

Ergunay, K., Aydogan, S., Ilhami Ozcebe, O., Cilek, E.E., Hacioglu, S., Karakaya, J., Ozkul, A. and Us, D. 2012. Toscana Virus (TOSV) exposure is confirmed in blood donors from Central, North and South/Southeast Anatolia, Turkey. *Zoonoses and Public Health*. **59**(2), pp.148–154.

Ergunay, K., Ayhan, N. and Charrel, R.N. 2017. Novel and emergent sandfly-borne phleboviruses in Asia Minor: a systematic review. *Reviews in Medical Virology*. **27**(2), p.e1898.

Ergunay, K., Kaplan, B., Okar, S., Akkutay-Yoldar, Z., Kurne, A., Arsava, E.M. and Ozkul, A. 2015. Urinary detection of toscana virus nucleic acids in neuroinvasive infections. *Journal of Clinical Virology*. **70**, pp.89–92.

Ergunay, K., Kasap, O.E., Orsten, S., Oter, K., Gunay, F., Yoldar, A.Z.A., Dincer, E., Alten, B. and Ozkul, A. 2014. Phlebovirus and Leishmania detection in sandflies from eastern Thrace and northern Cyprus. *Parasites and Vectors*. **7**(1), pp.1–13.

Ergünay, Koray, Litzba, N., Lo, M.M., Aydoğan, S., Saygan, M.B., Us, D., Weidmann, M. and Niedrig, M. 2011. Performance of various commercial assays for the detection of Toscana virus antibodies. *Vector-Borne and Zoonotic Diseases*. **11**(6), pp.781–787.

Ergünay, K., Saygan, M.B., Aydoğan, S., Lo, M.M., Weidmann, M., Dilcher, M., Şener, B., Hasçelik, G., Pinar, A. and Us, D. 2011. Sandfly fever virus activity in central/northern Anatolia, Turkey: First report of Toscana virus infections. *Clinical Microbiology and Infection*. **17**(4), pp.575–581.

Ergunay, Koray, Sayiner, A.A., Litzba, N., Lederer, S., Charrel, R., Kreher, P., Us, D., Niedrig, M., Ozkul, A. and Hascelik, G. 2012. Multicentre evaluation of central nervous system infections due to Flavi and Phleboviruses in Turkey. *Journal of Infection*. **65**(4), pp.343–349.

Es-Sette, N., Ajaoud, M., Anga, L., Mellouki, F. and Lemrani, M. 2015. Toscana virus isolated from sandflies, Morocco. *Parasites and Vectors*. **8**(1), pp.1–3.

Essink, B., Chu, L., Seger, W., Barranco, E., Le Cam, N., Bennett, H., Faughnan, V., Pajon, R., Paila, Y.D., Bollman, B., Wang, S., Dooley, J., Kalidindi, S. and Leav, B. 2023. The safety and immunogenicity of two Zika virus mRNA vaccine candidates in healthy flavivirus baseline seropositive and seronegative adults: the results of two randomised, placebo-controlled, dose-ranging, phase 1 clinical trials. *The Lancet Infectious Diseases*. **23**(5), pp.621–633.

European Centre for Disease Prevention and Control, C. 2023. Phlebotomine sandfly maps. Available from: https://www.ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps.

Farrugia, J., Maxwell-Scott, H., Brooks, T., Tang, J.W. and Pareek, M. 2020. Toscana virus as a cause of short-term fever and encephalitis in returning travellers from Mediterranean Europe. *Clinical Infection in Practice*. **6**, p.100018.

Fazakerley, J.K. 2002. Pathogenesis of Semliki Forest virus encephalitis. *Journal of NeuroVirology*. **8**(SUPPL. 2), pp.66–74.

Feldstein, L.R., Ellis, E.M., Rowhani-Rahbar, A., Hennessey, M.J., Staples, J.E., Halloran, M.E. and Weaver, M.R. 2019. Estimating the cost of illness and burden of disease associated with the 2014–2015 chikungunya outbreak in the U.S. Virgin Islands. *PLoS Neglected Tropical Diseases*. **13**(7), pp.1–14.

Ferguson, M.C., Saul, S., Fragkoudis, R., Weisheit, S., Cox, J., Patabendige, A., Sherwood, K., Watson, M., Merits, A. and Fazakerley, J.K. 2015. Ability of the Encephalitic Arbovirus Semliki Forest Virus To Cross the Blood-Brain Barrier Is Determined by the Charge of the E2 Glycoprotein. *Journal of Virology*. **89**(15), pp.7536–7549.

Ferreira, A.C., Reis, P.A., de Freitas, C.S., Sacramento, C.Q., Villas Bôas Hoelz, L., Bastos, M.M., Mattos, M., Rocha, N., Gomes de Azevedo Quintanilha, I., da Silva Gouveia Pedrosa, C., Rocha Quintino Souza, L., Correia Loiola, E., Trindade, P., Rangel Vieira, Y., Barbosa-Lima, G., de Castro Faria Neto, H.C., Boechat, N., Rehen, S.K., Brüning, K., Bozza, F.A., Bozza, P.T. and Souza, T.M.L. 2019. Beyond Members of the Flaviviridae Family, Sofosbuvir Also Inhibits Chikungunya Virus Replication. *Antimicrobial Agents and Chemotherapy*. **63**(2), pp.1–15.

Ferreira, F.V., Aguiar, E.R.G.R., Olmo, R.P., de Oliveira, K.P.V., Silva, E.G., Sant'Anna, M.R.V., Gontijo, N.D.F., Kroon, E.G., Imler, J.L. and Marques, J.T. 2018. The small non-coding RNA response to virus infection in the Leishmania vector Lutzomyia longipalpis P. Mireji, ed. *PLOS Neglected Tropical Diseases*. **12**(6), p.e0006569.

Fezaa, O., Bahri, O., Alaya Bouafif, N. Ben, Triki, H. and Bouattour, A. 2013. Seroprevalence of Toscana virus infection in Tunisia. *International Journal of Infectious Diseases*. **17**(12), pp.e1172–e1175.

Fezaa, O., M'ghirbi, Y., Savellini, G.G., Ammari, L., Hogga, N., Triki, H., Cusi, M.G. and Bouattour, A. 2014. Serological and molecular detection of Toscana and other Phleboviruses in patients and sandflies in Tunisia. *BMC Infectious Diseases*. **14**(1), pp.1–12.

Fragkoudis, R., Breakwell, L., McKimmie, C., Boyd, A., Barry, G., Kohl, A., Merits, A. and Fazakerley, J.K. 2007. The type I interferon system protects mice from Semliki Forest virus by preventing widespread virus dissemination in extraneural tissues, but does not mediate the restricted replication of avirulent virus in central nervous system neurons. *Journal of General Virology*. **88**(12), pp.3373–3384.

Fragkoudis, R., Tamberg, N., Siu, R., Kiiver, K., Kohl, A., Merits, A. and Fazakerley, J.K. 2009. Neurons and oligodendrocytes in the mouse brain differ in their ability to replicate Semliki Forest virus. *Journal of NeuroVirology*. **15**(1), pp.57–70.

Francisci, D., Papili, R., Camanni, G., Morosi, S., Ferracchiato, N., Valente, M., Ciufolini, M.G. and Baldelli, F. 2002. Evidence of Toscana virus circulation in Umbria: First report. *European Journal of Epidemiology*. **18**(5), pp.457–459.

Franco, E.J., Hanrahan, K.C. and Brown, A.N. 2023. Favipiravir Inhibits Zika Virus (ZIKV) Replication in HeLa Cells by Altering Viral Infectivity. *Microorganisms*. **11**(5).

Franz, A.W.E., Kantor, A.M., Passarelli, A.L. and Clem, R.J. 2015. Tissue barriers to arbovirus infection in mosquitoes. *Viruses*. **7**(7), pp.3741–3767.

Fritz, R., Stiasny, K. and Heinz, F.X. 2008. Identification of specific histidines as pH sensors in flavivirus membrane fusion. *Journal of Cell Biology*. **183**(2), pp.353–361.

Frumence, E., Roche, M., Krejbich-Trotot, P., El-Kalamouni, C., Nativel, B., Rondeau, P., Missé, D., Gadea, G., Viranaicken, W. and Desprès, P. 2016. The South Pacific epidemic strain of Zika virus replicates efficiently in human epithelial A549 cells leading to IFN-β production and apoptosis induction. *Virology*. **493**, pp.217–226.

Furuta, Y., Takahashi, K., Shiraki, K., Sakamoto, K., Smee, D.F., Barnard, D.L., Gowen, B.B., Julander, J.G. and Morrey, J.D. 2009. T-705 (favipiravir) and related compounds: Novel broad-spectrum inhibitors of RNA viral infections. *Antiviral Research*. **82**(3), pp.95–102.

Gabriel, M., Resch, C., Günther, S. and Schmidt-Chanasit, J. 2010. Toscana Virus Infection Imported from Elba into Switzerland. *Emerging Infectious Diseases*. **16**(6), pp.1034–1036.

Galli, S.J., Nakae, S. and Tsai, M. 2005. Mast cells in the development of adaptive immune responses. *Nature Immunology*. **6**(2), pp.135–142.

Gámbaro, F., Pérez, A.B., Prot, M., Agüera, E., Baidaliuk, A., Sánchez-Seco, M.P., Martínez-Martínez, L., Vázquez, A., Fernandez-Garcia, M.D. and Simon-Loriere, E. 2023. Untargeted metagenomic sequencing identifies Toscana virus in patients with idiopathic meningitis, southern Spain, 2015 to 2019. *Eurosurveillance*. **28**(45), pp.1–12.

García San Miguel, L., Sierra, M.J., Vazquez, A., Fernandez-Martínez, B., Molina, R., Sanchez-Seco, M.P., Lucientes, J., Figuerola, J., de Ory, F., Monge, S., Suarez, B. and Simón, F. 2021. Phlebovirus-associated diseases transmitted by phlebotominae in Spain: Are we at risk? *Enfermedades infecciosas y microbiologia clinica (English ed.)*. **39**(7), pp.345–351.

Gatherer, D. and Kohl, A. 2016. Zika virus: a previously slow pandemic spreads rapidly through the Americas. *Journal of General Virology*. **97**(2), pp.269–273.

Geevarghese, G., Arankalle, V.A., Jadi, R., Kanojia, P.C., Joshi, M. V. and Mishra, A.C. 2005. Detection of chandipura virus from sand flies in the genus Sergentomyia (Diptera: Phlebotomidae) at Karimnagar district, Andhra Pradesh, India. *Journal of Medical Entomology*. **42**(3), pp.495–496.

Geissmann, F., Jung, S. and Littman, D.R. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. **19**(1), pp.71–82.

Gerrard, S.R., Bird, B.H., Albariño, C.G. and Nichol, S.T. 2007. The NSm proteins of Rift Valley fever virus are dispensable for maturation, replication and infection. *Virology*. **359**(2), pp.459–465.

Gigon, L., Fettrelet, T., Yousefi, S., Simon, D. and Simon, H.U. 2023. Eosinophils from A to Z. *Allergy: European Journal of Allergy and Clinical Immunology*. **78**(7), pp.1810–1846.

Ginhoux, F. and Guilliams, M. 2016. Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity*. **44**(3), pp.439–449.

Giovanetti, M., Branda, F., Cella, E., Scarpa, F., Bazzani, L., Ciccozzi, A., Slavov, S.N., Benvenuto, D., Sanna, D., Casu, M., Santos, L.A., Lai, A., Zehender, G., Caccuri, F., Ianni, A., Caruso, A., Maroutti, A., Pascarella, S., Borsetti, A. and Ciccozzi, M. 2023. Epidemic history and evolution of an emerging threat of international concern, the severe acute respiratory syndrome coronavirus 2. *Journal of Medical Virology*. **95**(8).

Giraldo, M.I., Gonzalez-Orozco, M. and Rajsbaum, R. 2023. Pathogenesis of Zika Virus Infection. *Annual Review of Pathology: Mechanisms of Disease*. **18**(1), pp.181–203.

Giunchetti, R.C., Corrêa-Oliveira, R., Martins-Filho, O.A., Teixeira-Carvalho, A., Roatt, B.M., Aguiar-Soares, R.D. de O., Coura-Vital, W., Abreu, R.T. de, Malaquias, L.C.C., Gontijo, N.F., Brodskyn, C., Oliveira, C.I. de, Costa, D.J., de Lana, M. and Reis, A.B. 2008. A killed Leishmania vaccine with sand fly saliva extract and saponin adjuvant displays immunogenicity in dogs. *Vaccine*. **26**(5), pp.623–638.

Gladen, B.C. and Rogan, W.J. 1995. DDE and shortened duration of lactation in a northern Mexican town. *American Journal of Public Health*. **85**(4), pp.504–508.

Gligić, A., Miščević, Z., Tesh, R.B., Travassos da Rosa, A. and Živković, V. 1982. First isolation of Naples sandfly fever virus in Yugoslavia.

Goh, V.S.L., Mok, C.K. and Chu, J.J.H. 2020. Antiviral natural products for arbovirus infections. *Molecules*. **25**(12).

Gois, B.M., Peixoto, R.F., Guerra-Gomes, I.C., Palmeira, P.H. de S., Dias, C.N. de S., Araújo, J.M.G., Veras, R.C., Medeiros, I.A., Azevedo, F. de L.A.A. de, Boyton, R.J., Altmann, D.M. and Keesen, T.S.L. 2022. Regulatory T cells in acute and chronic human Chikungunya infection. *Microbes and Infection*. **24**(3), pp.2–5.

Gomes, B., Purkait, B., Deb, R.M., Rama, A., Singh, R.P., Foster, G.M., Coleman, M., Kumar, V., Paine, M., Das, P. and Weetman, D. 2017. Knockdown resistance mutations predict DDT resistance and pyrethroid tolerance in the visceral leishmaniasis vector Phlebotomus argentipes. *PLoS Neglected Tropical Diseases*. **11**(4), pp.1–14.

Gomez-Bris, R., Saez, A., Herrero-Fernandez, B., Rius, C., Sanchez-Martinez, H. and Gonzalez-Granado, J.M. 2023. CD4 T-Cell Subsets and the Pathophysiology of Inflammatory Bowel Disease. *International Journal of Molecular Sciences*. **24**(3), p.2696.

Gonen, O.M. and Sacagiu, T. 2013. Sensory polymyeloradiculopathy associated with Toscana virus infection. *Journal of NeuroVirology*. **19**(5), pp.508–510.

González, E., Molina, R., Aldea, I., Iriso, A., Tello, A. and Jiménez, M. 2020. Leishmania sp. detection and blood-feeding behaviour of Sergentomyia minuta collected in the human leishmaniasis focus of southwestern Madrid, Spain (2012–2017). *Transboundary and Emerging Diseases*. **67**(3), pp.1393–1400.

Gori Savellini, G., Gandolfo, C. and Cusi, M.G. 2020. Epidemiology of Toscana virus in South Tuscany over the years 2011-2019. *Journal of Clinical Virology*. **128**(March), p.104452.

Gori Savellini, G., Di Genova, G., Terrosi, C., Di Bonito, P., Giorgi, C., Valentini, M., Docquier, J.-D. and Cusi, M.G. 2008. Immunization with Toscana virus N-Gc proteins protects mice against virus challenge. *Virology*. **375**(2), pp.521–528.

Gori-Savellini, G., Valentini, M. and Cusi, M.G. 2013. Toscana Virus NSs Protein Inhibits the Induction of Type I Interferon by Interacting with RIG-I. *Journal of Virology*. **87**(12), pp.6660–6667.

Gorman, M.J., Caine, E.A., Zaitsev, K., Begley, M.C., Weger-Lucarelli, J., Uccellini, M.B., Tripathi, S., Morrison, J., Yount, B.L., Dinnon, K.H., Rückert, C., Young, M.C., Zhu, Z., Robertson, S.J., McNally, K.L., Ye, J., Cao, B., Mysorekar, I.U., Ebel, G.D., Baric, R.S., Best, S.M., Artyomov, M.N., Garcia-Sastre, A. and Diamond, M.S. 2018. An Immunocompetent Mouse Model of Zika Virus Infection. *Cell Host & Microbe*. **23**(5), pp.672-685.e6.

Goubau, D., Deddouche, S. and Reis e Sousa, C. 2013. Cytosolic Sensing of Viruses. *Immunity*. **38**(5), pp.855–869.

Gould, E. and Solomon, T. 2008. Pathogenic flaviviruses. *The Lancet*. **371**(9611), pp.500–509.

Goupil, B.A., McNulty, M.A., Martin, M.J., McCracken, M.K., Christofferson, R.C. and Mores, C.N. 2016. Novel Lesions of Bones and Joints Associated with Chikungunya Virus Infection in Two Mouse Models of Disease: New Insights into Disease Pathogenesis P. Roques, ed. *PLOS ONE*. **11**(5), p.e0155243.

Grandi, G., Chitimia-dobler, L., Choklikitumnuey, P., Strube, C., Springer, A., Albihn, A., Jaenson, T.G.T. and Omazic, A. 2020. First records of adult Hyalomma marginatum and H. rufipes ticks (Acari: Ixodidae) in Sweden. *Ticks and tick-borne diseases*. **11**(3), p.101403.

Grant, A., Ponia, S.S., Tripathi, S., Balasubramaniam, V., Miorin, L., Sourisseau, M., Schwarz, M.C., Sánchez-Seco, M.P., Evans, M.J., Best, S.M. and García-Sastre, A. 2016a. Zika Virus Targets Human STAT2 to Inhibit Type i Interferon Signaling. *Cell Host and Microbe*. **19**(6), pp.882–890.

Grant, A., Ponia, S.S., Tripathi, S., Balasubramaniam, V., Miorin, L., Sourisseau, M., Schwarz, M.C., Sánchez-Seco, M.P., Evans, M.J., Best, S.M. and García-Sastre, A. 2016b. Zika Virus Targets Human STAT2 to Inhibit Type i Interferon Signaling. *Cell Host and Microbe*. **19**(6), pp.882–890.

Grazia Cusi, M., Gori Savellini, G., Terrosi, C., Di Genova, G., Valassina, M., Valentini, M., Bartolommei, S. and Miracco, C. 2005. Development of a mouse model for the study of Toscana virus pathogenesis. *Virology*. **333**(1), pp.66–73.

Green, M.S., LeDuc, J., Cohen, D. and Franz, D.R. 2019. Confronting the threat of bioterrorism: realities, challenges, and defensive strategies. *The Lancet Infectious Diseases*. **19**(1), pp.e2–e13.

Griffin, L.F. and Knight, J.M. 2012. A review of the role of fish as biological control agents of disease vector mosquitoes in mangrove forests: Reducing human health risks while reducing environmental risk. *Wetlands Ecology and Management*. **20**(3), pp.243–252.

Groom, J.R. and Luster, A.D. 2011. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunology & Cell Biology*. **89**(2), pp.207–215.

Gubler, D.J. 2002. The Global Emergence/Resurgence of Arboviral Diseases As Public Health Problems. *Archives of Medical Research*. **33**(4), pp.330–342.

Gubler, D.J., Vasilakis, N. and Musso, D. 2017. History and Emergence of Zika Virus. *The Journal of Infectious Diseases*. **216**(suppl_10), pp.S860–S867.

Guerra-Gomes, I.C., Gois, B.M., Peixoto, R.F., Palmeira, P.H. de S., Dias, C.N. de S., Csordas, B.G., Araújo, J.M.G., Veras, R.C., de Medeiros, I.A., de Azevedo, F. de L.A.A., Boyton,

R.J., Altmann, D.M. and Keesen, T.S.L. 2021. Phenotypical characterization of regulatory T cells in acute Zika infection. *Cytokine*. **146**, p.155651.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada Peña, A., Horak, I.G., Shao, R. and Barker, S.C. 2010. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names.

Guimaraes, A., Wen, X., Castro, W., Townsend, S., Brzostowski, J., Meneses, C., Kamhawi, S., Valenzuela, J. and Oliveira, F. 2017. A sand fly salivary chemotactic protein drives neutrophil migration and exacerbates Leishmania infection. *The Journal of Immunology*. **198**(1_Supplement), 77.15-77.15.

Hacioglu, S., Dincer, E., Isler, C.T., Karapinar, Z., Ataseven, V.S., Ozkul, A. and Ergunay, K. 2017. A Snapshot Avian Surveillance Reveals West Nile Virus and Evidence of Wild Birds Participating in Toscana Virus Circulation. *Vector-Borne and Zoonotic Diseases*. **17**(10), pp.698–708.

Hacioglu, S. and Ozkul, A. 2023. Do birds play a role in the transmission of Toscana virus? Initial isolation results from birds in northernmost Türkiye. *Zoonoses and Public Health*. (November), pp.1–11.

Haese, N.N., Broeckel, R.M., Hawman, D.W., Heise, M.T., Morrison, T.E. and Streblow, D.N. 2016. Animal models of chikungunya virus infection and disease. *Journal of Infectious Diseases*. **214**(Suppl 5), pp.S482–S487.

Haist, K.C., Burrack, K.S., Davenport, B.J. and Morrison, T.E. 2017. Inflammatory monocytes mediate control of acute alphavirus infection in mice. *PLoS Pathogens*. **13**(12), pp.1–30.

Hamel, R., Dejarnac, O., Wichit, S., Ekchariyawat, P., Neyret, A., Luplertlop, N., Perera-Lecoin, M., Surasombatpattana, P., Talignani, L., Thomas, F., Cao-Lormeau, V.-M., Choumet, V., Briant, L., Desprès, P., Amara, A., Yssel, H. and Missé, D. 2015. Biology of Zika Virus Infection in Human Skin Cells. *Journal of Virology*. **89**(17), pp.8880–8896.

Hamers, A.A.J., Dinh, H.Q., Thomas, G.D., Marcovecchio, P., Blatchley, A., Nakao, C.S., Kim, C., McSkimming, C., Taylor, A.M., Nguyen, A.T., McNamara, C.A. and Hedrick, C.C. 2019. Human Monocyte Heterogeneity as Revealed by High-Dimensional Mass Cytometry. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **39**(1), pp.25–36.

Hamilton, J.G.C. 2022. Chapter 13: Sand fly sex/aggregation pheromones *In*: *Sensory ecology of disease vectors* [Online]. Brill | Wageningen Academic, pp.349–371. Available from: https://brill.com/view/book/9789086869329/BP000014.xml.

Hammon, W.McD., Rundnick, A. and Sather, G.E. 1960. Viruses Associated with Epidemic Hemorrhagic Fevers of the Philippines and Thailand. *Science*. **131**(3407), pp.1102–1103.

Hapuarachchi, H.C., Bandara, K.B.A.T., Sumanadasa, S.D.M., Hapugoda, M.D., Lai, Y.L., Lee, K.S., Tan, L.K., Lin, R.T.P., Ng, L.F.P., Bucht, G., Abeyewickreme, W. and Ng, L.C. 2010. Re-emergence of Chikungunya virus in South-east Asia: Virological evidence from Sri Lanka and Singapore. *Journal of General Virology*. **91**(4), pp.1067–1076.

Harris, A.F., McKemey, A.R., Nimmo, D., Curtis, Z., Black, I., Morgan, S.A., Oviedo, M.N., Lacroix, R., Naish, N., Morrison, N.I., Collado, A., Stevenson, J., Scaife, S., Dafa'Alla, T., Fu, G., Phillips, C., Miles, A., Raduan, N., Kelly, N., Beech, C., Donnelly, C.A., Petrie, W.D. and Alphey, L. 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology*. **30**(9), pp.828–830.

Hawman, D.W., Stoermer, K.A., Montgomery, S.A., Pal, P., Oko, L., Diamond, M.S. and Morrison, T.E. 2013. Chronic Joint Disease Caused by Persistent Chikungunya Virus Infection Is Controlled by the Adaptive Immune Response. *Journal of Virology*. **87**(24), pp.13878–13888.

Hay, Z.L.Z. and Slansky, J.E. 2022. Granzymes: The Molecular Executors of Immune-Mediated Cytotoxicity. *International Journal of Molecular Sciences*. **23**(3).

Hayward, J., Sanchez, J., Perry, A., Huang, C., Rodriguez Valle, M., Canals, M., Payne, R.J. and Stone, M.J. 2017. Ticks from diverse genera encode chemokine-inhibitory evasin proteins. *Journal of Biological Chemistry*. **292**(38), pp.15670–15680.

Helbig, K.J. and Beard, M.R. 2014. The Role of Viperin in the Innate Antiviral Response. *Journal of Molecular Biology*. **426**(6), pp.1210–1219.

Hemmersbach-Miller, M., Parola, P., Charrel, R.N., Paul Durand, J. and Brouqui, P. 2004. Sandfly fever due to Toscana virus: An emerging infection in southern France. *European Journal of Internal Medicine*. **15**(5), pp.316–317.

Hermance, M.E. and Thangamani, S. 2015. Tick Saliva Enhances Powassan Virus Transmission to the Host, Influencing Its Dissemination and the Course of Disease M. S. Diamond, ed. *Journal of Virology*. **89**(15), pp.7852–7860.

Hespel, C. and Moser, M. 2012. Role of inflammatory dendritic cells in innate and adaptive immunity. *European Journal of Immunology*. **42**(10), pp.2535–2543.

Higgs, S. and Vanlandingham, D.L. 2016. Influences of arthropod vectors on encephalitic arboviruses. *Neurotropic Viral Infections: Volume 2: Neurotropic Retroviruses, DNA Viruses, Immunity and Transmission*., pp.371–401.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. and Werren, J.H. 2008. How many species are infected with Wolbachia? - A statistical analysis of current data. *FEMS Microbiology Letters*. **281**(2), pp.215–220.

Hirahara, K. and Nakayama, T. 2016. CD4+ T-cell subsets in inflammatory diseases: Beyond the Th1/Th2 paradigm. *International Immunology*. **28**(4), pp.163–171.

Hoch, A.L., Turell, M.J. and Bailey, C.L. 1984. Replication of Rift Valley Fever Virus in the Sand Fly Lutzomyia Longipalpis. *The American Journal of Tropical Medicine and Hygiene*. **33**(2), pp.295–299.

Hoffmann, A.A., Montgomery, B.L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P.H., Muzzi, F., Greenfield, M., Durkan, M., Leong, Y.S., Dong, Y., Cook, H., Axford, J., Callahan, A.G., Kenny, N., Omodei, C., McGraw, E.A., Ryan, P.A., Ritchie, S.A., Turelli, M. and O'Neill, S.L. 2011. Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature*. **476**(7361), pp.454–459.

Holladay, A.J. and Poole, J.C.F. 1979. Thucydides and the Plague of Athens. *The Classical Quarterly*. **29**(2), pp.282–300.

Honda, K., Takaoka, A. and Taniguchi, T. 2006. Type I Inteferon Gene Induction by the Interferon Regulatory Factor Family of Transcription Factors. *Immunity*. **25**(3), pp.349–360.

Hornak, K., Lanchy, J.-M. and Lodmell, J. 2016. RNA Encapsidation and Packaging in the Phleboviruses. *Viruses*. **8**(7), p.194.

Horne, K. and Vanlandingham, D. 2014. Bunyavirus-Vector Interactions. *Viruses*. **6**(11), pp.4373–4397.

Hosseini, S.H., Allah-Kalteh, E. and Sofizadeh, A. 2021. The Effect of Geographical and Climatic Factors on the Distribution of Phlebotomus papatasi (Diptera: Psychodidae) in Golestan Province, an Endemic Focus of Zoonotic Cutaneous Leishmaniasis in Iran, 2014. *Journal of Arthropod-Borne Diseases*. **15**(2), pp.225–235.

Howell, B.A., Azar, M.M., Landry, M.L. and Shaw, A.C. 2015. Toscana Virus Encephalitis in a Traveler Returning to the United States M. J. Loeffelholz, ed. *Journal of Clinical Microbiology*. **53**(4), pp.1445–1447.

Hubálek, Z., Rudolf, I. and Nowotny, N. 2014. Arboviruses pathogenic for domestic and wild animals *In*: *Advances in virus research*. Elsevier, pp.201–275.

Hübschen, J.M., Gouandjika-Vasilache, I. and Dina, J. 2022. Measles. *The Lancet*. **399**(10325), pp.678–690.

Hukić, M. and Salimović-Bešić, I. 2009. Sandfly - Pappataci fever in Bosnia And Herzegovina: The new-old disease. *Bosnian Journal of Basic Medical Sciences*. **9**(1), pp.39–43.

van den Hurk, A.F., Hall-Mendelin, S., Pyke, A.T., Frentiu, F.D., McElroy, K., Day, A., Higgs, S. and O'Neill, S.L. 2012. Impact of Wolbachia on Infection with Chikungunya and Yellow Fever Viruses in the Mosquito Vector Aedes aegypti. *PLoS Neglected Tropical Diseases*. **6**(11).

Hwang, S.Y., Hertzog, P.J., Holland, K.A., Sumarsono, S.H., Tymms, M.J., Hamilton, J.A., Whitty, G., Bertoncello, I. and Kola, I. 1995. A null mutation in the gene encoding a type I interferon receptor component eliminates antiproliferative and antiviral responses to interferons alpha and beta and alters macrophage responses. *Proceedings of the National Academy of Sciences*. **92**(24), pp.11284–11288.

Ianevski, A., Ahmad, S., Anunnitipat, K., Oksenych, V., Zusinaite, E., Tenson, T., Bjørås, M. and Kainov, D.E. 2022. Seven classes of antiviral agents. *Cellular and Molecular Life Sciences*. **79**(12), p.605.

Iannacone, M., Moseman, E.A., Tonti, E., Bosurgi, L., Junt, T., Henrickson, S.E., Whelan, S.P., Guidotti, L.G. and Von Andrian, U.H. 2010. Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus. *Nature*. **465**(7301), pp.1079–1083.

IMF 2020. World Economic Outlook, April 2020: The Great Lockdown. IMF; 2020. Available from: https://www.imf.org/en/Publications/WEO/Issues/2020/ 04/14/weo-april-2020. .

Imirzalioglu, C., Schaller, M. and Bretzel, R.G. 2006. Sandfly fever Naples virus (serotype Toscana) infection with meningeal involvement after a vacation in Italy. *Deutsche Medizinische Wochenschrift (1946)*. **131**(50), pp.2838–2840.

Indran, S. V., Lihoradova, O.A., Phoenix, I., Lokugamage, N., Kalveram, B., Head, J.A., Tigabu, B., Smith, J.K., Zhang, L., Juelich, T.L., Gong, B., Freiberg, A.N. and Ikegami, T. 2013. Rift Valley fever virus MP-12 vaccine encoding Toscana virus NSs retains neuroinvasiveness in mice. *Journal of General Virology*. **94**(7), pp.1441–1450.

Ito, T., Nishiyama, C., Nakano, N., Nishiyama, M., Usui, Y., Takeda, K., Kanada, S., Fukuyama, K., Akiba, H., Tokura, T., Hara, M., Tsuboi, R., Ogawa, H. and Okumura, K. 2009. Roles of PU.1 in monocyte- and mast cell-specific gene regulation: PU.1 transactivates CIITA pIV in cooperation with IFN-γ. *International Immunology*. **21**(7), pp.803–816.

Ivashkiv, L.B. and Donlin, L.T. 2014. Regulation of type I interferon responses. *Nature Reviews Immunology*. **14**(1), pp.36–49.

Jancarova, M., Bichaud, L., Hlavacova, J., Priet, S., Ayhan, N., Spitzova, T., Volf, P. and Charrel, R. 2019. Experimental Infection of Sand Flies by Massilia Virus and Viral Transmission by Co-Feeding on Sugar Meal. *Viruses*. **11**(4), p.332.

Janeway, C.A. 1989. Approaching the asymptote? Evolution and revolution in immunology *In*: *Cold Spring Harbor symposia on quantitative biology*. Cold Spring Harbor Laboratory Press, pp.1–13.

Jenner, E. 1802. An inquiry into the causes and effects of the variolae vaccinae: a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox. *Springfield [Mass.] : Re-printed for Dr. Samuel Cooley, by Ashley & Brewer, 1802*., p.134.

Jennings, M. and Boorman, J. 1980. The susceptibility of Lutzomyia longipalpis (Lutz and Neiva), Diptera, Psychodidae, to artificial infection with three viruses of the Phlebotomus fever group. *Annals of Tropical Medicine & Parasitology*. **74**(4), pp.455–462.

Ji, L., Yang, X. and Qi, F. 2022. Distinct Responses to Pathogenic and Symbionic Microorganisms: The Role of Plant Immunity. *International Journal of Molecular Sciences*. **23**(18).

Johnson, N.P.A.S. and Mueller, J. 2002. Updating the accounts: global mortality of the 1918-1920" Spanish" influenza pandemic. *Bulletin of the History of Medicine*., pp.105–115.

Jose, J., Snyder, J.E. and Kuhn, R.J. 2009. A structural and functional perspective of alphavirus replication and assembly. *Future Microbiology*. **4**(7), pp.837–856.

Joshua Lederberg, Robert E. Shope, and S.C., Oaks, Jr., Joshua Lederberg, Robert E. Shope, and S.C. and Oaks, Jr. 1992. *Emerging Infections* [Online]. Washington, D.C.: National Academies Press. Available from: http://www.nap.edu/catalog/2008.

Kalveram, B. and Ikegami, T. 2013. Toscana Virus NSs Protein Promotes Degradation of Double-Stranded RNA-Dependent Protein Kinase. *Journal of Virology*. **87**(7), pp.3710–3718.

Kamhawi, S. 2000. The biological and immunomodulatory properties of sand fly saliva and its role in the establishment of infections. *Microbes and Infection*. **2**(14), pp.1765–1773.

Kamhawi, S., Ramalho-Ortigao, M., Van, M.P., Kumar, S., Lawyer, P.G., Turco, S.J., Barillas-Mury, C., Sacks, D.L. and Valenzuela, J.G. 2004. A role for insect galectins in parasite survival. *Cell*. **119**(3), pp.329–341.

Kamijo, R., Shapiro, D., Le, J., Huang, S., Aguet, M. and Vilček, J. 1993. Generation of nitric oxide and induction of major histocompatibility complex class II antigen in macrophages from mice lacking the interferon γ receptor. *Proceedings of the National Academy of Sciences of the United States of America*. **90**(14), pp.6626–6630.

Kantha, S.S. 1991. A Centennial Review; the 1890 Tetanus Antitoxin Paper of von Behring and Kitasato and the Related Developments. *The Keio Journal of Medicine*. **40**(1), pp.35–39.

Karabatsos, N. 1978. Supplement to International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates. *The American Journal of Tropical Medicine and Hygiene*. **27**(2_Part_2), pp.372–372.

Karmakar, S., Ismail, N., Oliveira, F., Oristian, J., Zhang, W.W., Kaviraj, S., Singh, K.P., Mondal, A., Das, S., Pandey, K., Bhattacharya, P., Volpedo, G., Gannavaram, S., Satoskar, M., Satoskar, S., Sastry, R.M., Oljuskin, T., Sepahpour, T., Meneses, C., Hamano, S., Das, P., Matlashewski, G., Singh, S., Kamhawi, S., Dey, R., Valenzuela, J.G., Satoskar, A. and

Nakhasi, H.L. 2021. Preclinical validation of a live attenuated dermotropic Leishmania vaccine against vector transmitted fatal visceral leishmaniasis. *Communications Biology*. **4**(1), pp.1–14.

Katze, M.G., He, Y. and Gale, M. 2002. Viruses and interferon: A fight for supremacy. *Nature Reviews Immunology*. **2**(9), pp.675–687.

Kaufmann, S.H.E. and Winau, F. 2005. From bacteriology to immunology: The dualism of specificity. *Nature Immunology*. **6**(11), pp.1063–1066.

Kawai, T. and Akira, S. 2010. The role of pattern-recognition receptors in innate immunity: Update on toll-like receptors. *Nature Immunology*. **11**(5), pp.373–384.

Kawakami, T. and Galli, S.J. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nature Reviews Immunology*. **2**(10), pp.773–786.

Kay, M.K., Gibney, K.B., Riedo, F.X., Kosoy, O.L., Lanciotti, R.S. and Lambert, A.J. 2010. Toscana Virus Infection in American Traveler Returning from Sicily, 2009. *Emerging Infectious Diseases*. **16**(9), pp.1498–1500.

Kell, A.M. and Gale, M. 2015. RIG-I in RNA virus recognition. *Virology*. **479–480**(January), pp.110–121.

Kelley, N., Jeltema, D., Duan, Y. and He, Y. 2019. The Inflammasome signaling pathway responds to pathogens or danger signals by assembling inflammasomes, with NLRP3 serving as a prominent sensor. Activation of the inflammasome triggers caspase-1 activation, leading to the cleavage of pro-IL-1β and pro-IL. *International Journal of Molecular Sciences*. **20**(13), pp.1–24.

Kelly, P.H., Bahr, S.M., Serafim, T.D., Ajami, N.J., Petrosino, J.F., Meneses, C., Kirby, J.R., Valenzuela, J.G., Kamhawi, S. and Wilson, M.E. 2017. The gut microbiome of the vector lutzomyia longipalpis is essential for survival of leishmania infantum. *mBio*. **8**(1), pp.1–12.

Kemp, G.E., Causey, O.R., Setzer, H.W., Moore, D. and L 1974. Isolation of Viruses from Wild Mammals in West Africa, 1966-1970. *Journal of Wildlife Diseases*. **10**(3), pp.279–293.

Kemper, C., Ferreira, V.P., Paz, J.T., Holers, V.M., Lionakis, M.S. and Alexander, J.J. 2023. Complement: The Road Less Traveled. *The Journal of Immunology*. **210**(2), pp.119–125.

Keskek Turk, Y., Barningham, L.D. and McKimmie, C.S. 2024. Sensing the danger in mosquito spit. *The EMBO Journal*. **43**(9), pp.1687–1689.

Khanam, A., Gutiérrez-Barbosa, H., Lyke, K.E. and Chua, J. V. 2022. Immune-Mediated Pathogenesis in Dengue Virus Infection. *Viruses*. **14**(11), pp.1–19.

Killick-Kendrick, R. 1999. The biology and control of Phlebotomine sand flies. *Clinics in Dermatology*. **17**(3), pp.279–289.

Killick-Kendrick, R., Wilkes, T.J., Bailly, M., Bailly, I. and Righton, L.A. 1986. Preliminary field observations on the flight speed of a phlebotomine sandfly. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **80**(1), pp.138–142.

Kim, A.S. and Diamond, M.S. 2023. A molecular understanding of alphavirus entry and antibody protection. *Nature Reviews Microbiology*. **21**(6), pp.396–407.

King, A. 2023. In search of a vaccine for leishmaniasis. *Nature*.

King, A.M.Q., Lefkowitz, E.J., Mushegian, A.R., Adams, M.J., Dutilh, B.E., Gorbalenya, A.E., Harrach, B., Harrison, R.L., Junglen, S., Knowles, N.J., Kropinski, A.M., Krupovic, M.,

Kuhn, J.H., Nibert, M.L., Rubino, L., Sabanadzovic, S., Sanfaçon, H., Siddell, S.G., Simmonds, P., Varsani, A., Zerbini, F.M. and Davison, A.J. 2018. *Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2018)* [Online]. Springer Vienna. Available from: https://doi.org/10.1007/s00705-018-3847-1.

Klimstra, W.B., Nangle, E.M., Smith, M.S., Yurochko, A.D. and Ryman, K.D. 2004. DC-SIGN and L-SIGN Can Act as Attachment Receptors for Alphaviruses and Distinguish between Mosquito Cell- and Mammalian Cell-Derived Viruses. *Journal of Virology*. **78**(14), pp.7862–7862.

Knapp, J., Macdonald, M., Malone, D., Hamon, N. and Richardson, J.H. 2015. Disruptive technology for vector control: The Innovative Vector Control Consortium and the US Military join forces to explore transformative insecticide application technology for mosquito control programmes. *Malaria Journal*. **14**(1), pp.1–5.

Knechtli, R. and Jenni, L. 1989. Distribution and relative density of three sandfly (Diptera: Phlebotominae) species in southern Switzerland. *Annales de Parasitologie Humaine et Comparee*. **64**(1), pp.53–63.

Koch, J., Xin, Q., Obr, M., Schäfer, A., Rolfs, N., Anagho, H.A., Kudulyte, A., Woltereck, L., Kummer, S., Campos, J., Uckeley, Z.M., Bell-Sakyi, L., Kräusslich, H.G., Schur, F.K.M., Acuna, C. and Lozach, P.Y. 2023. *The phenuivirus Toscana virus makes an atypical use of vacuolar acidity to enter host cells*.

Koch, J., Xin, Q., Tischler, N.D. and Lozach, P.Y. 2021. Entry of phenuiviruses into mammalian host cells. *Viruses*. **13**(2), pp.11–14.

Kondo, M. 2010. Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *Immunological Reviews*. **238**(1), pp.37–46.

Koper, O., Kamińska, J., Sawicki, K. and Kemona, H. 2018. CXCL9, CXCL10, CXCL11, and their receptor (CXCR3) in neuroinflammation and neurodegeneration. *Advances in Clinical and Experimental Medicine*. **27**(6), pp.849–856.

Kotenko, S. V. and Durbin, J.E. 2017. Contribution of type III interferons to antiviral immunity: location, location, location. *Journal of Biological Chemistry*. **292**(18), pp.7295–7303.

Kraemer, M.U.G., Reiner, R.C., Brady, O.J., Messina, J.P., Gilbert, M., Pigott, D.M., Yi, D., Johnson, K., Earl, L., Marczak, L.B., Shirude, S., Davis Weaver, N., Bisanzio, D., Perkins, T.A., Lai, S., Lu, X., Jones, P., Coelho, G.E., Carvalho, R.G., Van Bortel, W., Marsboom, C., Hendrickx, G., Schaffner, F., Moore, C.G., Nax, H.H., Bengtsson, L., Wetter, E., Tatem, A.J., Brownstein, J.S., Smith, D.L., Lambrechts, L., Cauchemez, S., Linard, C., Faria, N.R., Pybus, O.G., Scott, T.W., Liu, Q., Yu, H., Wint, G.R.W., Hay, S.I. and Golding, N. 2019. Past and future spread of the arbovirus vectors Aedes aegypti and Aedes albopictus. *Nature Microbiology*. **4**(5), pp.854–863.

Krystel-Whittemore, M., Dileepan, K.N. and Wood, J.G. 2016. Mast cell: A multi-functional master cell. *Frontiers in Immunology*. **6**(JAN), pp.1–12.

Kuhn, J.H., Abe, J., Adkins, S., Alkhovsky, S. V., Avšič-Županc, T., Ayllón, M.A., Bahl, J., Balkema-Buschmann, A., Ballinger, M.J., Baranwal, V.K., Beer, M., Bejerman, N., Bergeron, É., Biedenkopf, N., Blair, C.D., Blasdell, K.R., Blouin, A.G., Bradfute, S.B., Briese, T., Brown, P.A., Buchholz, U.J., Buchmeier, M.J., Bukreyev, A., Burt, F., Büttner, C., Calisher, C.H., Cao, M., Casas, I., Chandran, K., Charrel, R.N., Chaturvedi, K.K., Chooi, K.M., Crane, A., Bó, E.D., de la Torre, J.C., de Souza, W.M., de Swart, R.L., Debat, H., Dheilly, N.M., Di Paola, N., Di Serio, F., Dietzgen, R.G., Digiaro, M., Drexler,

J.F., Duprex, W.P., Dürrwald, R., Easton, A.J., Elbeaino, T., Ergünay, K., Feng, G., Firth, A.E., Fooks, A.R., Formenty, P.B.H., Freitas-Astúa, J., Gago-Zachert, S., García, M.L., García-Sastre, A., Garrison, A.R., Gaskin, T.R., Gong, W., Gonzalez, J.P.J., de Bellocq, J.G., Griffiths, A., Groschup, M.H., Günther, I., Günther, S., Hammond, J., Hasegawa, Y., Hayashi, K., Hepojoki, J., Higgins, C.M., Hongō, S., Horie, M., Hughes, H.R., Hume, A.J., Hyndman, T.H., Ikeda, K., Jiāng, D., Jonson, G.B., Junglen, S., Klempa, B., Klingström, J., Kondō, H., Koonin, E. V., Krupovic, M., Kubota, K., Kurath, G., Laenen, L., Lambert, A.J., Lǐ, J., Li, J.M., Liu, R., Lukashevich, I.S., MacDiarmid, R.M., Maes, P., Marklewitz, M., Marshall, S.H., Marzano, S.Y.L., McCauley, J.W., Mirazimi, A., Mühlberger, E., Nabeshima, T., Naidu, R., Natsuaki, T., Navarro, B., Navarro, J.A., Neriya, Y., Netesov, S. V., Neumann, G., Nowotny, N., Nunes, M.R.T., Ochoa-Corona, F.M., Okada, T., Palacios, G., Pallás, V., Papa, A., Paraskevopoulou, S., Parrish, C.R., Pauvolid-Corrêa, A., Pawęska, J.T., Pérez, D.R., Pfaff, F., Plemper, R.K., Postler, T.S., Rabbidge, L.O., Radoshitzky, S.R., Ramos-González, P.L., Rehanek, M., Resende, R.O., Reyes, C.A., Rodrigues, T.C.S., Romanowski, V., Rubbenstroth, D., Rubino, L., Runstadler, J.A., Sabanadzovic, S., Sadiq, S., Salvato, M.S., Sasaya, T., Schwemmle, M., Sharpe, S.R., Shi, M., Shimomoto, Y., Sidharthan, V.K., Sironi, M., Smither, S., Song, J.W., Spann, K.M., Spengler, J.R., Stenglein, M.D., Takada, A., Takeyama, S., Tatara, A., Tesh, R.B., Thornburg, N.J., Tian, X., Tischler, N.D., Tomitaka, Y., Tomonaga, K., Tordo, N., Tu, C., Turina, M., Tzanetakis, I.E., Vaira, A.M., van den Hoogen, B., Vanmechelen, B., Vasilakis, N., Verbeek, M., von Bargen, S., Wada, J., Wahl, V., Walker, P.J., Waltzek, T.B., Whitfield, A.E., Wolf, Y.I., Xia, H., Xylogianni, E., Yanagisawa, H., Yano, K., Ye, G., Yuan, Z., Zerbini, F.M., Zhang, G., Zhang, S., Zhang, Y.Z., Zhao, L. and Økland, A.L. 2023. Annual (2023) taxonomic update of RNA-directed RNA polymerase-encoding negative-sense RNA viruses (realm Riboviria: kingdom Orthornavirae: phylum Negarnaviricota). *Journal of General Virology*. **104**(8), pp.1–55.

Kuhn, J.H., Adkins, S., Alioto, D., Alkhovsky, S. V., Amarasinghe, G.K., Anthony, S.J., Avšič-Županc, T., Ayllón, M.A., Bahl, J., Balkema-Buschmann, A., Ballinger, M.J., Bartonička, T., Basler, C., Bavari, S., Beer, M., Bente, D.A., Bergeron, É., Bird, B.H., Blair, C., Blasdell, K.R., Bradfute, S.B., Breyta, R., Briese, T., Brown, P.A., Buchholz, U.J., Buchmeier, M.J., Bukreyev, A., Burt, F., Buzkan, N., Calisher, C.H., Cao, M., Casas, I., Chamberlain, J., Chandran, K., Charrel, R.N., Chen, B., Chiumenti, M., Choi, I.R., Clegg, J.C.S., Crozier, I., da Graça, J. V., Dal Bó, E., Dávila, A.M.R., de la Torre, J.C., de Lamballerie, X., de Swart, R.L., Di Bello, P.L., Di Paola, N., Di Serio, F., Dietzgen, R.G., Digiaro, M., Dolja, V. V., Dolnik, O., Drebot, M.A., Drexler, J.F., Dürrwald, R., Dufkova, L., Dundon, W.G., Duprex, W.P., Dye, J.M., Easton, A.J., Ebihara, H., Elbeaino, T., Ergünay, K., Fernandes, J., Fooks, A.R., Formenty, P.B.H., Forth, L.F., Fouchier, R.A.M., Freitas-Astúa, J., Gago-Zachert, S., Gāo, G.F., García, M.L., García-Sastre, A., Garrison, A.R., Gbakima, A., Goldstein, T., Gonzalez, J.P.J., Griffiths, A., Groschup, M.H., Günther, S., Guterres, A., Hall, R.A., Hammond, J., Hassan, M., Hepojoki, J., Hepojoki, S., Hetzel, U., Hewson, R., Hoffmann, B., Hongo, S., Höper, D., Horie, M., Hughes, H.R., Hyndman, T.H., Jambai, A., Jardim, R., Jiāng, D., Jin, Q., Jonson, G.B., Junglen, S., Karadağ, S., Keller, K.E., Klempa, B., Klingström, J., Kobinger, G., Kondō, H., Koonin, E. V., Krupovic, M., Kurath, G., Kuzmin, I. V., Laenen, L., Lamb, R.A., Lambert, A.J., Langevin, S.L., Lee, B., Lemos, E.R.S., Leroy, E.M., Li, D., Lǐ, J., Liang, M., Liú, W., Liú, Y., Lukashevich, I.S., Maes, P., Marciel de Souza, W., Marklewitz, M., Marshall, S.H., Martelli, G.P., Martin, R.R., Marzano, S.Y.L., Massart, S., McCauley, J.W., Mielke-Ehret, N., Minafra, A., Minutolo, M., Mirazimi, A., Mühlbach, H.P., Mühlberger, E., Naidu, R., Natsuaki, T., Navarro, B., Navarro, J.A., Netesov, S. V., Neumann, G., Nowotny, N., Nunes, M.R.T., Nylund, A., Økland, A.L., Oliveira, R.C., Palacios, G., Pallas, V., Pályi, B., Papa, A., Parrish, C.R., Pauvolid-Corrêa, A., Pawęska, J.T., Payne, S., Pérez, D.R., Pfaff, F., Radoshitzky, S.R., Rahman, A. ul, Ramos-González, P.L., Resende, R.O., Reyes, C.A.,

Rima, B.K., Romanowski, V., Robles Luna, G., Rota, P., Rubbenstroth, D., Runstadler, J.A., Ruzek, D., Sabanadzovic, S., Salát, J., Sall, A.A., Salvato, M.S., Sarpkaya, K., Sasaya, T., Schwemmle, M., Shabbir, M.Z., Shí, X., Shí, Z., Shirako, Y., Simmonds, P., Širmarová, J., Sironi, M., Smither, S., Smura, T., Song, J.W., Spann, K.M., Spengler, J.R., Stenglein, M.D., Stone, D.M., Straková, P., Takada, A., Tesh, R.B., Thornburg, N.J., Tomonaga, K., Tordo, N., Towner, J.S., Turina, M., Tzanetakis, I., Ulrich, R.G., Vaira, A.M., van den Hoogen, B., Varsani, A., Vasilakis, N., Verbeek, M., Wahl, V., Walker, P.J., Wang, H., Wang, J., Wang, X., Wang, L.F., Wèi, T., Wells, H., Whitfield, A.E., Williams, J. V., Wolf, Y.I., Wú, Z., Yang, X., Yáng, X., Yu, X., Yutin, N., Zerbini, F.M., Zhang, T., Zhang, Y.Z., Zhou, G. and Zhou, X. 2020. 2020 taxonomic update for phylum Negarnaviricota (Riboviria: Orthornavirae), including the large orders Bunyavirales and Mononegavirales. *Archives of Virology*. **165**(12), pp.3023–3072.

Kumar, H., Kawai, T. and Akira, S. 2011. Pathogen recognition by the innate immune system. *International Reviews of Immunology*. **30**(1), pp.16–34.

Kuşçu, F., Menemenlioğlu, D., Öztürk, D.B., Korukluoğlu, G. and Uyar, Y. 2014. Anti-HIV Pozitif Bir Hastada Saptanan Akut Toskana Virus Enfeksiyonu*. *Mikrobiyoloji Bulteni*. **48**(1), pp.168–173.

Kutz, F.W., Yobs, A.R., Strassman, S.C. and Viar, J.F. 1977. Pesticides in people. Effects of reducing DDT usage on total DDT storage in humans. *Pesticides Monitor. J.* **11**(2), pp.61–63.

Lacey, L.A. 2007. Bacillus thuringiensis serovariety israelensis and Bacillus sphaericus for mosquito control. *Journal of the American Mosquito Control Association*. **23**(sp2), pp.133–163.

Lane, R.P. and Crosskey, R.W. 2012. *Medical insects and arachnids*. Springer Science & Business Media.

Lang, K.S., Burow, A., Kurrer, M., Lang, P.A. and Recher, M. 2007. The role of the innate immune response in autoimmune disease. *Journal of Autoimmunity*. **29**(4), pp.206–212.

Laroche, L., Ayhan, N., Charrel, R., Bañuls, A.-L. and Prudhomme, J. 2023. Persistence of Toscana virus in sugar and blood meals of phlebotomine sand flies: epidemiological and experimental consequences. *Scientific Reports 2023 13:1*. **13**(1), pp.1–7.

Laroche, L., Jourdain, F., Ayhan, N., Bañuls, A.L., Charrel, R. and Prudhomme, J. 2021. Incubation period for neuroinvasive toscana virus infections. *Emerging Infectious Diseases*. **27**(12), pp.3147–3150.

Latrofa, M.S., Iatta, R., Dantas-Torres, F., Annoscia, G., Gabrielli, S., Pombi, M., Gradoni, L. and Otranto, D. 2018. Detection of Leishmania infantum DNA in phlebotomine sand flies from an area where canine leishmaniosis is endemic in southern Italy. *Veterinary Parasitology*. **253**(February), pp.39–42.

Lazear, H.M., Govero, J., Smith, A.M., Platt, D.J., Fernandez, E., Miner, J.J. and Diamond, M.S. 2016. A Mouse Model of Zika Virus Pathogenesis. *Cell Host and Microbe*. **19**(5), pp.720–730.

Lee, M.F., Wu, Y.S. and Poh, C.L. 2023. Molecular Mechanisms of Antiviral Agents against Dengue Virus. *Viruses*. **15**(3), p.705.

Lefteri, D.A., Bryden, S.R., Pingen, M., Terry, S., McCafferty, A., Beswick, E.F., Georgiev, G., Van der Laan, M., Mastrullo, V., Campagnolo, P., Waterhouse, R.M., Varjak, M., Merits, A., Fragkoudis, R., Griffin, S., Shams, K., Pondeville, E. and McKimmie, C.S. 2022.

Mosquito saliva enhances virus infection through sialokinin-dependent vascular leakage. *Proceedings of the National Academy of Sciences*. **119**(24).

Leitner, W.W., Wali, T., Kincaid, R. and Costero-Saint Denis, A. 2015. Arthropod Vectors and Disease Transmission: Translational Aspects. *PLoS Neglected Tropical Diseases*. **9**(11), pp.1–11.

Lestinova, T., Rohousova, I., Sima, M., de Oliveira, C.I. and Volf, P. 2017. Insights into the sand fly saliva: Blood-feeding and immune interactions between sand flies, hosts, and Leishmania G. Milon, ed. *PLOS Neglected Tropical Diseases*. **11**(7), p.e0005600.

van Leur, S.W., Heunis, T., Munnur, D. and Sanyal, S. 2021. Pathogenesis and virulence of flavivirus infections. *Virulence*. **12**(1), pp.2814–2838.

Lewis, D.J. 1971. Phlebotomid sandflies. *Bulletin of the World Health Organization*. **44**(4), pp.535–551.

Lewis, S.M., Williams, A. and Eisenbarth, S.C. 2019. Structure and function of the immune system in the spleen. *Science Immunology*. **4**(33), pp.100–106.

Li, H., Gang, Z., Yuling, H., Luokun, X., Jie, X., Hao, L., Li, W., Chunsong, H., Junyan, L., Mingshen, J., Youxin, J., Feili, G., Boquan, J. and Jinquan, T. 2006. Different Neurotropic Pathogens Elicit Neurotoxic CCR9- or Neurosupportive CXCR3-Expressing Microglia. *The Journal of Immunology*. **177**(6), pp.3644–3656.

Li, P., Spolski, R., Liao, W. and Leonard, W.J. 2014. Complex interactions of transcription factors in mediating cytokine biology in T cells. *Immunological Reviews*. **261**(1), pp.141–156.

Liang, G., Gao, X. and Gould, E.A. 2015. Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. *Emerging Microbes and Infections*. **4**(3).

Liao, W., Lin, J.-X. and Leonard, W.J. 2013. Interleukin-2 at the Crossroads of Effector Responses, Tolerance, and Immunotherapy. *Immunity*. **38**(1), pp.13–25.

Lieskovská, J., Páleníková, J., Širmarová, J., Elsterová, J., Kotsyfakis, M., Campos Chagas, A., Calvo, E., Růžek, D. and Kopecký, J. 2015. Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells. *Parasite Immunology*. **37**(2), pp.70–78.

Liljeström, P., Lusa, S., Huylebroeck, D. and Garoff, H. 1991. In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *Journal of Virology*. **65**(8), pp.4107–4113.

Lim, P.-Y., Behr, M.J., Chadwick, C.M., Shi, P.-Y. and Bernard, K.A. 2011. Keratinocytes Are Cell Targets of West Nile Virus In Vivo. *Journal of Virology*. **85**(10), pp.5197–5201.

Lima, H.C. and Titus, R.G. 1996. Effects of sand fly vector saliva on development of cutaneous lesions and the immune response to Leishmania braziliensis in BALB/c mice. *Infection and Immunity*. **64**(12), pp.5442–5445.

Lin, C.Y., Wang, W.H., Urbina, A.N., Tseng, S.P., Lu, P.L., Chen, Y.H., Yu, M.L. and Wang, S.F. 2020. Importation of SARS-CoV-2 infection leads to major COVID-19 epidemic in Taiwan. *International Journal of Infectious Diseases*. **97**, pp.240–244.

Lin, J.-X. and Leonard, W.J. 2018. The Common Cytokine Receptor γ Chain Family of Cytokines. *Cold Spring Harbor Perspectives in Biology*. **10**(9), p.a028449.

Lindsey, N.P., Horton, J., Barrett, A.D.T., Demanou, M., Monath, T.P., Tomori, O., Van Herp, M., Zeller, H., Fall, I.S., Cibrelus, L. and Erin Staples, J. 2022. Yellow fever resurgence: An avoidable crisis? *npj Vaccines*. **7**(1), pp.2–4.

Link, V.M., Duttke, S.H., Chun, H.B., Holtman, I.R., Westin, E., Hoeksema, M.A., Abe, Y., Skola, D., Romanoski, C.E., Tao, J., Fonseca, G.J., Troutman, T.D., Spann, N.J., Strid, T., Sakai, M., Yu, M., Hu, R., Fang, R., Metzler, D., Ren, B. and Glass, C.K. 2018. Analysis of Genetically Diverse Macrophages Reveals Local and Domain-wide Mechanisms that Control Transcription Factor Binding and Function. *Cell*. **173**(7), pp.1796-1809.e17.

Liu, J., Zhang, X., Cheng, Y. and Cao, X. 2021. Dendritic cell migration in inflammation and immunity. *Cellular and Molecular Immunology*. **18**(11), pp.2461–2471.

Liu, M., Guo, S., Hibbert, J.M., Jain, V., Singh, N., Wilson, N.O. and Stiles, J.K. 2011. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine and Growth Factor Reviews*. **22**(3), pp.121–130.

Liu, T., Zhang, L., Joo, D. and Sun, S.C. 2017. NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*. **2**(April).

Liu, X., Poo, Y.-S., Alves, J.C., Almeida, R.P., Mostafavi, H., Tang, P.C.H., Bucala, R., Teixeira, M.M., Taylor, A., Zaid, A. and Mahalingam, S. 2022. Interleukin-17 Contributes to Chikungunya Virus-Induced Disease D. E. Griffin, ed. *mBio*. **13**(2).

Lopez-Castejon, G. and Brough, D. 2011. Understanding the mechanism of IL-1β secretion. *Cytokine & Growth Factor Reviews*. **22**(4), pp.189–195.

Lozach, P.-Y., Kühbacher, A., Meier, R., Mancini, R., Bitto, D., Bouloy, M. and Helenius, A. 2011. DC-SIGN as a Receptor for Phleboviruses. *Cell Host & Microbe*. **10**(1), pp.75–88.

Lozach, P.Y., Mancini, R., Bitto, D., Meier, R., Oestereich, L., Överby, A.K., Pettersson, R.F. and Helenius, A. 2010. Entry of bunyaviruses into mammalian cells. *Cell Host and Microbe*. **7**(6), pp.488–499.

Ludlow, M., Kortekaas, J., Herden, C., Hoffmann, B., Tappe, D., Trebst, C., Griffin, D.E., Brindle, H.E., Solomon, T., Brown, A.S., van Riel, D., Wolthers, K.C., Pajkrt, D., Wohlsein, P., Martina, B.E.E., Baumgärtner, W., Verjans, G.M. and Osterhaus, A.D.M.E. 2016. Neurotropic virus infections as the cause of immediate and delayed neuropathology. *Acta Neuropathologica*. **131**(2), pp.159–184.

MacNamara, F.N. 1954. Zika virus: A report on three cases of human infection during an epidemic of jaundice in Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **48**(2), pp.139–145.

Maia, C., Alwassouf, S., Cristóvão, J.M., Ayhan, N., Pereira, A., Charrel, R.N. and Campino, L. 2017. Serological association between Leishmania infantum and sand fly fever Sicilian (but not Toscana) virus in sheltered dogs from southern Portugal. *Parasites & Vectors*. **10**(1), p.92.

Maia, C., Ayhan, N., Cristóvão, J.M., Pereira, A. and Charrel, R. 2022. Human seroprevalence of Toscana virus and Sicilian phlebovirus in the southwest of Portugal. *European Journal of Clinical Microbiology and Infectious Diseases*. **41**(1), pp.137–141.

Maia, C. and Depaquit, J. 2016. Can Sergentomyia (Diptera, Psychodidae) play a role in the transmission of mammal-infecting Leishmania ? *Parasite*. **23**.

Maia, C., Parreira, R., Cristóvão, J.M., Freitas, F.B., Afonso, M.O. and Campino, L. 2015. Molecular detection of Leishmania DNA and identification of blood meals in wild caught

phlebotomine sand flies (Diptera: Psychodidae) from southern Portugal. *Parasites and Vectors*. **8**(1), pp.1–10.

Manwaring, W.H. 1945. Semliki Forest Virus. *California and Western Medicine*. **62**(1), p.4.

Mapalagamage, M., Weiskopf, D., Sette, A. and De Silva, A.D. 2022. Current Understanding of the Role of T Cells in Chikungunya, Dengue and Zika Infections. *Viruses*. **14**(2), p.242.

Marchi, S., Trombetta, C.M., Kistner, O. and Montomoli, E. 2017. Seroprevalence study of Toscana virus and viruses belonging to the Sandfly fever Naples antigenic complex in central and southern Italy. *Journal of Infection and Public Health*. **10**(6), pp.866–869.

Marklewitz, M., Tchouassi, D.P., Hieke, C., Heyde, V., Torto, B., Sang, R. and Junglen, S. 2020. Insights into the Evolutionary Origin of Mediterranean Sandfly Fever Viruses C. F. Spiropoulou, ed. *mSphere*. **5**(5), pp.1–12.

Maroli, M., Ciufolini, M.G. and Verani, P. 1993. Vertical transmission of Toscana virus in the sandfly, Phlebotomus perniciosus, via the second gonotrophic cycle. *Medical and veterinary entomology*. **7**(3), pp.283–286.

Maroli, M., Feliciangeli, M.D., Bichaud, L., Charrel, R.N. and Gradoni, L. 2013. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. *Medical and Veterinary Entomology*. **27**(2), pp.123–147.

Marou, V., Vardavas, C.I., Aslanoglou, K., Nikitara, K., Plyta, Z., Leonardi-Bee, J., Atkins, K., Condell, O., Lamb, F. and Suk, J.E. 2024. The impact of conflict on infectious disease: a systematic literature review. *Conflict and Health*. **18**(1), p.27.

Marovich, M., Grouard-Vogel, G.N., Louder, M., Eller, M., Sun, W., Wu, S.J., Putvatana, R., Murphy, G., Tassaneetrithep, B., Burgess, T., Birx, D., Hayes, C., Schlesinger-Frankel, S. and Mascola, J. 2001. Human dendritic cells as targets of dengue virus infection. *Journal of Investigative Dermatology Symposium Proceedings*. **6**(3), pp.219–224.

Martin, M.-F., Maarifi, G., Abiven, H., Seffals, M., Mouchet, N., Beck, C., Bodet, C., Lévèque, N., Arhel, N.J., Blanchet, F.P., Simonin, Y. and Nisole, S. 2022. Usutu Virus escapes langerin-induced restriction to productively infect human Langerhans cells, unlike West Nile virus. *Emerging Microbes & Infections*. **11**(1), pp.761–774.

Martinez, F.O. and Gordon, S. 2014. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Reports*. **6**(March), pp.1–13.

Martinez, J., Garcia, S., Walter, S., Gil- Prieto, R., Lacomba, D., Marin-Garcia, P., Gil-De-Miguel, A. and Gonzalez-Escalada, A. 2022. Seroprevalence against Toscana virus in Spain: The case of the autonomous community of Madrid. *Journal of Vector Borne Diseases*. **59**(2), pp.172–177.

Martínez-García, F.A., Moreno-Docón, A., Segovia-Hernández, M. and Fernández-Barreiro, A. 2008. [Deafness as a sequela of Toscana virus meningitis]. *Medicina clinica*. **130**(16), p.639.

Marzouki, S., Abdeladhim, M., Abdessalem, C. Ben, Oliveira, F., Ferjani, B., Gilmore, D., Louzir, H., Valenzuela, J.G. and Ahmed, M. Ben 2012. Salivary Antigen SP32 Is the Immunodominant Target of the Antibody Response to Phlebotomus papatasi Bites in Humans. *PLoS Neglected Tropical Diseases*. **6**(11), pp.1–11.

Marzouki, S., Kammoun-Rebai, W., Bettaieb, J., Abdeladhim, M., Hadj Kacem, S., Abdelkader, R., Gritli, S., Chemkhi, J., Aslan, H., Kamhawi, S., Ben Salah, A., Louzir, H., Valenzuela, J.G. and Ben Ahmed, M. 2015. Validation of Recombinant Salivary Protein PpSP32 as a

Suitable Marker of Human Exposure to Phlebotomus papatasi, the Vector of Leishmania major in Tunisia. *PLoS Neglected Tropical Diseases*. **9**(9), pp.1–14.

Mascitti, H., Calin, R., Dinh, A., Makhloufi, S. and Davido, B. 2020. Testicular pain associated with clear fluid meningitis: How many cases of Toscana virus are we missing? *International Journal of Infectious Diseases*. **93**, pp.198–200.

Masse, S., Ayhan, N., Capai, L., Bosseur, F., De Lamballerie, X., Charrel, R. and Falchi, A. 2019. Circulation of toscana virus in a sample population of corsica, France. *Viruses*. **11**(9), pp.1–8.

Mathiot, C.C., Grimaud, G., Garry, P., Bouquety, J.C., Mada, A., Daguisy, A.M. and Georges, A.J. 1990. An outbreak of human semliki forest virus infections in Central African Republic. *American Journal of Tropical Medicine and Hygiene*. **42**(4), pp.386–393.

Mathur, A., Khanna, N., Kulshreshtha, R., Maitra, S.C. and Chaturvedi, U.C. 1995. Viruria during acute Japanese encephalitis virus infection. *International journal of experimental pathology*. **76**(2), pp.103–9.

Matlock, B. 2015. Assessment of Nucleic Acid Purity. *Thermoscientific Technical Bulletin NanoDrop Spectrophotometers*., pp.1–2.

Matusali, G., D'Abramo, A., Terrosi, C., Carletti, F., Colavita, F., Vairo, F., Savellini, G.G., Gandolfo, C., Anichini, G., Lalle, E., Bordi, L., Corpolongo, A., Maritti, M., Marchioni, L., Capobianchi, M.R., Castilletti, C., Cusi, M.G. and Nicastri, E. 2022. Infectious Toscana Virus in Seminal Fluid of Young Man Returning from Elba Island, Italy. *Emerging Infectious Diseases*. **28**(4), pp.865–869.

Mbow, M.L., Bleyenberg, J.A., Hall, L.R. and Titus, R.G. 1998. Phlebotomus papatasi Sand Fly Salivary Gland Lysate Down-Regulates a Th1, but Up-Regulates a Th2, Response in Mice Infected with Leishmania major. *The Journal of Immunology*. **161**(10), pp.5571–5577.

McCracken, M.K., Christofferson, R.C., Chisenhall, D.M. and Mores, C.N. 2014. Analysis of Early Dengue Virus Infection in Mice as Modulated by Aedes aegypti Probing. *Journal of Virology*. **88**(4), pp.1881–1889.

McKimmie, C.S. and Fazakerley, J.K. 2016. Encephalitic Alphaviruses *In*: *Alphaviruses: Current Biology* [Online]. Caister Academic Press, pp.139–156. Available from: https://www.caister.com/alpha.

McNab, F., Mayer-Barber, K., Sher, A., Wack, A. and O'Garra, A. 2015. Type I interferons in infectious disease. *Nature Reviews Immunology*. **15**(2), pp.87–103.

Medzhitov, R. and Janeway, C. 2000. Innate immune recognition: Mechanisms and pathways. *Immunological Reviews*. **173**, pp.89–97.

Medzhitov, R. and Janeway, C.A. 1997. Innate immunity: impact on the adaptive immune response. *Current Opinion in Immunology*. **9**(1), pp.4–9.

Di Meglio, P., Perera, G.K. and Nestle, F.O. 2011. The Multitasking Organ: Recent Insights into Skin Immune Function. *Immunity*. **35**(6), pp.857–869.

Mehlhop, E. and Diamond, M.S. 2006. Protective immune responses against West Nile virus are primed by distinct complement activation pathways. *Journal of Experimental Medicine*. **203**(5), pp.1371–1381.

Mellor, P.S. 2000. Replication of arboviruses in insect vectors. *Journal of Comparative Pathology*. **123**(4), pp.231–247.

Mendoza-Montero, J., Gamez-Rueda, M.-I., Navarro-Mari, J.-M., de la Rosa-Fraile, M. and Oyonarte-Gomez, S. 1998. Infections Due to Sandfly Fever Virus Serotype Toscana in Spain. *Clinical Infectious Diseases*. **27**(3), pp.434–436.

Messahel, N.E., Benallal, K.E., Halada, P., Lafri, I., Manseur, H., Hakem, A., Houali, K., Harrat, Z., Volf, P. and Dvorak, V. 2022. Identification of blood source preferences and Leishmania infection in sand flies (Diptera: Psychodidae) in north-eastern Algeria. *Veterinary Parasitology: Regional Studies and Reports*. **31**(March), p.100729.

Messina, J.P., Pigott, D.M., Golding, N., Duda, K.A., Brownstein, J.S., Weiss, D.J., Gibson, H., Robinson, T.P., Gilbert, M., Wint, G.R.W., Nuttall, P.A., Gething, P.W., Myers, M.F., George, D.B. and Hay, S.I. 2015. The global distribution of Crimean-Congo hemorrhagic fever. . (July), pp.503–513.

Michlmayr, D., Andrade, P., Gonzalez, K., Balmaseda, A. and Harris, E. 2017. CD14+CD16+ monocytes are the main target of Zika virus infection in peripheral blood mononuclear cells in a paediatric study in Nicaragua. *Nature Microbiology*. **2**(11), pp.1462–1470.

Michlmayr, D., McKimmie, C.S., Pingen, M., Haxton, B., Mansfield, K., Johnson, N., Fooks, A.R. and Graham, G.J. 2014. Defining the Chemokine Basis for Leukocyte Recruitment during Viral Encephalitis M. S. Diamond, ed. *Journal of Virology*. **88**(17), pp.9553–9567.

Miller, L.S. 2008. Toll-Like Receptors in Skin. *Advances in Dermatology*. **24**(C), pp.71–87.

Miller, M.C. and Mayo, K.H. 2017. Chemokines from a structural perspective. *International Journal of Molecular Sciences*. **18**(10), pp.1–16.

Miner, J.J. 2017. Congenital Zika virus infection: More than just microcephaly. *Science translational medicine*. **9**(393), p.eaan8195.

Miner, J.J. and Diamond, M.S. 2017. Zika Virus Pathogenesis and Tissue Tropism. *Cell Host & Microbe*. **21**(2), pp.134–142.

Mitchell, A.J., Roediger, B. and Weninger, W. 2014. Monocyte homeostasis and the plasticity of inflammatory monocytes. *Cellular Immunology*. **291**(1–2), pp.22–31.

Mittrücker, H.W., Visekruna, A. and Huber, M. 2014. Heterogeneity in the Differentiation and Function of CD8+ T Cells. *Archivum Immunologiae et Therapiae Experimentalis*. **62**(6), pp.449–458.

Moafi, M., Rezvan, H., Sherkat, R. and Taleban, R. 2019. Leishmania vaccines entered in clinical trials: A review of literature. *International Journal of Preventive Medicine*. **10**(1), p.95.

Molgora, M., Supino, D., Mavilio, D., Santoni, A., Moretta, L., Mantovani, A. and Garlanda, C. 2018. The yin-yang of the interaction between myelomonocytic cells and NK cells. *Scandinavian Journal of Immunology*. **88**(3), pp.1–17.

Monath, T.P. 2005. Yellow fever vaccine. *Expert Review of Vaccines*. **4**(4), pp.553–574.

Moncaz, A., Kirstein, O., Gebresellassie, A., Lemma, W., Gebre-Michael, T., Balkew, M., Belay, S., Hailu, A. and Warburg, A. 2014. Sergentomyia spp.: Breeding sites in vertisols and peri-domestic habitats in North West Ethiopia. *Acta Tropica*. **137**, pp.88–94.

Moratelli, R. and Calisher, C.H. 2015. Bats and zoonotic viruses: Can we confidently link bats with emerging deadly viruses? *Memorias do Instituto Oswaldo Cruz*. **110**(1), pp.1–22.

Morens, D.M., Breman, J.G., Calisher, C.H., Doherty, P.C., Hahn, B.H., Keusch, G.T., Kramer, L.D., LeDuc, J.W., Monath, T.P. and Taubenberger, J.K. 2020. The Origin of COVID-19

and Why It Matters. *The American Journal of Tropical Medicine and Hygiene*. **103**(3), pp.955–959.

Mori, A., Matucci, A., Pomari, E., Accordini, S., Piubelli, C., Donini, A., Nicolini, L. and Castilletti, C. 2024. Urine: A Pitfall for Molecular Detection of Toscana Virus? An Analytical Proof-of-Concept Study. *Viruses*. **16**(1).

Morrison, T.E. and Diamond, M.S. 2017. Animal Models of Zika Virus Infection, Pathogenesis, and Immunity T. C. Pierson, ed. *Journal of Virology*. **91**(8), pp.419–426.

Morrison, T.E., Oko, L., Montgomery, S.A., Whitmore, A.C., Lotstein, A.R., Gunn, B.M., Elmore, S.A. and Heise, M.T. 2011. A mouse model of chikungunya virus-induced musculoskeletal inflammatory disease: Evidence of arthritis, tenosynovitis, myositis, and persistence. *American Journal of Pathology*. **178**(1), pp.32–40.

Morrison, T.E., Whitmore, A.C., Shabman, R.S., Lidbury, B.A., Mahalingam, S. and Heise, M.T. 2006. Characterization of Ross River Virus Tropism and Virus-Induced Inflammation in a Mouse Model of Viral Arthritis and Myositis. *Journal of Virology*. **80**(2), pp.737–749.

Morse, S.S. 2004. Factors and determinants of disease emergence. *Revue Scientifique et Technique de l'OIE*. **23**(2), pp.443–451.

Moser, L.A., Lim, P.-Y., Styer, L.M., Kramer, L.D. and Bernard, K.A. 2016. Parameters of Mosquito-Enhanced West Nile Virus Infection. *Journal of Virology*. **90**(1), pp.292–299.

Mosnier, E., Charrel, R., Vidal, B., Ninove, L., Schleinitz, N., Harlé, J.R. and Bernit, E. 2013. Toscana virus myositis and fasciitis. *Medecine et Maladies Infectieuses*. **43**(5), pp.208–210.

Mosser, D.M. and Edwards, J.P. 2008. Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology*. **8**(12), pp.958–969.

Mostafavi, H., Tharmarajah, K., Vider, J., West, N.P., Freitas, J.R., Cameron, B., Foster, P.S., Hueston, L.P., Lloyd, A.R., Mahalingam, S. and Zaid, A. 2022. *Interleukin-17 contributes to Ross River virus-induced arthritis and myositis* [Online]. Available from: http://dx.doi.org/10.1371/journal.ppat.1010185.

Moureau, G., Cook, S., Lemey, P., Nougairede, A., Forrester, N.L., Khasnatinov, M., Charrel, R.N., Firth, A.E., Gould, E.A. and de Lamballerie, X. 2015. New Insights into Flavivirus Evolution, Taxonomy and Biogeographic History, Extended by Analysis of Canonical and Alternative Coding Sequences Y.-M. Lee, ed. *PLOS ONE*. **10**(2), p.e0117849.

Mowat, A.M. and Agace, W.W. 2014. Regional specialization within the intestinal immune system. *Nature Reviews Immunology*. **14**(10), pp.667–685.

Mueller, C.G. and Cao-Lormeau, V.-M. 2018. Insect-Borne Viruses and Host Skin Interface *In*: *Skin and Arthropod Vectors* [Online]. Elsevier, pp.275–292. Available from: https://doi.org/10.1016/B978-0-12-811436-0.00008-3.

Muñoz, C., Ayhan, N., Ortuño, M., Ortiz, J., Gould, E.A., Maia, C., Berriatua, E. and Charrel, R.N. 2020. Experimental Infection of Dogs with Toscana Virus and Sandfly Fever Sicilian Virus to Determine Their Potential as Possible Vertebrate Hosts. *Microorganisms*. **8**(4), p.596.

Murphy, P.M., Baggiolini, M., Charo, I.F., Hebert, C.A., Horuk, R., Matsushima, K., Miller, L.H., Oppenheim, J.J. and Power, C.A. 2000. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacological Reviews*. **52**(1), pp.145–176.

Musso, D. and Gubler, D.J. 2016. Zika Virus. *Clinical Microbiology Reviews*. **29**(3), pp.487–524.

Musso, D., Rousset, D. and Peyrefitte, C. 2022. Special Issue “Endemic Arboviruses”. *Viruses*. **14**(3), p.645.

Nanyingi, M.O., Munyua, P., Kiama, S.G., Muchemi, G.M., Thumbi, S.M., Bitek, A.O., Bett, B., Muriithi, R.M. and Njenga, M.K. 2015. A systematic review of Rift Valley Fever epidemiology 1931–2014. *Infection Ecology and Epidemiology*. **5**(1), pp.1–12.

Nasci, R.S. and Mutebi, J.P. 2019. Reducing West Nile Virus Risk Through Vector Management. *Journal of Medical Entomology*. **56**(6), pp.1516–1521.

Naucke, T.J., Lorentz, S., Rauchenwald, F. and Aspöck, H. 2011. Phlebotomus (Transphlebotomus) mascittii Grassi, 1908, in Carinthia: First record of the occurrence of sandflies in Austria (Diptera: Psychodidae: Phlebotominae). *Parasitology Research*. **109**(4), pp.1161–1164.

Naucke, T.J., Menn, B., Massberg, D. and Lorentz, S. 2008. Sandflies and leishmaniasis in Germany. *Parasitology Research*. **103**(SUPPL. 1), pp.65–68.

Navarro, E., Serrano-Heras, G., Castaño, M.J. and Solera, J. 2015. Real-time PCR detection chemistry. *Clinica Chimica Acta*. **439**, pp.231–250.

Navarro, J.M., Fernández-Roldán, C., Pérez-Ruiz, M., Sanbonmatsu, S., de la Rosa, M. and Sánchez-Seco, M.P. 2004. Meningitis by Toscana virus in Spain: description of 17 cases. *Medicina clinica*. **122**(11), pp.420–422.

Navarro-Marí, J.M., Palop-Borrás, B., Pérez-Ruiz, M. and Sanbonmatsu-Gámez, S. 2011. Serosurvey study of toscana virus in domestic animals, Granada, Spain. *Vector-Borne and Zoonotic Diseases*. **11**(5), pp.583–587.

Nestle, F.O., Di Meglio, P., Qin, J.-Z. and Nickoloff, B.J. 2009. Skin immune sentinels in health and disease. *Nature Reviews Immunology*. **9**(10), pp.679–691.

Nguyen, A. V. and Soulika, A.M. 2019. The Dynamics of the Skin’s Immune System. *International Journal of Molecular Sciences*. **20**(8), p.1811.

Nicoletti, L., Ciufolini, M.G. and Verani, P. 1996. Sandfly fever viruses in Italy *In*: *Imported Virus Infections* [Online]. Vienna, Austria: Springer Vienna, pp.41–47. Available from: http://link.springer.com/10.1007/978-3-7091-7482-1_5.

Nicoletti, L., Verani, P., Caciolli, S., Ciufolini, M.G., Renzi, A., Bartolozzi, D., Paci, P., Leoncini, F., Padovani, P., Traini, E., Baldereschi, M. and Balducci, M. 1991. Central Nervous System Involvement during Infection by Phlebovirus Toscana of Residents in Natural Foci in Central Italy (1977–1988). *The American Journal of Tropical Medicine and Hygiene*. **45**(4), pp.429–434.

Di Nicuolo, G., Pagliano, P., Battisti, S., Starace, M., Mininni, V., Attanasio, V. and Faella, F.S. 2005. Toscana virus central nervous system infections in Southern Italy. *Journal of Clinical Microbiology*. **43**(12), pp.6186–6188.

Nii-Trebi, N.I. 2017. Emerging and Neglected Infectious Diseases: Insights, Advances, and Challenges. *BioMed Research International*. **2017**, pp.1–15.

Niklasson, B. and Eitrem, R. 1985. Sandfly fever among Swedish UN troops in Cyprus. *The Lancet*. **325**(8439), p.1212.

Nussenzweig, M.C. 2011. Ralph Steinman and the discovery of dendritic cells. *Nobel lecture*., pp.1–31.

Nuttall, P.A. 2019a. Tick saliva and its role in pathogen transmission. *Wiener klinische Wochenschrift*. **135**(7–8), pp.165–176.

Nuttall, P.A. 2019b. Wonders of tick saliva. *Ticks and Tick-borne Diseases*. **10**(2), pp.470–481.

Obeng-Kusi, M., Martin, J. and Abraham, I. 2024. The economic burden of Ebola virus disease: a review and recommendations for analysis. *Journal of Medical Economics*. **27**(1), pp.309–323.

O'Brien, R.L. and Born, W.K. 2015. Dermal γδ T cells – What have we learned? *Cellular Immunology*. **296**(1), pp.62–69.

Ocal, M., Orsten, S., Inkaya, A.C., Yetim, E., Acar, N.P., Alp, S., Erisoz Kasap, O., Gunay, F., Arsava, E.M., Alten, B., Ozkul, A., Us, D., Niedrig, M. and Ergunay, K. 2014. Ongoing Activity of Toscana Virus Genotype A and West Nile Virus Lineage 1 Strains in Turkey: A Clinical and Field Survey. *Zoonoses and Public Health*. **61**(7), pp.480–491.

O'Connor, T., Borsig, L. and Heikenwalder, M. 2015. CCL2-CCR2 Signaling in Disease Pathogenesis. *Endocrine, Metabolic & Immune Disorders-Drug Targets*. **15**(2), pp.105–118.

Oechtering, J. and Petzold, G.C. 2012. Acute hydrocephalus due to impaired CSF resorption in Toscana virus meningoencephalitis. *Neurology*. **79**(8), pp.829–831.

Oerther, S. 2020. Entomology , Ornithology & Herpetology : Phlebotomus mascittii as Potential Vector of Human Pathogens in Southwest Germany. . **9**(4), pp.1–3.

Okar, S.V., Bekircan-Kurt, C.E., Hacıoğlu, S., Erdem-Özdamar, S., Özkul, A. and Ergünay, K. 2021. Toscana virus associated with Guillain–Barré syndrome: a case–control study. *Acta Neurologica Belgica*. **121**(3), pp.661–668.

Oker-Blom, N., Salminen, A., Brummer-Korvenkontio, M., Kaariainen, L. and Weckstrom, P. 1964. Isolation of some viruses other than typical tick-borne encephalitis viruses from Ixodes ricinus ticks in Finland.

Okwor, I. and Uzonna, J. 2016. Social and economic burden of human leishmaniasis. *American Journal of Tropical Medicine and Hygiene*. **94**(3), pp.489–493.

Oliveira, F., Lawyer, P.G., Kamhawi, S. and Valenzuela, J.G. 2008. Immunity to Distinct Sand Fly Salivary Proteins Primes the Anti-Leishmania Immune Response towards Protection or Exacerbation of Disease M. J. Lehane, ed. *PLoS Neglected Tropical Diseases*. **2**(4), p.e226.

Ortuño, M., Muñoz, C., Spitzová, T., Sumova, P., Iborra, M.A., Pérez-Cutillas, P., Ayhan, N., Charrel, R.N., Volf, P. and Berriatua, E. 2022. Exposure to Phlebotomus perniciosus sandfly vectors is positively associated with Toscana virus and Leishmania infantum infection in human blood donors in Murcia Region, southeast Spain. *Transboundary and Emerging Diseases*. **69**(5), pp.e1854–e1864.

Oryan, A. and Akbari, M. 2016. Worldwide risk factors in leishmaniasis. *Asian Pacific Journal of Tropical Medicine*. **9**(10), pp.925–932.

de Ory-Manchón, F., Carlos Sanz-Moreno, J., Aranguez-Ruiz, E. and Ramírez-Fernández, R. 2007. Seroprevalencia edad dependiente frente al virus Toscana en la Comunidad de Madrid: años 1993-1994 y 1999-2000. *Enfermedades Infecciosas y Microbiología Clínica*. **25**(3), pp.187–189.

Paaijmans, K.P. and Thomas, M.B. 2011. The influence of mosquito resting behaviour and associated microclimate for malaria risk. *Malaria Journal*. **10**, pp.1–7.

Paixao, E.S., Cardim, L.L., Costa, M.C.N., Brickley, E.B., de Carvalho-Sauer, R.C.O., Carmo, E.H., Andrade, R.F.S., Rodrigues, M.S., Veiga, R. V., Costa, L.C., Moore, C.A., França, G.V.A., Smeeth, L., Rodrigues, L.C., Barreto, M.L. and Teixeira, M.G. 2022. Mortality from Congenital Zika Syndrome — Nationwide Cohort Study in Brazil. *New England Journal of Medicine*. **386**(8), pp.757–767.

Pal, M., Ejeta, I., Girma, A., Dave, K. and Dave, P. 2022. Etiology, Clinical Spectrum, Epidemiology, Diagnosis, Public Health Significance and Control of Leishmaniasis: A Comprehensive Review. *Acta Scientific Microbiology*. (May), pp.110–121.

Palacios, G., Tesh, R., Travassos da Rosa, A., Savji, N., Sze, W., Jain, K., Serge, R., Guzman, H., Guevara, C., Nunes, M.R.T., Nunes-Neto, J.P., Kochel, T., Hutchison, S., Vasconcelos, P.F.C. and Lipkin, W.I. 2011. Characterization of the Candiru Antigenic Complex (Bunyaviridae: Phlebovirus), a Highly Diverse and Reassorting Group of Viruses Affecting Humans in Tropical America. *Journal of Virology*. **85**(8), pp.3811–3820.

Palomino, D.C. arolina T. and Marti, L.C. avalheiro 2015. Chemokines and immunity. *Einstein (São Paulo)*. **13**(3), pp.469–473.

Paludan, S.R. and Bowie, A.G. 2013. Immune Sensing of DNA. *Immunity*. **38**(5), pp.870–880.

Papa, A., Andriotis, V. and Tzilianos, M. 2010. Prevalence of Toscana virus antibodies in residents of two Ionian islands, Greece. *Travel Medicine and Infectious Disease*. **8**(5), pp.302–304.

Papa, A., Kesisidou, C., Kontana, A., Arapidou, Z. and Petropoulou, D. 2015. Phlebovirus infection in Greece: A case report. *Hippokratia*. **19**(2), pp.189–191.

Papa, A., Kontana, A. and Tsergouli, K. 2015. Phlebovirus infections in Greece. *Journal of Medical Virology*. **87**(7), pp.1072–1076.

Papa, A., Mallias, J., Tsergouli, K., Markou, F., Poulou, A. and Milidis, T. 2014. Neuroinvasive phlebovirus infection in greece: A case report. *Intervirology*. **57**(6), pp.393–395.

Papa, A., Paraforou, T., Papakonstantinou, I., Pagdatoglou, K., Kontana, A. and Koukoubani, T. 2014. Severe encephalitis caused by Toscana virus, Greece. *Emerging Infectious Diseases*. **20**(8), pp.1417–1419.

Paul, A.M., Acharya, D., Duty, L., Thompson, E.A., Le, L., Stokic, D.S., Leis, A.A. and Bai, F. 2017. Osteopontin facilitates West Nile virus neuroinvasion via neutrophil ‘trojan horse’ transport. *Scientific Reports*. **7**(1), pp.1–11.

Paul, W.E. 2015. History of interleukin-4. *Cytokine*. **75**(1), pp.3–7.

Pauli, C., Schwarz, T.F., Meyer, C.G. and Jäger, G. 2008. Neurologische Symptome nach Infektion durch Sandfliegenfieber-Virus. *DMW - Deutsche Medizinische Wochenschrift*. **120**(43), pp.1468–1472.

Pepin, M., Bouloy, M., Bird, B.H., Kemp, A. and Paweska, J. 2010. Rift Valley fever virus (Bunyaviridae: Phlebovirus): An update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Veterinary Research*. **41**(6).

Pereira, A., Ayhan, N., Cristóvão, J.M., Vilhena, H., Martins, Â., Cachola, P., Henriques, J., Coimbra, M., Catarino, A., Lestinova, T., Spitzova, T., Volf, P., Campino, L., Charrel, R. and Maia, C. 2019. Antibody Response to Toscana Virus and Sandfly Fever Sicilian Virus

in Cats Naturally Exposed to Phlebotomine Sand Fly Bites in Portugal. *Microorganisms*. **7**(9), p.339.

Pérez-Cutillas, P., Muñoz, C., Martínez-De La Puente, J., Figuerola, J., Navarro, R., Ortuño, M., Bernal, L.J., Ortiz, J., Soriguer, R.C. and Berriatua, E. 2020. A spatial ecology study in a high-diversity host community to understand blood-feeding behaviour in Phlebotomus sandfly vectors of Leishmania. *Medical and Veterinary Entomology*. **34**(2), pp.164–174.

Pérez-Ruiz, M., Collao, X., Navarro-Marí, J.M. and Tenorio, A. 2007. Reversetranscription, real-time PCR assay for detection of Toscana virus. *Journal of Clinical Virology*. **39**(4), pp.276–281.

Perry, M. and Whyte, A. 1998. Immunology of the tonsils. *Immunology today*. **19**(9), pp.414–421.

Perveen, N., Muhammad, K., Muzaffar, S. Bin, Zaheer, T., Munawar, N., Gajic, B., Sparagano, O.A., Kishore, U. and Willingham, A.L. 2023. Host-pathogen interaction in arthropod vectors: Lessons from viral infections. *Frontiers in Immunology*. **14**(January), pp.1–11.

Pestka, S., Krause, C.D. and Walter, M.R. 2004. Interferons, interferon-like cytokines, and their receptors. *Immunological Reviews*. **202**(1), pp.8–32.

Peters, N.C., Egen, J.G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., Lawyer, P., Fay, M.P., Germain, R.N. and Sacks, D. 2008. In Vivo Imaging Reveals an Essential Role for Neutrophils in Leishmaniasis Transmitted by Sand Flies. *Science*. **321**(5891), pp.970–974.

Petersen, L.R., Jamieson, D.J., Powers, A.M. and Honein, M.A. 2016. Zika Virus L. R. Baden, ed. *New England Journal of Medicine*. **374**(16), pp.1552–1563.

Peterson, A.T., Campbell, L.P., Moo-Llanes, D.A., Travi, B., González, C., Ferro, M.C., Ferreira, G.E.M., Brandão-Filho, S.P., Cupolillo, E., Ramsey, J., Leffer, A.M.C., Pech-May, A. and Shaw, J.J. 2017. Influences of climate change on the potential distribution of Lutzomyia longipalpis sensu lato (Psychodidae: Phlebotominae). *International Journal for Parasitology*. **47**(10–11), pp.667–674.

Peyre, M., Chevalier, V., Abdo-Salem, S., Velthuis, A., Antoine-Moussiaux, N., Thiry, E. and Roger, F. 2015. A Systematic Scoping Study of the Socio-Economic Impact of Rift Valley Fever: Research Gaps and Needs. *Zoonoses and Public Health*. **62**(5), pp.309–325.

Peyrefitte, C.N., Devetakov, I., Pastorino, B., Villeneuve, L., Bessaud, M., Stolidi, P., Depaquit, J., Segura, L., Gravier, P., Tock, F., Durand, F., Vagneur, J.-P., Tolou, H.J. and Grandadam, M. 2005. Toscana Virus and Acute Meningitis, France. *Emerging Infectious Diseases*. **11**(5), pp.778–780.

Pichlmair, A., Lassnig, C., Eberle, C.-A., Górna, M.W., Baumann, C.L., Burkard, T.R., Bürckstümmer, T., Stefanovic, A., Krieger, S., Bennett, K.L., Rülicke, T., Weber, F., Colinge, J., Müller, M. and Superti-Furga, G. 2011. IFIT1 is an antiviral protein that recognizes 5′-triphosphate RNA. *Nature Immunology*. **12**(7), pp.624–630.

Pieper, K., Grimbacher, B. and Eibel, H. 2013. B-cell biology and development. *Journal of Allergy and Clinical Immunology*. **131**(4), pp.959–971.

Pierro, A., Landini, M.P., Gaibani, P., Rossini, G., Vocale, C., Finarelli, A.C., Cagarelli, R., Sambri, V. and Varani, S. 2014. A model of laboratory surveillance for neuro-arbovirosis applied during 2012 in the Emilia-Romagna region, Italy. *Clinical Microbiology and Infection*. **20**(7), pp.672–677.

Pingen, M., Bryden, S.R., Pondeville, E., Schnettler, E., Kohl, A., Merits, A., Fazakerley, J.K., Graham, G.J. and McKimmie, C.S. 2016. Host Inflammatory Response to Mosquito Bites Enhances the Severity of Arbovirus Infection. *Immunity*. **44**(6), pp.1455–1469.

Pingen, M., Schmid, M.A., Harris, E. and McKimmie, C.S. 2017. Mosquito Biting Modulates Skin Response to Virus Infection. *Trends in Parasitology*. **33**(8), pp.645–657.

Piper, M.E., Sorenson, D.R. and Gerrard, S.R. 2011. Efficient cellular release of rift valley fever virus requires genomic RNA. *PLoS ONE*. **6**(3).

Platanitis, E. and Decker, T. 2018. Regulatory networks involving STATs, IRFs, and NFκB in inflammation. *Frontiers in Immunology*. **9**(NOV), pp.1–16.

Pletnev, A.G., Maximova, O.A., Liu, G., Kenney, H., Nagata, B.M., Zagorodnyaya, T., Moore, I., Chumakov, K. and Tsetsarkin, K.A. 2021. Epididymal epithelium propels early sexual transmission of Zika virus in the absence of interferon signaling. *Nature Communications*. **12**(1).

Poeppl, W., Obwaller, A.G., Weiler, M., Burgmann, H., Mooseder, G., Lorentz, S., Rauchenwald, F., Aspöck, H., Walochnik, J. and Naucke, T.J. 2013. Emergence of sandflies (Phlebotominae) in Austria, a Central European country. *Parasitology Research*. **112**(12), pp.4231–4237.

Pombi, M., Giacomi, A., Barlozzari, G., Mendoza-Roldan, J., Macrì, G., Otranto, D. and Gabrielli, S. 2020. Molecular detection of Leishmania (Sauroleishmania) tarentolae in human blood and Leishmania (Leishmania) infantum in Sergentomyia minuta: unexpected host-parasite contacts. *Medical and Veterinary Entomology*. **34**(4), pp.470–475.

Ponte-Sucre, A., Gamarro, F., Dujardin, J.C., Barrett, M.P., López-Vélez, R., García-Hernández, R., Pountain, A.W., Mwenechanya, R. and Papadopoulou, B. 2017. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLoS Neglected Tropical Diseases*. **11**(12), pp.1–24.

Popescu, C.P., Cotar, A.I., Dinu, S., Zaharia, M., Tardei, G., Ceausu, E., Badescu, D., Ruta, S., Ceianu, C.S. and Florescu, S.A. 2021. Emergence of toscana virus, romania, 2017-2018. *Emerging Infectious Diseases*. **27**(5), pp.1482–1485.

Portolani, M., Sabbatini, A.M., Beretti, F., Gennari, W., Tamassia, M.G. and Pecorari, M. 2002. Symptomatic infections by toscana virus in the Modena province in the triennium 1999-2001. *The New microbiologica*. **25**(4), pp.485–488.

Punda-Polić, V., Jerončić, A., Mohar, B. and Kraljevićc, K.Š. 2012. Prevalence of Toscana virus antibodies in residents of Croatia. *Clinical Microbiology and Infection*. **18**(6), pp.E200–E203.

Pusztai, R., Gould, E.A. and Smith, H. 1971. Infection patterns in mice of an avirulent and virulent strain of Semliki Forest virus. *British Journal of Experimental Pathology*. **52**(6), pp.669–677.

Quattrone, F., Mazzetti, P., Aquino, F., Sani, S., Carneglia, L., Pistello, M., Lopalco, P.L. and Tavoschi, L. 2020. Two clusters of Toscana virus meningo-encephalitis in Livorno Province and Elba Island, July-September 2018. *Annali di Igiene Medicina Preventiva e di Comunita*. **32**(6), pp.674–681.

Qureshi, S.T., Larivière, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P. and Malo, D. 1999. Endotoxin-tolerant mice have mutations in toll-like receptor 4 (Tlr4). *Journal of Experimental Medicine*. **189**(4), pp.615–625.

Rahman, Md.T., Sobur, M.A., Islam, M.S., Ievy, S., Hossain, M.J., El Zowalaty, M.E., Rahman, A.T. and Ashour, H.M. 2020. Zoonotic Diseases: Etiology, Impact, and Control. *Microorganisms*. **8**(9), p.1405.

Ranaldi, R., Goteri, G., Biagetti, S., Cusi, M.G. and Rossini, S. 2011. Histological description of the lymphadenopathy related to Toscana virus infection. Report of a case. *Pathology Research and Practice*. **207**(3), pp.197–201.

Randall, T.D., Carragher, D.M. and Rangel-Moreno, J. 2008. Development of secondary lymphoid organs. *Annual Review of Immunology*. **26**, pp.627–650.

Rao, D., Meade-White, K., Leventhal, S., Mihalakakos, E., Carmody, A., Feldmann, H. and Hawman, D.W. 2023. CD8+ T-cells target the Crimean-Congo haemorrhagic fever virus Gc protein to control the infection in wild-type mice. *eBioMedicine*. **97**, p.104839.

Ratcliffe, D.A. 1970. Changes Attributable to Pesticides in Egg Breakage Frequency and Eggshell Thickness in Some British Birds. *The Journal of Applied Ecology*. **7**(1), p.67.

Ready, P.D. 2013. Biology of phlebotomine sand flies as vectors of disease agents. *Annual Review of Entomology*. **58**, pp.227–250.

Ready, P.D. and Croset, H. 1980. Diapause and laboratory breeding of Phlebotomus perniciosus Newstead and Phlebotomus ariasi Tonnoir (Diptera: Psychodidae) from southern France. *Bulletin of Entomological Research*. **70**(3), pp.511–523.

Rego, F.D. and Soares, R.P. 2021. Lutzomyia longipalpis: an update on this sand fly vector. *Anais da Academia Brasileira de Ciências*. **93**(3), p.e20200254.

Reikine, S., Nguyen, J.B. and Modis, Y. 2014. Pattern recognition and signaling mechanisms of RIG-I and MDA5. *Frontiers in Immunology*. **5**(JUL), pp.1–7.

Remadi, L., Farjallah, D., Chargui, N., Belgacem, S., Baba, H., Zrieq, R., Alzain, M.A., Babba, H. and Haouas, N. 2023. Blood meal analysis and molecular detection of mammalian Leishmania DNA in wild-caught Sergentomyia spp. from Tunisia and Saudi Arabia. *Parasitology Research*. (0123456789).

Remoli, M.E., Fiorentini, C., Marchi, A., Di Renzi, S., Vonesch, N., Peri, V.M., Bastianini, L., Rossi, S., Bartoccini, G., Kuttappasery, M.L., Ciufolini, M.G. and Tomao, P. 2018. Seroprevalence survey of arboviruses in workers from Tuscany, Italy. *Medicina del Lavoro*. **109**(2), pp.125–131.

Rezza, G., Nicoletti, L., Angelini, R., Romi, R., Finarelli, A., Panning, M., Cordioli, P., Fortuna, C., Boros, S., Magurano, F., Silvi, G., Angelini, P., Dottori, M., Ciufolini, M., Majori, G. and Cassone, A. 2007. Infection with chikungunya virus in Italy: an outbreak in a temperate region. *The Lancet*. **370**(9602), pp.1840–1846.

Ribeiro, C.M.S., Sarrami-Forooshani, R., Setiawan, L.C., Zijlstra-Willems, E.M., Van Hamme, J.L., Tigchelaar, W., Van Der Wel, N.N., Kootstra, N.A., Gringhuis, S.I. and Geijtenbeek, T.B.H. 2016. Receptor usage dictates HIV-1 restriction by human TRIM5α in dendritic cell subsets. *Nature*. **540**(7633), pp.448–452.

Ribeiro, J.M.C. and Francischetti, I.M.B. 2003. Role of Arthropod Saliva in Blood Feeding: Sialome and Post-Sialome Perspectives. *Annual Review of Entomology*. **48**(1), pp.73–88.

Richardson, C.D. and Vance, D.E. 1976. Biochemical evidence that Semliki Forest virus obtains its envelope from the plasma membrane of the host cell. *Journal of Biological Chemistry*. **251**(18), pp.5544–5550.

Rinkevich, Y., Walmsley, G.G., Hu, M.S., Maan, Z.N., Newman, A.M., Drukker, M., Januszyk, M., Krampitz, G.W., Gurtner, G.C., Lorenz, H.P., Weissman, I.L. and Longaker, M.T. 2015. Identification and isolation of a dermal lineage with intrinsic fibrogenic potential. *Science*. **348**(6232).

Rivera-Serrano, E.E., Gizzi, A.S., Arnold, J.J., Grove, T.L., Almo, S.C. and Cameron, C.E. 2020. Viperin Reveals Its True Function. *Annual Review of Virology*. **7**(1), pp.421–446.

Rocklöv, J. and Dubrow, R. 2020. Climate change: an enduring challenge for vector-borne disease prevention and control. *Nature Immunology*. **21**(5), pp.479–483.

Rodriguez-Andres, J., Rani, S., Varjak, M., Chase-Topping, M.E., Beck, M.H., Ferguson, M.C., Schnettler, E., Fragkoudis, R., Barry, G., Merits, A., Fazakerley, J.K., Strand, M.R. and Kohl, A. 2012. Phenoloxidase Activity Acts as a Mosquito Innate Immune Response against Infection with Semliki Forest Virus. *PLoS Pathogens*. **8**(11).

Rohoušová, I., Subrahmanyam, S., Volfová, V., Mu, J., Volf, P., Valenzuela, J.G. and Jochim, R.C. 2012. Salivary Gland Transcriptomes and Proteomes of Phlebotomus tobbi and Phlebotomus sergenti, Vectors of Leishmaniasis C.-C. Chen, ed. *PLoS Neglected Tropical Diseases*. **6**(5), p.e1660.

Rohoušová, I. and Volf, P. 2006. Sand fly saliva: Effects on host immune response and Leishmania transmission. *Folia Parasitologica*. **53**(3), pp.161–171.

Roiz, D., Boussès, P., Simard, F., Paupy, C. and Fontenille, D. 2015. Autochthonous Chikungunya Transmission and Extreme Climate Events in Southern France M. S. Carvalho, ed. *PLOS Neglected Tropical Diseases*. **9**(6), p.e0003854.

Rosadini, C. V. and Kagan, J.C. 2017. Early innate immune responses to bacterial LPS. *Current Opinion in Immunology*. **44**, pp.14–19.

Rosales, C. 2018. Neutrophil: A cell with many roles in inflammation or several cell types? *Frontiers in Physiology*. **9**(FEB), pp.1–17.

Ross, R.W. 1955. The Newala Epidemic and Relationship to The Epidemic. *Virus Research Institute, Entebbe*., pp.177–191.

Ross, R.W. 1956. The newala epidemic: III. The virus: Isolation, pathogenic properties and relationship to the epidemic. *Journal of Hygiene*. **54**(2), pp.177–191.

Rossi, S.L., Tesh, R.B., Azar, S.R., Muruato, A.E., Hanley, K.A., Auguste, A.J., Langsjoen, R.M., Paessler, S., Vasilakis, N. and Weaver, S.C. 2016. Characterization of a novel murine model to study zika virus. *American Journal of Tropical Medicine and Hygiene*. **94**(6), pp.1362–1369.

Rota, E., Morelli, N., Immovilli, P., De Mitri, P. and Guidetti, D. 2017. Guillain-Barré-like axonal polyneuropathy associated with Toscana virus infection. *Medicine*. **96**(38), p.e8081.

Roy, C.J., Reed, D.S. and Hutt, J.A. 2010. Aerobiology and inhalation exposure to biological select agents and toxins. *Veterinary Pathology*. **47**(5), pp.779–789.

Ruddle, N.H. and Akirav, E.M. 2009. Secondary Lymphoid Organs: Responding to Genetic and Environmental Cues in Ontogeny and the Immune Response. *The Journal of Immunology*. **183**(4), pp.2205–2212.

Ruktanonchai, D.J., Stonecipher, S., Lindsey, N., McAllister, J., Pillai, S.K., Horiuchi, K., Delorey, M., Biggerstaff, B.J., Sidwa, T., Zoretic, J., Nasci, R., Fischer, M. and Hills, S.L. 2014. Effect of aerial insecticide spraying on West Nile virus disease-north-central Texas, 2012. *American Journal of Tropical Medicine and Hygiene*. **91**(2), pp.240–245.

Rupp, J.C., Sokoloski, K.J., Gebhart, N.N. and Hardy, R.W. 2015. Alphavirus RNA synthesis and non-structural protein functions. *Journal of General Virology*. **96**(9), pp.2483–2500.

Saadawi, W., Abozaid, F., Almukhtar, M., Annajar, B. and Shaibi, T. 2022. Seroprevalence study of Toscana virus in Yafran area, Libya. *Journal of Vector Borne Diseases*. **59**(2), pp.186–189.

Sabin, A.B. 1944. PHLEBOTOMUS (PAPPATACI OR SANDFLY) FEVER. *Journal of the American Medical Association*. **125**(9), p.603.

Sabin, A.B. 1955. Recent Advances in Our Knowledge of Dengue and Sandfly Fever. *The American Journal of Tropical Medicine and Hygiene*. **4**(2), pp.198–207.

Sabin, A.B., Philip, C.B. and Paul, J.R. 1944. Phlebotomus (Pappataci or Sandfly) fever: a disease of military importance summary of existing knowledge and preliminary report of original investigations. *Journal of the American Medical Association*. **125**(10), pp.693–699.

Sah, R., Siddiq, A., Al-Ahdal, T., Maulud, S.Q., Mohanty, A., Padhi, B.K., El-Shall, N.A., Chandran, D., Emran, T. Bin, Hussein, N.R., Dhama, K. and Satapathy, P. 2023. The emerging scenario for the Eastern equine encephalitis virus and mitigation strategies to counteract this deadly mosquito-borne zoonotic virus, the cause of the most severe arboviral encephalitis in humans—an update. *Frontiers in Tropical Diseases*. **3**(January).

Sakhria, S., Alwassouf, S., Fares, W., Bichaud, L., Dachraoui, K., Alkan, C., Zoghlami, Z., De Lamballerie, X., Zhioua, E. and Charrel, R.N. 2014. Presence of sandfly-borne phleboviruses of two antigenic complexes (Sandfly fever Naples virus and Sandfly fever Sicilian virus) in two different bio-geographical regions of Tunisia demonstrated by a microneutralisation-based seroprevalence study in dogs. *Parasites and Vectors*. **7**(1), pp.1–5.

Sakhria, S., Bichaud, L., Mensi, M., Salez, N., Dachraoui, K., Thirion, L., Cherni, S., Chelbi, I., De Lamballerie, X., Zhioua, E. and Charrel, R.N. 2013. Co-Circulation of Toscana Virus and Punique Virus in Northern Tunisia: A Microneutralisation-Based Seroprevalence Study. *PLoS Neglected Tropical Diseases*. **7**(9), pp.1–6.

Salazar, M.I., del Angel, R.M., Lanz-Mendoza, H., Ludert, J.E. and Pando-Robles, V. 2014. The role of cell proteins in dengue virus infection. *Journal of Proteomics*. **111**, pp.6–15.

Ben Salem, A., Ben Aicha, E., Kalthoum, S., Dhaouadi, A., Hajlaoui, H., Bel Haj Mohamed, B., Ben Slimen, I., Khalfaoui, W., Gharbi, R., Guesmi, K., Ben Ali, M., Fatnassi, N., Seghaier, C., Ben Hassine, T. and Gharbi, M. 2024. Estimation of the economic impact of a bluetongue serotype 4 outbreak in Tunisia. *Frontiers in Veterinary Science*. **11**(February).

Salim Abadi, Y., Telmadarraiy, Z., Vatandoost, H., Chinikar, S., Oshaghi, M.A., Moradi, M., Mirabzadeh Ardakan, E., Hekmat, S. and Nasiri, A. 2010. Hard ticks on domestic ruminants and their seasonal population pynamics in Yazd Province, Iran. *Iranian Journal of Arthropod-Borne Diseases*. **4**(1), pp.66–71.

Samuelson, J., Lerner, E., Tesh, R. and Titus, R. 1991. A mouse model of Leishmania braziliensis braziliensis infection produced by coinjection with sand fly saliva. *The Journal of experimental medicine*. **173**(1), pp.49–54.

Sanbonmatsu-Gámez, S., Pérez-Ruiz, M., Palop-Borrás, B. and Navarro-Marí, J.M. 2009. Unusual Manifestation of Toscana Virus Infection, Spain. *Emerging Infectious Diseases*. **15**(2), pp.347–348.

Sánchez, O.A., Portillo, K.M., Reyes-Garcia, S.Z., England, J.D. and Medina, M.T. 2021. Characterization of adult patients with Guillain–Barré syndrome during the arboviral infection outbreaks in Honduras. *Journal of the Neurological Sciences*. **427**(August 2020), p.117551.

Sánchez-Seco, M.P., Echevarría, J.M., Hernández, L., Estévez, D., Navarro-Marí, J.M. and Tenorio, A. 2003. Detection and identification of Toscana and other phleboviruses by RT-nested-PCR assays with degenerated primers. *Journal of Medical Virology*. **71**(1), pp.140–149.

Sant'Anna, M.R. V., Darby, A.C., Brazil, R.P., Montoya-Lerma, J., Dillon, V.M., Bates, P.A. and Dillon, R.J. 2012. Investigation of the Bacterial Communities Associated with Females of Lutzomyia Sand Fly Species from South America L. A. Moreira, ed. *PLoS ONE*. **7**(8), p.e42531.

Santos, D.O., Coutinho, C.E.R., Madeira, M.F., Bottino, C.G., Vieira, R.T., Nascimento, S.B., Bernardino, A., Bourguignon, S.C., Corte-Real, S., Pinho, R.T., Rodrigues, C.R. and Castro, H.C. 2008. Leishmaniasis treatment - A challenge that remains: A review. *Parasitology Research*. **103**(1), pp.1–10.

Santos, L., Simões, J., Costa, R., Martins, S. and Lecour, H. 2007. Toscana virus meningitis in Portugal, 2002-2005. *Eurosurveillance*. **12**(6), pp.3–4.

Santos Souza, H.F., da Silva Almeida, B. and Boscardin, S.B. 2016. Early dengue virus interactions: the role of dendritic cells during infection. *Virus Research*. **223**, pp.88–98.

Sarkar, S.N. and Sen, G.C. 2004. Novel functions of proteins encoded by viral stress-inducible genes. *Pharmacology & Therapeutics*. **103**(3), pp.245–259.

Sasaya, T., Palacios, G., Briese, T., Serio, F. Di, Groschup, M.H., Neriya, Y., Song, J.W. and Tomitaka, Y. 2023. ICTV Virus Taxonomy Profile: Phenuiviridae 2023. *Journal of General Virology*. **104**(9), pp.1–2.

Savellini, G.G., Anichini, G., Gandolfo, C., Prathyumnan, S. and Cusi, M.G. 2019. Toscana virus non-structural protein NSs acts as E3 ubiquitin ligase promoting RIG-I degradation. *PLoS Pathogens*. **15**(12), pp.1–19.

Savellini, G.G., Weber, F., Terrosi, C., Habjan, M., Martorelli, B. and Cusi, M.G. 2011. Toscana virus induces interferon although its NSs protein reveals antagonistic activity. *Journal of General Virology*. **92**(1), pp.71–79.

Schilte, C., Couderc, T., Chretien, F., Sourisseau, M., Gangneux, N., Guivel-Benhassine, F., Kraxner, A., Tschopp, J., Higgs, S., Michault, A., Arenzana-Seisdedos, F., Colonna, M., Peduto, L., Schwartz, O., Lecuit, M. and Albert, M.L. 2010. Type I IFN controls chikungunya virus via its action on nonhematopoietic cells. *Journal of Experimental Medicine*. **207**(2), pp.429–442.

Schleimer, R.P., Kato, A., Kern, R., Kuperman, D. and Avila, P.C. 2007. Epithelium: At the interface of innate and adaptive immune responses. *Journal of Allergy and Clinical Immunology*. **120**(6), pp.1279–1284.

Schmid, M.A., Glasner, D.R., Shah, S., Michlmayr, D., Kramer, L.D. and Harris, E. 2016. Mosquito Saliva Increases Endothelial Permeability in the Skin, Immune Cell Migration, and Dengue Pathogenesis during Antibody-Dependent Enhancement M. T. Heise, ed. *PLOS Pathogens*. **12**(6), p.e1005676.

Schneider, B.S. and Higgs, S. 2008. The enhancement of arbovirus transmission and disease by mosquito saliva is associated with modulation of the host immune response. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **102**(5), pp.400–408.

Schneider, C.A., Leung, J.M., Valenzuela-Leon, P.C., Golviznina, N.A., Toso, E.A., Bosnakovski, D., Kyba, M., Calvo, E. and Peterson, K.E. 2024. Skin muscle is the initial site of viral replication for arboviral bunyavirus infection. *Nature Communications*. **15**(1), p.1121.

Schoggins, J.W. 2014. Interferon-stimulated genes: roles in viral pathogenesis. *Current Opinion in Virology*. **6**(January), pp.40–46.

Schoggins, J.W. 2019. Interferon-Stimulated Genes: What Do They All Do? *Annual Review of Virology*. **6**(1), pp.567–584.

Schoggins, J.W., Wilson, S.J., Panis, M., Murphy, M.Y., Jones, C.T., Bieniasz, P. and Rice, C.M. 2011. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature*. **472**(7344), pp.481–485.

Scholte, E.-J., Knols, B.G.J., Samson, R.A. and Takken, W. 2004. Entomopathogenic fungi for mosquito control: a review. *Journal of insect science*. **4**(1), p.19.

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M.C., Lavenir, R., Pardigon, N., Reynes, J.M., Pettinelli, F., Biscornet, L., Diancourt, L., Michel, S., Duquerroy, S., Guigon, G., Frenkiel, M.P., Bréhin, A.C., Cubito, N., Desprès, P., Kunst, F., Rey, F.A., Zeller, H. and Brisse, S. 2006. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Medicine*. **3**(7), pp.1058–1070.

Schultz, K.T. and Grieder, F. 1987. Structure and Function of the Immune System. *Toxicologic Pathology*. **15**(3), pp.262–264.

Schwartz, C., Eberle, J.U. and Voehringer, D. 2016. Basophils in inflammation. *European Journal of Pharmacology*. **778**, pp.90–95.

Schwarz, T., Gilch, S. and Jäger, G. 1993. Travel-related Toscana virus infection. *The Lancet*. **342**(8874), pp.803–804.

Schwarz, Tino F., Gilch, S. and Jäger, G. 1995. Aseptic meningitis caused by sandfly fever virus, serotype toscana. *Clinical Infectious Diseases*. **21**(3), pp.669–671.

Schwarz, T. F., Jäger, G., Gilch, S. and Nitschko, H. 1995. Nested RT-PCR for detection of sandfly fever virus, serotype Toscana, in clinical specimens, with confirmation by nucleotide sequence analysis. *Research in Virology*. **146**(5), pp.355–362.

Schwarz, T. F., Jäger, G., Gilch, S. and Pauli, C. 1995. Serosurvey and laboratory diagnosis of imported sandfly fever virus, serotype Toscana, infection in Germany. *Epidemiology and Infection*. **114**(3), pp.501–510.

Schwarz, T.F., Jäger, G., Gilch, S., Pauli, C., Eisenhut, M., Nitschko, H. and Hegenscheid, B. 1996. Travel-related vector-borne virus infections in Germany *In*: *Imported Virus Infections* [Online]. Vienna: Springer Vienna, pp.57–65. Available from: http://link.springer.com/10.1007/978-3-7091-7482-1_7.

Šedý, J., Bekiaris, V. and Ware, C.F. 2015. Tumor Necrosis Factor Superfamily in Innate Immunity and Inflammation. *Cold Spring Harbor Perspectives in Biology*. **7**(4), p.a016279.

Segura, E. and Amigorena, S. 2013. Inflammatory dendritic cells in mice and humans. *Trends in Immunology*. **34**(9), pp.440–445.

Seifert, L., Wiechmann, I., Harbeck, M., Thomas, A., Grupe, G., Projahn, M., Scholz, H.C. and Riehm, J.M. 2016. Genotyping Yersinia pestis in Historical Plague: Evidence for Long-Term Persistence of Y. pestis in Europe from the 14th to the 17th Century X. Yu, ed. *PLOS ONE*. **11**(1), p.e0145194.

Sellali, S., Lafri, I., Hachid, A., Ayhan, N., Benbetka, C., Medrouh, B., Messahel, N.E., El Amine Bekara, M., Lafri, M., Charrel, R.N. and Bitam, I. 2022. Presence of the sandfly-borne phlebovirus (Toscana virus) in different bio-geographical regions of Algeria demonstrated by a microneutralisation-based seroprevalence study in owned dogs. *Comparative Immunology, Microbiology and Infectious Diseases*. **88**(February), p.101861.

Serata, D., Rapinesi, C., Del Casale, A., Simonetti, A., Mazzarini, L., Ambrosi, E., Kotzalidis, G.D., Fensore, C., Girardi, P. and Tatarelli, R. 2011. Personality changes after Toscana virus (TOSV) encephalitis in a 49-year-old man: A case report. *International Journal of Neuroscience*. **121**(3), pp.165–169.

Sergon, K., Njuguna, C., Kalani, R., Ofula, V., Onyango, C., Konongoi, L.S., Bedno, S., Burke, H., Dumilla, A.M., Konde, J., Kariuki Njenga, M., Sang, R. and Breiman, R.F. 2008. Seroprevalence of Chikungunya Virus (CHIKV) Infection on Lamu Island. *Am. J. Trop. Med. Hyg*. **78(2)**(October 2004), pp.333–337.

Sharma, P., Sharma, S., Maurya, R.K., De, T. Das, Thomas, T., Lata, S., Singh, N., Pandey, K.C., Valecha, N. and Dixit, R. 2014. Salivary glands harbor more diverse microbial communities than gut in Anopheles culicifacies. *Parasites and Vectors*. **7**(1), pp.1–7.

Sharp, P.M. and Hahn, B.H. 2011. Origins of HIV and the AIDS pandemic. *Cold Spring Harbor Perspectives in Medicine*. **1**(1), pp.1–22.

Shaw, W.R. and Catteruccia, F. 2018. Vector biology meets disease control: using basic research to fight vector-borne diseases. *Nature Microbiology*. **4**(1), pp.20–34.

Shearer, W.T., Rosenwasser, L.J., Bochner, B.S., Martinez-Moczygemba, M. and Huston, D.P. 2003. Biology of common β receptor–signaling cytokines: IL-3, IL-5, and GM-CSF. *Journal of Allergy and Clinical Immunology*. **112**(4), pp.653–665.

Shi, C., Jia, T., Mendez-Ferrer, S., Hohl, T.M., Serbina, N. V., Lipuma, L., Leiner, I., Li, M.O., Frenette, P.S. and Pamer, E.G. 2011. Bone Marrow Mesenchymal Stem and Progenitor Cells Induce Monocyte Emigration in Response to Circulating Toll-like Receptor Ligands. *Immunity*. **34**(4), pp.590–601.

Shi, Y.J., Li, J.Q., Zhang, H.Q., Deng, C.L., Zhu, Q.X., Zhang, B. and Li, X.D. 2023. A high throughput antiviral screening platform for alphaviruses based on Semliki Forest virus expressing eGFP reporter gene. *Virologica Sinica*. **38**(4), pp.585–594.

Shipman, W.D., Dasoveanu, D.C. and Lu, T.T. 2017. Tertiary lymphoid organs in systemic autoimmune diseases: pathogenic or protective? *F1000Research*. **6**(0), p.196.

Shiraly, R., Khosravi, A. and Farahangiz, S. 2017. Seroprevalence of sandfly fever virus infection in military personnel on the western border of Iran. *Journal of Infection and Public Health*. **10**(1), pp.59–63.

Shrestha, B., Wang, T., Samuel, M.A., Whitby, K., Craft, J., Fikrig, E. and Diamond, M.S. 2006. Gamma Interferon Plays a Crucial Early Antiviral Role in Protection against West Nile Virus Infection. *Journal of Virology*. **80**(11), pp.5338–5348.

Shu, J., Ma, X., Zhang, Y., Zou, J., Yuan, Z. and Yi, Z. 2021. NS5-independent Ablation of STAT2 by Zika virus to antagonize interferon signalling. *Emerging Microbes and Infections*. **10**(1), pp.1609–1625.

Sichien, D., Lambrecht, B.N., Guilliams, M. and Scott, C.L. 2017. Development of conventional dendritic cells: From common bone marrow progenitors to multiple subsets in peripheral tissues. *Mucosal Immunology*. **10**(4), pp.831–844.

Silverstein, A.M. and Bialasiewicz, A.A. 1980. A history of theories of acquired immunity. *Cellular Immunology*. **51**(1), pp.151–167.

Simpson, D.I.H., Knight, E.M., Courtois, G.H., Williams, M.C., Weinbren, M.P. and Kibukamusoke, J.W. 1967. Congo virus: a hitherto undescribed virus occurring in Africa. Part 1. Human isolations-clinical notes. *East African medical journal*. **44**(2), pp.87–92.

Singh, S.P., Reddy, D.C.S., Rai, M. and Sundar, S. 2006. Serious underreporting of visceral leishmaniasis through passive case reporting in Bihar, India. *Tropical Medicine and International Health*. **11**(6), pp.899–905.

Skaug, B. and Chen, Z.J. 2010. Emerging Role of ISG15 in Antiviral Immunity. *Cell*. **143**(2), pp.187–190.

Smith, K.A. 2012. Louis Pasteur, the father of immunology? *Frontiers in Immunology*. **3**(APR), pp.1–10.

Smithburn, K.C. and Haddow, A.J. 1944. Semliki Forest virus: I. Isolation and pathogenic properties. *The Journal of Immunology*. **49**(3), pp.141–157.

Smoots, A.N., Olson, S.M., Cragan, J., Delaney, A., Roth, N.M., Godfred-Cato, S., Jones, A.M., Nahabedian, J.F., Fornoff, J., Sandidge, T., Yazdy, M.M., Higgins, C., Olney, R.S., Eckert, V., Forkner, A., Fox, D.J., Stolz, A., Crawford, K., Cho, S.J., Knapp, M., Ahmed, M.F., Lake-Burger, H., Elmore, A.L., Langlois, P., Breidenbach, R., Nance, A., Denson, L., Caton, L., Forestieri, N., Bergman, K., Humphries, B.K., Leedom, V.O., Tran, T., Johnston, J., Valencia-Prado, M., Pérez-González, S., Romitti, P.A., Fall, C., Bryan, J.M., Barton, J., Arias, W., St. John, K., Mann, S., Kimura, J., Orantes, L., Martin, B., de Wilde, L., Ellis, E.M., Song, Z., Akosa, A., Goodroe, C., Ellington, S.R., Tong, V.T., Gilboa, S.M., Moore, C.A. and Honein, M.A. 2020. Population-Based Surveillance for Birth Defects Potentially Related to Zika Virus Infection — 22 States and Territories, January 2016–June 2017. *MMWR. Morbidity and Mortality Weekly Report*. **69**(3), pp.67–71.

Soares, M.B.P., Titus, R.G., Shoemaker, C.B., David, J.R. and Bozza, M. 1998. The Vasoactive Peptide Maxadilan from Sand Fly Saliva Inhibits TNF-α and Induces IL-6 by Mouse Macrophages Through Interaction with the Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Receptor. *The Journal of Immunology*. **160**(4), pp.1811–1816.

Soares, V.Y.R., da Silva, J.C., da Silva, K.R., Pires e Cruz, M. do S., Santos, M.P.D., Ribolla, P.E.M., Alonso, D.P., Coelho, L.F.L., Costa, D.L. and Costa, C.H.N. 2014. Identification of blood meal sources of Lutzomyia longipalpis using polymerase chain reaction-restriction fragment length polymorphism analysis of the cytochrome B gene. *Memorias do Instituto Oswaldo Cruz*. **109**(3), pp.379–383.

Socha, W., Kwasnik, M., Larska, M., Rola, J. and Rozek, W. 2022. Vector-Borne Viral Diseases as a Current Threat for Human and Animal Health—One Health Perspective. *Journal of Clinical Medicine*. **11**(11).

Sonderegger, B., Hachler, H., Dobler, G. and Frei, M. 2009. Imported aseptic meningitis due to Toscana virus acquired on the island of Elba, Italy, August 2008. *Euro surveillance : bulletin européen sur les maladies transmissibles = European communicable disease bulletin*. **14**(1), pp.9–10.

Song, Y. and Buchwald, P. 2015. TNF Superfamily Protein-Protein Interactions: Feasibility of Small- Molecule Modulation. *Current Drug Targets*. **16**(4), pp.393–408.

Souissi, C., Marzouki, S., Elbini-Dhouib, I., Jebali, J., Oliveira, F., Valenzuela, J.G., Srairi-Abid, N., Kamhawi, S. and Ben Ahmed, M. 2023. PpSP32, the Phlebotomus papatasi immunodominant salivary protein, exerts immunomodulatory effects on human monocytes, macrophages, and lymphocytes. *Parasites & Vectors*. **16**(1), p.1.

Soumahoro, M.K., Boelle, P.Y., Gaüzere, B.A., Atsou, K., Pelat, C., Lambert, B., La Ruche, G., Gastellu-Etchegorry, M., Renault, P., Sarazin, M., Yazdanpanah, Y., Flahault, A., Malvy, D. and Hanslik, T. 2011. The Chikungunya epidemic on La Réunion Island in 2005-2006: A cost-of-illness study. *PLoS Neglected Tropical Diseases*. **5**(6).

Spence, J.S., He, R., Hoffmann, H.H., Das, T., Thinon, E., Rice, C.M., Peng, T., Chandran, K. and Hang, H.C. 2019. IFITM3 directly engages and shuttles incoming virus particles to lysosomes. *Nature Chemical Biology*. **15**(3), pp.259–268.

Spernovasilis, N., Tsiodras, S. and Poulakou, G. 2022. Emerging and Re-Emerging Infectious Diseases: Humankind's Companions and Competitors. *Microorganisms*. **10**(1), p.98.

Spiegel, M., Plegge, T. and Pöhlmann, S. 2016. The Role of Phlebovirus Glycoproteins in Viral Entry, Assembly and Release. *Viruses*. **8**(7), p.202.

Spielman, A. and Turell, M.J. 1992. Nonvascular Delivery of Rift Valley Fever Virus by Infected Mosquitoes. *The American Journal of Tropical Medicine and Hygiene*. **47**(2), pp.190–194.

Spolski, R., Gromer, D. and Leonard, W.J. 2017. The γc family of cytokines: fine-tuning signals from IL-2 and IL-21 in the regulation of the immune response. *F1000Research*. **6**, p.1872.

Srivastava, K.S., Jeswani, V., Pal, N., Bohra, B., Vishwakarma, V., Bapat, A.A., Patnaik, Y.P., Khanna, N. and Shukla, R. 2023. Japanese Encephalitis Virus: An Update on the Potential Antivirals and Vaccines. *Vaccines*. **11**(4), pp.1–25.

Stavnezer, J., Guikema, J.E.J. and Schrader, C.E. 2008. Mechanism and regulation of class switch recombination. *Annual Review of Immunology*. **26**, pp.261–292.

Steele, E.J. 2009. Mechanism of somatic hypermutation: Critical analysis of strand biased mutation signatures at A:T and G:C base pairs. *Molecular Immunology*. **46**(3), pp.305–320.

Strauss, J.H. and Strauss, E.G. 1994. The alphaviruses: gene expression, replication, and evolution. *Microbiological Reviews*. **58**(3), pp.491–562.

Styer, L.M., Kent, K.A., Albright, R.G., Bennett, C.J., Kramer, L.D. and Bernard, K.A. 2007. Mosquitoes Inoculate High Doses of West Nile Virus as They Probe and Feed on Live Hosts M. J. Buchmeier, ed. *PLoS Pathogens*. **3**(9), p.e132.

Styer, L.M., Lim, P.-Y., Louie, K.L., Albright, R.G., Kramer, L.D. and Bernard, K.A. 2011. Mosquito Saliva Causes Enhancement of West Nile Virus Infection in Mice. *Journal of Virology*. **85**(4), pp.1517–1527

Surasombatpattana, P., Patramool, S., Luplertlop, N., Yssel, H. and Missé, D. 2012. Aedes aegypti Saliva Enhances Dengue Virus Infection of Human Keratinocytes by Suppressing Innate Immune Responses. *Journal of Investigative Dermatology*. **132**(8), pp.2103–2105.

Swamy, M., Abeler-Dörner, L., Chettle, J., Mahlakõiv, T., Goubau, D., Chakravarty, P., Ramsay, G., Reis E Sousa, C., Staeheli, P., Blacklaws, B.A., Heeney, J.L. and Hayday, A.C. 2015. Intestinal intraepithelial lymphocyte activation promotes innate antiviral resistance. *Nature Communications*. **6**(May).

Swiecki, M. and Colonna, M. 2015. The multifaceted biology of plasmacytoid dendritic cells. *Nature Reviews Immunology*. **15**(8), pp.471–485.

Swirski, F.K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi, P., Figueiredo, J.-L., Kohler, R.H., Chudnovskiy, A., Waterman, P., Aikawa, E., Mempel, T.R., Libby, P., Weissleder, R. and Pittet, M.J. 2009. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. *Science*. **325**(5940), pp.612–616.

Tahir, D., Alwassouf, S., Loudahi, A., Davoust, B. and Charrel, R.N. 2016. Seroprevalence of Toscana virus in dogs from Kabylia (Algeria). *Clinical Microbiology and Infection*. **22**(3), pp.e16–e17.

Takeuchi, O. and Akira, S. 2010. Pattern Recognition Receptors and Inflammation. *Cell*. **140**(6), pp.805–820.

Tamoutounour, S., Guilliams, M., Montanana Sanchis, F., Liu, H., Terhorst, D., Malosse, C., Pollet, E., Ardouin, L., Luche, H., Sanchez, C., Dalod, M., Malissen, B. and Henri, S. 2013. Origins and Functional Specialization of Macrophages and of Conventional and Monocyte-Derived Dendritic Cells in Mouse Skin. *Immunity*. **39**(5), pp.925–938.

Tangena, J.A.A., Marcombe, S., Thammavong, P., Chonephetsarath, S., Somphong, B., Sayteng, K., Grandadam, M., Sutherland, I.W., Lindsay, S.W. and Brey, P.T. 2018. Bionomics and insecticide resistance of the arboviral vector Aedes albopictus in northern Lao PDR. *PLoS ONE*. **13**(10), pp.1–21.

Tauber, A.I. 1992. The birth of immunology. *Cellular Immunology*. **139**(2), pp.505–530.

Teixeira, C.R., Santos, C. da S., Prates, D.B., dos Santos, R.T., Araújo-Santos, T., de Souza-Neto, S.M., Borges, V.M., Barral-Netto, M. and Brodskyn, C.I. 2018. Lutzomyia longipalpis saliva drives interleukin-17-induced neutrophil recruitment favoring Leishmania infantum infection. *Frontiers in Microbiology*. **9**(MAY), pp.1–10.

Teixeira, C.R., Teixeira, M.J., Gomes, R.B.B., Santos, C.S., Andrade, B.B., Raffaele-Netto, I., Silva, J.S., Guglielmotti, A., Miranda, J.C., Barral, A., Brodskyn, C. and Barral-Netto, M. 2005. Saliva from Lutzomyia longipalpis Induces CC Chemokine Ligand 2/Monocyte Chemoattractant Protein-1 Expression and Macrophage Recruitment. *The Journal of Immunology*. **175**(12), pp.8346–8353.

Telleria, E.L., Martins-da-Silva, A., Tempone, A.J. and Traub-Csekö, Y.M. 2018. Leishmania , microbiota and sand fly immunity. *Parasitology*. **145**(10), pp.1336–1353.

Teng, T., Chin, K., Ng, L.F.P., Teng, T., Foo, S., Simamarta, D., Lum, F., Teo, T., Merits, A., Chin, K. and Ng, L.F.P. 2012. Viperin restricts chikungunya virus replication and pathology Find the latest version : Viperin restricts chikungunya virus replication and pathology. *Journal of Clinical Investigation*. **122**(12), pp.4447–4460.

Teo, T.-H., Lum, F.-M., Claser, C., Lulla, V., Lulla, A., Merits, A., Rénia, L. and Ng, L.F.P. 2013. A Pathogenic Role for CD4+ T Cells during Chikungunya Virus Infection in Mice. *The Journal of Immunology*. **190**(1), pp.259–269.

Terrosi, C., Olivieri, R., Bianco, C., Cellesi, C. and Cusi, M.G. 2009. Age-dependent seroprevalence of toscana virus in central Italy and correlation with the clinical profile. *Clinical and Vaccine Immunology*. **16**(8), pp.1251–1252.

Terry, R.L., Deffrasnes, C., Getts, D.R., Minten, C., van Vreden, C., Ashhurst, T.M., Getts, M.T., Xie, R.D.V., Campbell, I.L. and King, N.J.C. 2015. Defective Inflammatory Monocyte Development in IRF8-Deficient Mice Abrogates Migration to the West Nile Virus-Infected Brain. *Journal of Innate Immunity*. **7**(1), pp.102–112.

Tesh, R. B., Chaniotis, B. N., Peralta, P. H., & Johnson, K.M. 1974. Ecology of Viruses Isolated From Panamanian. *The American journal of tropical medicine and hygiene*. **23**(2), pp.258–269.

Tesh, R.B., Lubroth, J. and Guzman, H. 1992. Simulation of arbovirus overwintering: survival of Toscana virus (Bunyaviridae: Phlebovirus) in its natural sand fly vector Phlebotomus perniciosus. *The American journal of tropical medicine and hygiene*. **47**(5), pp.574–581.

Tesh, R.B. and Modi, G.B. 1983. Development of a Continuous Cell Line from the Sand Fly Lutzomyia Longipalpis (Diptera: Psychodidae), and its Susceptibility to Infection with Arboviruses1. *Journal of Medical Entomology*. **20**(2), pp.199–202.

Tesh, R.B. and Modi, G.B. 1987. Maintenance of Toscana virus in phlebotomus perniciosus by vertical transmission. *American Journal of Tropical Medicine and Hygiene*. **36**(1), pp.189–193.

TEZCAN, S., DİNÇER, E., ÜLGER, M., ÖZGÜR, D., ERDOĞAN, S., ÖZKUL, A., EMEKDAŞ, G. and ERGÜNAY, K. 2015. Serological Investigation of Phlebovirus Exposure in Blood Donors from the Mediterranean Province of Mersin, Turkey. *Mikrobiyoloji Bulteni*. **49**(3), pp.403–413.

Theodos, C.M., Ribeiro, J.M.C. and Titus, R.G. 1991. Analysis of enhancing effect of sand fly saliva on Leishmania infection in mice. *Infection and Immunity*. **59**(5), pp.1592–1598.

Thirion, L., Pezzi, L., Pedrosa-Corral, I., Sanbonmatsu-Gamez, S., de Lamballerie, X., Falchi, A., Perez-Ruiz, M. and Charrel, R.N. 2021. Evaluation of a trio toscana virus real-time rt-pcr assay targeting three genomic regions within nucleoprotein gene. *Pathogens*. **10**(3), pp.1–11.

Ticha, L., Volfova, V., Mendoza-Roldan, J.A., Bezerra-Santos, M.A., Maia, C., Sadlova, J., Otranto, D. and Volf, P. 2023. Experimental feeding of Sergentomyia minuta on reptiles and mammals: comparison with Phlebotomus papatasi. *Parasites and Vectors*. **16**(1), pp.1–9.

Titus, R.G. and Ribeiro, J.M.C. 1988. Salivary Gland Lysates from the Sand Fly Lutzomyia longipalpis Enhance Leishmania Infectivity. *Science*. **239**(4845), pp.1306–1308.

Topluoglu, S., Taylan-Ozkan, A. and Alp, E. 2023. Impact of wars and natural disasters on emerging and re-emerging infectious diseases. *Frontiers in Public Health*. **11**(September).

Tschumi, F., Schmutz, S., Kufner, V., Heider, M., Pigny, F., Schreiner, B., Capaul, R., Achermann, Y. and Huber, M. 2019. Meningitis and epididymitis caused by Toscana virus infection imported to Switzerland diagnosed by metagenomic sequencing: A case report. *BMC Infectious Diseases*. **19**(1), pp.2–5.

Tsetsarkin, K.A., Vanlandingham, D.L., McGee, C.E. and Higgs, S. 2007. A single mutation in Chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathogens*. **3**(12), pp.1895–1906.

Tu, Y. 2011. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. *Nature Medicine*. **17**(10), pp.1217–1220.

Tunalı, V. and Özbilgin, A. 2023. Knock, knock, knocking on Europe's door: Threat of leishmaniasis in Europe with a focus on Turkey. *Current Research in Parasitology and Vector-Borne Diseases*. **4**(June).

Turell, M.J., Tammariello, R.F. and Spielman, A. 1995. Nonvascular delivery of St. Louis encephalitis and Venezuelan equine encephalitis viruses by infected mosquitoes (Diptera:

Culicidae) feeding on a vertebrate host. *Journal of medical entomology*. **32**(4), pp.563–568.

UNAIDS 2022. Global HIV & AIDS statistics — 2022 fact sheet. Available from: https://www.unaids.org/en/resources/fact-sheet.

Valassina, M., Cuppone, A.M., Bianchi, S., Santini, L. and Cusi, M.G. 1998. Evidence of toscana virus variants circulating in Tuscany, Italy, during the summers of 1995 to 1997. *Journal of Clinical Microbiology*. **36**(7), pp.2103–2104.

Valassina, M., Cusi, M.G. and Valensin, P.E. 1996. Rapid identification of Toscana virus by nested PCR during an outbreak in the Siena area of Italy. *Journal of Clinical Microbiology*. **34**(10), pp.2500–2502.

Valassina, M., Meacci, F., Valensin, P.E. and Cusi, M.G. 2000. Detection of neurotropic viruses circulating in Tuscany: The incisive role of Toscana virus. *Journal of Medical Virology*. **60**(1), pp.86–90.

Valassina, M., Valentini, M., Pugliese, A., Valensin, P.E. and Cusi, M.G. 2003. Serological Survey of Toscana Virus Infections in a High-Risk Population in Italy. *Clinical and Vaccine Immunology*. **10**(3), pp.483–484.

Valassina, M., Valentini, M., Valensin, P.E. and Cusi, M.G. 2002. Fast duplex one-step RT-PCR for rapid differential diagnosis of entero- or toscana virus meningitis. *Diagnostic Microbiology and Infectious Disease*. **43**(3), pp.201–205.

Valenzuela, J.G., Belkaid, Y., Garfield, M.K., Mendez, S., Kamhawi, S., Rowton, E.D., Sacks, D.L. and Ribeiro, J.M.C. 2001. Toward a defined anti-Leishmania vaccine targeting vector antigens: Characterization of a protective salivary protein. *Journal of Experimental Medicine*. **194**(3), pp.331–342.

Varani, S., Gelsomino, F., Bartoletti, M., Viale, P., Mastroianni, A., Briganti, E., Ortolani, P., Albertini, F., Calzetti, C., Prati, F., Cenni, P., Castellani, G., Morini, S., Rossini, G., Landini, M.P. and Sambri, V. 2015. Meningitis caused by toscana virus is associated with strong antiviral response in the CNS and altered frequency of blood antigen-presenting cells. *Viruses*. **7**(11), pp.5831–5843.

Vega-Rúa, A., Marconcini, M., Madec, Y., Manni, M., Carraretto, D., Gomulski, L.M., Gasperi, G., Failloux, A.-B. and Malacrida, A.R. 2020. Vector competence of Aedes albopictus populations for chikungunya virus is shaped by their demographic history. *Communications Biology*. **3**(1), p.326.

Venturi, G., El-Sawaf, G., Arpino, C., Madeddu, G., Fiorentini, C., Benedetti, E., Marchi, A., Helaly, G., El Ghazouly, K., Ghazal, A., Farchi, F., Soddu, A., Cacciatore, D., El Zalabani, M., Mura, M.S., Rezza, G. and Ciufolini, M.G. 2009. Arboviral infections in Egyptian and Sardinian children and adults with aseptic meningitis and meningo-encephalitis. *Scandinavian Journal of Infectious Diseases*. **41**(11–12), pp.898–899.

Venturi, G., Di Luca, M., Fortuna, C., Remoli, M.E., Riccardo, F., Severini, F., Toma, L., Del Manso, M., Benedetti, E., Caporali, M.G., Amendola, A., Fiorentini, C., De Liberato, C., Giammattei, R., Romi, R., Pezzotti, P., Rezza, G. and Rizzo, C. 2017. Detection of a chikungunya outbreak in central Italy, August to September 2017. *Eurosurveillance*. **22**(39), pp.11–14.

Venturi, G., Madeddu, G., Rezza, G., Ciccozzi, M., Pettinato, M.L., Cilliano, M., Fiorentini, C., Mura, M.S. and Ciufolini, M.G. 2007. Detection of Toscana virus central nervous system infections in Sardinia Island, Italy. *Journal of Clinical Virology*. **40**(1), pp.90–91.

Venturi, G., Marchi, A., Fiorentini, C., Ramadani, N., Quaglio, G., Kalaveshi, A., Bertinato, L., Putoto, G., Benedetti, E., Rezza, G. and Ciufolini, M.G. 2011. Prevalence of antibodies to phleboviruses and flaviviruses in Peja, Kosovo. *Clinical Microbiology and Infection*. **17**(8), pp.1180–1182.

Verani, P., Ciufolini, M.G., Caciolli, S., Renzi, A., Nicoletti, L., Sabatinelli, G., Bartolozzi, D., Volpi, G., Amaducci, L., Coluzzi, M., Paci, P. and Balducci, M. 1988. Ecology of viruses isolated from sand flies in Italy and characterization of a new Phlebovirus (Arbia virus). *American Journal of Tropical Medicine and Hygiene*. **38**(2), pp.433–439.

Verani, P., Ciufolini, M.G., Nicoletti, L., Balducci, M., Sabatinelli, G., Coluzzi, M., Paci, P. and Amaducci, L. 1982. Ecological and epidemiological studies of Toscana virus, an arbovirus isolated from Phlebotomus. *Annali dell'Istituto Superiore di Sanita*. **18**(3), p.119443.

Verani, P., Lopes, M.C., Nicoletti, L. and Balducci, M. 1980. Studies on Phlebotomus-transmitted viruses in Italy. I. Isolation and characterization of a sandfly fever Naples-like virus. *Arboviruses in the Mediterranean countries. Stuttgart: Gustav Fischer Verlag*., pp.195–201.

Vilibic-Cavlek, T., Zidovec-Lepej, S., Ledina, D., Knezevic, S., Savic, V., Tabain, I., Ivic, I., Slavuljica, I., Bogdanic, M., Grgic, I., Gorenec, L., Stevanovic, V. and Barbic, L. 2020. Clinical, virological, and immunological findings in patients with toscana neuroinvasive disease in Croatia: Report of three cases. *Tropical Medicine and Infectious Disease*. **5**(3).

Villani, A.-C., Satija, R., Reynolds, G., Sarkizova, S., Shekhar, K., Fletcher, J., Griesbeck, M., Butler, A., Zheng, S., Lazo, S., Jardine, L., Dixon, D., Stephenson, E., Nilsson, E., Grundberg, I., McDonald, D., Filby, A., Li, W., De Jager, P.L., Rozenblatt-Rosen, O., Lane, A.A., Haniffa, M., Regev, A. and Hacohen, N. 2017. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science*. **356**(6335), pp.139–148.

Vivier, E., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P., Eberl, G., Koyasu, S., Locksley, R.M., McKenzie, A.N.J., Mebius, R.E., Powrie, F. and Spits, H. 2018. Innate Lymphoid Cells: 10 Years On. *Cell*. **174**(5), pp.1054–1066.

Vlkova, M., Rohousova, I., Drahota, J., Stanneck, D., Kruedewagen, E.M., Mencke, N., Otranto, D. and Volf, P. 2011. Canine antibody response to Phlebotomus perniciosus bites negatively correlates with the risk of Leishmania infantum transmission. *PLoS Neglected Tropical Diseases*. **5**(10), pp.1–9.

Vocale, C., Bartoletti, M., Rossini, G., Macini, P., Pascucci, M.G., Mori, F., Tampieri, A., Lenzi, T., Pavoni, M. and Giorgi, C. 2012. Toscana virus infections in northern Italy: laboratory and clinical evaluation. *Vector-Borne and Zoonotic Diseases*. **12**(6), pp.526–529.

Volf, P., Hajmova, M., Sadlova, J. and Votypka, J. 2004. Blocked stomodeal valve of the insect vector: similar mechanism of transmission in two trypanosomatid models. *International Journal for Parasitology*. **34**(11), pp.1221–1227.

Volf, P., Kiewegová, A. and Nemec, A. 2002. Bacterial colonisation in the gut of Phlebotomus duboscqi (Diptera: Psychodidae): transtadial passage and the role of female diet. *Folia Parasitologica*. **49**(1), pp.73–77.

Volf, P., Tesařová, P. and Nohýnkova, E. 2000. Salivary proteins and glycoproteins in phlebotomine sandflies of various species, sex and age. *Medical and Veterinary Entomology*. **14**(3), pp.251–256.

Volf, P. and Volfova, V. 2011. Establishment and maintenance of sand fly colonies. *Journal of Vector Ecology*. **36**(SUPPL.1), pp.S1–S9.

Volk, S.M., Chen, R., Tsetsarkin, K.A., Adams, A.P., Garcia, T.I., Sall, A.A., Nasar, F., Schuh, A.J., Holmes, E.C., Higgs, S., Maharaj, P.D., Brault, A.C. and Weaver, S.C. 2010. Genome-Scale Phylogenetic Analyses of Chikungunya Virus Reveal Independent Emergences of Recent Epidemics and Various Evolutionary Rates. *Journal of Virology*. **84**(13), pp.6497–6504.

Vreysen, M.J.B., Saleh, K., Mramba, F., Parker, A., Feldmann, U., Dyck, V.A., Msangi, A. and Bouyer, J. 2014. Sterile Insects to Enhance Agricultural Development: The Case of Sustainable Tsetse Eradication on Unguja Island, Zanzibar, Using an Area-Wide Integrated Pest Management Approach. *PLoS Neglected Tropical Diseases*. **8**(5), pp.1–5.

Walker, P.J., Siddell, S.G., Lefkowitz, E.J., Mushegian, A.R., Adriaenssens, E.M., Alfenas-Zerbini, P., Davison, A.J., Dempsey, D.M., Dutilh, B.E., García, M.L., Harrach, B., Harrison, R.L., Hendrickson, R.C., Junglen, S., Knowles, N.J., Krupovic, M., Kuhn, J.H., Lambert, A.J., Łobocka, M., Nibert, M.L., Oksanen, H.M., Orton, R.J., Robertson, D.L., Rubino, L., Sabanadzovic, S., Simmonds, P., Smith, D.B., Suzuki, N., Van Dooerslaer, K., Vandamme, A.M., Varsani, A. and Zerbini, F.M. 2021. Changes to virus taxonomy and to the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2021). *Archives of Virology*. **166**(9), pp.2633–2648.

Walker, T., Johnson, P.H., Moreira, L.A., Iturbe-Ormaetxe, I., Frentiu, F.D., McMeniman, C.J., Leong, Y.S., Dong, Y., Axford, J., Kriesner, P., Lloyd, A.L., Ritchie, S.A., O'Neill, S.L. and Hoffmann, A.A. 2011. The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. *Nature*. **476**(7361), pp.450–455.

Walter, C.T. and Barr, J.N. 2011. Recent advances in the molecular and cellular biology of bunyaviruses. *Journal of General Virology*. **92**(11), pp.2467–2484.

Wang, Y., Ren, K., Li, S., Yang, C. and Chen, L. 2020. Interferon stimulated gene 15 promotes Zika virus replication through regulating Jak/STAT and ISGylation pathways. *Virus Research*. **287**(July), p.198087.

Wang, Z., Nie, K., Liang, Y., Niu, J., Yu, X., Zhang, O., Liu, L., Shi, X., Wang, Y., Feng, X., Zhu, Y., Wang, P. and Cheng, G. 2024. A mosquito salivary protein-driven influx of myeloid cells facilitates flavivirus transmission. *EMBO Journal*. **43**(9).

Watts, D.M., Ksiazek, T.G., Linthicum, K.J. and Hoogstraal, H. 2019. Crimean-Congo hemorrhagic fever. *The Arboviruses*., pp.177–222.

Weaver, S.C. and Barrett, A.D.T. 2004. Transmission cycles, host range, evolution and emergence of arboviral disease. *Nature Reviews Microbiology*. **2**(10), pp.789–801.

Weaver, Scott C, Charlier, C., Nikos, V. and Lecuit, M. 2018. Viral, Other Emerging Vector-borne. *Annu Rev Med.* **69**, pp.395–408.

Weaver, Scott C., Charlier, C., Vasilakis, N. and Lecuit, M. 2018. Zika, Chikungunya, and Other Emerging Vector-Borne Viral Diseases. *Annual Review of Medicine*. **69**, pp.395–408.

Weaver, S.C. and Lecuit, M. 2015. Chikungunya Virus and the Global Spread of a Mosquito-Borne Disease. *New England Journal of Medicine*. **372**(13), pp.1231–1239.

Weaver, S.C. and Reisen, W.K. 2010. *Present and future arboviral threats*.

Wegner, G.I., Murray, K.A., Springmann, M., Muller, A., Sokolow, S.H., Saylors, K. and Morens, D.M. 2022. Averting wildlife-borne infectious disease epidemics requires a focus on socio-ecological drivers and a redesign of the global food system. *eClinicalMedicine*. **47**, p.101386.

Weidmann, M., Sanchez-Seco, M.P., Sall, A.A., Ly, P.O., Thiongane, Y., Lô, M.M., Schley, H. and Hufert, F.T. 2008. Rapid detection of important human pathogenic Phleboviruses. *Journal of Clinical Virology*. **41**(2), pp.138–142.

Welch, S.R., Ritter, J.M., McElroy, A.K., Harmon, J.R., Coleman-McCray, J.A.D., Scholte, F.E.M., Kobinger, G.P., Bergeron, É., Zaki, S.R., Nichol, S.T., Spengler, J.R. and Spiropoulou, C.F. 2019. Fluorescent Crimean-Congo hemorrhagic fever virus illuminates tissue tropism patterns and identifies early mononuclear phagocytic cell targets in IFNAR-/- mice. *PLoS Pathogens*. **15**(12), pp.1–23.

Werneke, S.W., Schilte, C., Rohatgi, A., Monte, K.J., Michault, A., Arenzana-Seisdedos, F., Vanlandingham, D.L., Higgs, S., Fontanet, A., Albert, M.L. and Lenschow, D.J. 2011. ISG15 is critical in the control of chikungunya virus infection independent of UbE1l mediated conjugation. *PLoS Pathogens*. **7**(10).

Wheeler, C.M. 1946. Control of typhus in Italy 1943-1944 by use of DDT. *American Journal of Public Health and the Nations Health*. **36**(2), pp.119–129.

WHO 2024. WHO News, accessed on 5 March 2024. Available from: https://www.who.int/news/item/05-03-2024-new-report-documents-increase-in-hiv-drug-resistance-to-dolutegravir.

WHO 2022. WHO The Global Health Observatory, accessed on 2022. Available from: https://www.who.int/data/gho/data/themes/hiv-aids/hiv-aids.

WHO, W.H.O. 2023. WHO Coronavirus Disease (COVID-19) Dashboard, accessed on 26 November 2023. Available from: https://covid19.who.int/ .

Willems, W.R., Kaluza, G., Boschek, C.B., Bauer, H., Hager, H., Schütz, H.J. and Feistner, H. 1979. Semliki forest virus: Cause of a fatal case of human encephalitis. *Science*. **203**(4385), pp.1127–1129.

Wilson, A.L., Courtenay, O., Kelly-Hope, L.A., Scott, T.W., Takken, W., Torr, S.J. and Lindsay, S.W. 2020. *The importance of vector control for the control and elimination of vector-borne diseases*.

Woelfl, F., Léger, P., Oreshkova, N., Pahmeier, F., Windhaber, S., Koch, J., Stanifer, M., Sosa, G.R., Uckeley, Z.M., Rey, F.A., Boulant, S., Kortekaas, J., Wichgers Schreur, P.J. and Lozach, P.Y. 2020. Novel toscana virus reverse genetics system establishes NSS as an antagonist of type I interferon responses. *Viruses*. **12**(4).

Wolf, A.A., Yáñez, A., Barman, P.K. and Goodridge, H.S. 2019. The Ontogeny of Monocyte Subsets. *Frontiers in Immunology*. **10**(JULY).

World Health Organization 2022. Crimean-Congo haemorrhagic fever. Accessed February 25, 2022. Available from: https://www.who.int/news-room/fact-sheets/detail/crimean-congo-haemorrhagic-fever.

World Health Organization (WHO) 2024. COVID-19 report. Available from: https://covid19.who.int/, accessed on 24 March 2024.

World Health Organization (WHO) 2023. Global burden of Dengue virus (DENV), accessed on 17 March 2023. Available from: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue.

Wu, P., Yu, X., Wang, P. and Cheng, G. 2019. Arbovirus lifecycle in mosquito: acquisition, propagation and transmission. *Expert Reviews in Molecular Medicine*. **21**, p.e1.

Wu, S.J.L., Grouard-Vogel, G., Sun, W., Mascola, J.R., Brachtel, E., Putvatana, R., Louder, M.K., Filgueira, L., Marovich, M.A., Wong, H.K., Blauvelt, A., Murphy, G.S., Robb, M.L., Innes, B.L., Birx, D.L., Hayes, C.G. and Frankel, S.S. 2000. Human skin Langerhans cells are targets of dengue virus infection. *Nature Medicine*. **6**(7), pp.816–820.

Wu, Y., Liu, Q., Zhou, J., Xie, W., Chen, C., Wang, Z., Yang, H. and Cui, J. 2017. Zika virus evades interferon-mediated antiviral response through the co-operation of multiple nonstructural proteins in vitro. *Cell Discovery*. **3**, pp.1–14.

Wuerth, J. and Weber, F. 2016. Phleboviruses and the Type I Interferon Response. *Viruses*. **8**(6), p.174.

Xu, X., Oliveira, F., Chang, B.W., Collin, N., Gomes, R., Teixeira, C., Reynoso, D., My Pham, V., Elnaiem, D.E., Kamhawi, S., Ribeiro, J.M.C., Valenzuela, J.G. and Andersen, J.F. 2011. Structure and function of a 'yellow' protein from saliva of the sand fly Lutzomyia longipalpis that confers protective immunity against Leishmania major infection. *Journal of Biological Chemistry*. **286**(37), pp.32383–32393.

Yadav, P., Chakraborty, P., Jha, N.K., Dewanjee, S., Jha, A.K., Panda, S.P., Mishra, P.C., Dey, A. and Jha, S.K. 2022. Molecular Mechanism and Role of Japanese Encephalitis Virus Infection in Central Nervous System-Mediated Diseases. *Viruses*. **14**(12), pp.1–25.

Yatim, K.M. and Lakkis, F.G. 2015. A brief journey through the immune system. *Clinical Journal of the American Society of Nephrology*. **10**(7), pp.1274–1281.

Ye, L., Schnepf, D. and Staeheli, P. 2019. Interferon-λ orchestrates innate and adaptive mucosal immune responses. *Nature Reviews Immunology*. **19**(10), pp.614–625.

Ylösmäki, E., Martikainen, M., Hinkkanen, A. and Saksela, K. 2013. Attenuation of Semliki Forest Virus Neurovirulence by MicroRNA-Mediated Detargeting. *Journal of Virology*. **87**(1), pp.335–344.

Yona, S., Kim, K.W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M., Misharin, A., Hume, D.A., Perlman, H., Malissen, B., Zelzer, E. and Jung, S. 2013. Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. *Immunity*. **38**(1), pp.79–91.

Zaid, A., Burt, F.J., Liu, X., Poo, Y.S., Zandi, K., Suhrbier, A., Weaver, S.C., Texeira, M.M. and Mahalingam, S. 2021. Arthritogenic alphaviruses: epidemiological and clinical perspective on emerging arboviruses. *The Lancet Infectious Diseases*. **21**(5), pp.e123–e133.

Zanelli, G., Bianco, C. and Cusi, M.G. 2013. Testicular involvement during Toscana virus infection: An unusual manifestation? *Infection*. **41**(3), pp.735–736.

Zhang, J.-M. and An, J. 2007. Cytokines, Inflammation, and Pain. *International Anesthesiology Clinics*. **45**(2), pp.27–37.

Zhang, M., Li, J., Yan, H., Huang, J., Wang, F., Liu, T., Zeng, L. and Zhou, F. 2021. ISGylation in Innate Antiviral Immunity and Pathogen Defense Responses: A Review. *Frontiers in Cell and Developmental Biology*. **9**(November), pp.1–8.

Zhang, Y., Hinojosa, M.E., Yoo, N. and Holtzman, M.J. 2010. Viral and host strategies to take advantage of the innate immune response. *American Journal of Respiratory Cell and Molecular Biology*. **43**(5), pp.507–510.

Zhioua, E., Moureau, G., Chelbi, I., Ninove, L., Bichaud, L., Derbali, M., Champs, M., Cherni, S., Salez, N., Cook, S., De Lamballerie, X. and Charrel, R.N. 2010. Punique virus, a novel

phlebovirus, related to sandfly fever Naples virus, isolated from sandflies collected in Tunisia. *Journal of General Virology*. **91**(5), pp.1275–1283.

Zhu, Y.P., Thomas, G.D. and Hedrick, C.C. 2016. 2014 Jeffrey M. Hoeg Award Lecture. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **36**(9), pp.1722–1733.

Zúñiga-Pflücker, J.C. 2004. T-cell development made simple. *Nature Reviews Immunology*. **4**(1), pp.67–72.

Züst, R., Toh, Y.-X., Valdés, I., Cerny, D., Heinrich, J., Hermida, L., Marcos, E., Guillén, G., Kalinke, U., Shi, P.-Y. and Fink, K. 2014. Type I Interferon Signals in Macrophages and Dendritic Cells Control Dengue Virus Infection: Implications for a New Mouse Model To Test Dengue Vaccines. *Journal of Virology*. **88**(13), pp.7276–7285.