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In vitro immune responses to Sindbis virus

Matefo Millicent Litabe

HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE
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February 2020

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In vitro immune responses to Sindbis virus

Matefo Millicent Litabe

Submitted in fulfilment of the requirements in respect of the M. Med. Sc. Virology degree completed in the Division of Virology in the Faculty of Health Sciences at the University of the Free State

Supervisor: Professor Felicity Burt, Division of Virology, Faculty of Health Science, University of the Free State Bloemfontein

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Declaration

I, Matefo Millicent Litabe declare that the Master's Research Dissertation that I herewith submit for MMed Sc. Degree qualification in Medical Virology at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

Matefo Millicent Litabe

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''I will praise you, LORD, with all my heart; I will tell of the wonderful things you have done. I will sing with joy because of you. I will sing praises to you, Almighty God.''

- **Psalms 9:1-2**

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Oral Presentations

Litabe MM & Burt FJ. *In vitro* immune responses to Sindbis virus. 4th Tofo Advanced Study Week, Praia do Tofo, Inhambane, Mozambique, 1-5 September 2019.

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Table of abbreviations

Abbreviation	Meaning
Ab	Antibody
Ae.	*Aedes*
Ag	Antigen
AURAV	Aura virus
BEBV	Bebaru virus
BFV	Barmah Forest virus
BSL2	Biosafety level 2
C	Capsid
CABV	Cabassou virus
cDNA	Complementary DNA
CHIKV	Chikungunya virus
CP	Crossing point
CPE	Cytopathic effects
Ct	Cycle threshold
Cx.	*Culex*
DC	Dendritic cells
DMEM	Dulbeccos modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Envelope
EDTA	Ethylenediminetetraacetate
EEEV	Eastern equine encephalitis virus
EILV	Eilat virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EVEV	Everglades virus
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FMV	Fort Morgan virus
FRET	Fluoresence resonance energy transfer
GETV	Getah virus
GM-CSF	Granulocyte-macrophage colony-stimulating factor

HI SINV	Heat inactivated Sindbis virus
HJV	Highlands J virus
HM-CSF	Human-macrophage colony-stimulating factor
HRP	Horseradish peroxidase
IFA	Immunofluorescence assay
IFN	Interferon
IFNAR	Interferon-alpha receptor
Ig	Immunoglobulin
IRES	Internal ribosome energy transfer
ISGs	Interferon stimulated genes
IL	Interleukin
JAK	Janus kinase
L-glut	L-glutamine
MADV	Madariage virus
MAYV	Mayaro virus
MCP-I	Monocyte chemoattractant protein-1
MDPV	Mosso das Pedras virus
MIDV	Middelburg virus
MIF	Macrophage migration inhibitor factor
MOI	Multiplicity of infection
MUCV	Mucambo virus
NDUV	Ndumu virus
NFW	Nuclease free water
NICD	National Institute of Communicable Diseases
NK	Natural killer
NSAID	Nonsteroidal anti-inflammatory drugs
Ns	Non-structural
NsP	Non-structural protein
OD	Optical density
ONNV	O'nyong nyong virus
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction

Pen/step	Penicillin/ streptomycin
pEnv	Enveloped protein
PFU	Plaque forming units
PIXV	Pixuna virus
PRNT	Plaque reduction neutralization test
qRT-PCT	Real-time reverse transcriptase-polymerase chain reaction
RA	Rheumatoid arthritis
RdRp	RNA-dependent RNA-polymerase
RNA	Ribonucleic acid
RNV	Rio Negro virus
RRV	Ross River virus
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription polymerase chain reaction
SESV	Southern elephant seal virus
SFV	Semliki Forest virus
SINV	Sindbis virus
SIRS	Systemic inflammatory response syndrome
SPDV	Salmon pancreas disease virus
STAT	Signal transducers and activators of transcription
TAE	Tris-Acetate-Ethylenediminetetraacetate
$TCID_{50}$	Tissue culture infectious dose
Taq	*Thermus aquaticus*
TNF-α	Tumor necrosis factor-alpha
TONV	Tonate virus
TROV	Trocara virus
TYK2	Tyrosine kinase 2
UNAV	Una virus
VEEV	Venezuelan equine encephalitis virus
VLP	Virus-like particle
WEEV	Western equine encephalitis virus
WHAV	Whataroa virus

Abstract

Sindbis virus (SINV) is an arthritogenic alphavirus belonging to the *Togaviridae* family. SINV was initially isolated in Sindbis, Egypt in 1952, since then the virus has been isolated in different parts of the world including Africa, Asia, Australia and Europe. Infection with SINV results in febrile self-limiting symptoms that are usually of short duration. However, some individuals develop prolonged incapacitating joint pain that may persist for months to years. The mechanism by which SINV and other arthritogenic alphaviruses cause chronic arthritis is poorly understood however previous studies suggest the involvement of monocytes and macrophages which result in the secretion of pro-inflammatory cytokines which are induced by virus replicating in or around joint tissue. The aim of the study was to investigate the innate immune response to *in vitro* infection of macrophages with SINV in order to determine if human macrophages from different individuals differ in their susceptibility to SINV infection and to determine the role of interferon (IFN) in SINV infected macrophages.

Peripheral blood mononuclear cells (PBMCs) were isolated from ten SINV antibody naïve individuals using ficoll-paque density gradient method. The cells were stimulated with human-macrophage colony-stimulating factor (HM-CSF) to differentiate from monocytes to macrophages. Post-stimulation the macrophages were infected with SINV at a multiplicity of infection (MOI) of 0.1. Additionally, some macrophages cultures were pre-treated with ruxolitinib, an IFN inhibitor 2 hours or immediately prior to infection with SINV at an MOI of 0.1. Virus replication was determined at different time intervals for 24 hours using a two-step quantitative RT-PCR. To determine the secretion levels of pro-inflammatory cytokines post-SINV infection, cell-free supernatant was collected at different intervals post-infection and levels of pro-inflammatory cytokines including IFN-α, TNF-α, IL-1β, IL-6, IL-8 and IL-12 in the supernatant were tested by ELISA.

A primer pair that targets the nsP2 region of SINV nsP2 protein together with the TaqMan hydrolysis probe were used to quantify viral loads of infected macrophages by qRT-PCR. An increase in viral loads was detected in macrophages of 5/10 participants, suggestive of viral replication. A decrease in viral load over time was observed in macrophages of the remaining five participants, suggestive of little to no viral replication. IFN inhibition resulted in SINV replication in macrophages from 7/10

and 8/10 participants when treated at time 0 of infection and 2 hours prior to infection, respectively. Infection elicited a strong innate immune response demonstrated by secretion of pro-inflammatory cytokines including IFN-α. IL-6 and IL-8.

Infection with SINV results in a strong pro-inflammatory response which seems to control viral load. Results suggest that macrophages are among SINV targeted cells during human infection and macrophages from different individuals appear to differ in their susceptibility to SINV replication. The study also shows that type I IFN may play an important role in the protection of SINV as its inhibition rendered macrophages from more participants susceptible to viral replication.

Chapter 1: Literature review

1.1 Introduction and history

Viruses belonging to the family *Togaviridae*, genus *Alphavirus,* are enveloped, positive-sense, single-stranded RNA viruses transmitted by mosquitoes. They are referred to as either Old World or New World viruses depending on where the viruses were first isolated and are circulating. Old World viruses include viruses such as chikungunya virus (CHIKV), Ross River virus (RRV), Barmah Forest virus (BFV), o'nyong-nyong virus (ONNV) and Sindbis virus (SINV). The Old World viruses are commonly associated with rheumatic disease in humans, and they have been isolated in Africa, Europe, Australia, and Asia (Norder et al., 1996). New World alphaviruses include Eastern, Venezuelan and Western equine encephalitis viruses. These viruses are associated with potentially fatal encephalitic disease and are found in the Americas (Suhrbier and Gasque, 2012). Unlike encephalitic alphaviruses, arthritogenic alphaviruses have a low mortality rate but a high morbidity rate. In some individuals, infection results in disabling joint pain that may persist for months or years.

SINV and other Old World alphaviruses are emerging or re-emerging viruses causing massive epidemics throughout the world commonly associated with outbreaks of acute and persistent arthritis in humans (Heise et al., 2000). The mechanism through which these viruses cause arthritis is diverse and poorly understood, however previous studies suggest that arthritis is due to inflammatory responses induced by viruses replicating in or around joint tissue (Chen et al., 2015). The global distribution of these viruses has increased due to international travel, economic development, and adaptation to different mosquito vectors. One of the significant alphaviruses that have emerged is CHIKV and has been increasingly spreading throughout the world, causing major outbreaks from 2003.

SINV is a mosquito-borne alphavirus found in the Western equine encephalitis serocomplex. SINV was initially isolated from a pool of *Culex (Cx.) univittatus and Cx. pipiens* mosquitoes in Sindbis, Egypt in 1952 (Taylor et al., 1953). The first human cases of SINV were reported in Uganda in 1961, where the virus was isolated from patients that presented with fever, malaise, and headache (Haddow, 1961). Since then, the virus has been isolated in different parts of the world, including Africa,

Australia, Asia, and Europe causing major epidemics (Norder et al. 1996; Medlock et al., 2007). In Europe, SINV is the etiological agent of diseases that are referred to as Ockelbo disease in Sweden, Pogosta disease in Finland, and Karelian fever in Russian (Laine et al., 2000).

1.2 Virus classification

There are currently 31 known alphaviruses with a broad host range (https://talk.ictvonline.org/files/master-species-lists/m/msl/6776). Alphaviruses are grouped into eight antigenic complexes based on serological cross-reactivity. They include Barmah Forest, Eastern equine encephalitis, Middelburg, Ndumu, Semliki Forest, Venezuelan equine encephalitis, Western equine encephalitis, and the last complex which is unclassified. Table 1.1 shows the grouping of the viruses in the eight antigenic complexes.

Table 1.1: Alphaviruses are grouped into eight antigenic complexes based on serological cross-reactivity. (https://talk.ictvonline.org/files/master-species-lists/m/msl/6776).

Antigenic complex	Virus species
Barmah Forest	Barmah Forest virus (BFV)
Eastern equine encephalitis	Eastern equine encephalitis virus (EEEV)
Middelburg	Middelburg virus (MIDV)
Ndumu	Ndumu virus (NDUV)
Semliki Forest	Bebaru virus (BEBV) Chikungunya virus (CHIKV) Getah virus (GETV) Mayaro virus (MAYV) O'nyong nyong virus (ONNV) Ross River virus (RRV) Semliki Forest virus (SFV) Una virus (UNAV)
Venezuelan equine encephalitis	Venezuelan equine encephalitis virus (VEEV) Mosso das Pedras virus (MDPV) Everglades virus (EVEV) Mucambo virus (MUCV)

	Tonate virus (TONV) Pixuna virus (PIXV) Cabassou virus (CABV) Rio Negro virus (RNV) Madariage virus (MADV)
Western equine encephalitis	Aura virus (AURAV) Sindbis virus (SINV) Whataroa virus (WHAV) Fort Morgan virus (FMV) Highlands J virus (HJV) Western equine encephalitis virus (WEEV)
Unclassified	Eilat virus (EILV) Trocara virus (TROV) Salmon pancreas disease virus (SPDV) Southern elephant seal virus (SESV)

1.3 Alphavirus genome

The genome of alphaviruses is about 11.7 kb in length and is contained within a small, icosahedral, enveloped virion (Strauss et al., 1984). Figure 1.1 represents the alphaviral genome. The heterodimeric protein spikes are composed of the two immunodominant proteins, E1 and E2, enclosed by a lipid bilayer that is derived from the host cell (Jose et al., 2009). The coding region of alphaviruses consists of two open reading frames (ORF), the N-terminal ORF, which is translated from genomic RNA and encodes the nonstructural polyprotein, and the C-terminal ORF which is translated from subgenomic 26S RNA and encodes the structural polyprotein. The polyproteins are posttranslationally cleaved by viral and host proteases (Leung et al., 2011). Polyprotein of the genomic RNA is processed into nonstructural (ns) nsP1-4 proteins whereas the polyprotein translated from the subgenomic 26S RNA is processed into the capsid protein (C), E1 and E2 glycoproteins, and two peptides 6K and E3 (Thiberville et al., 2013).

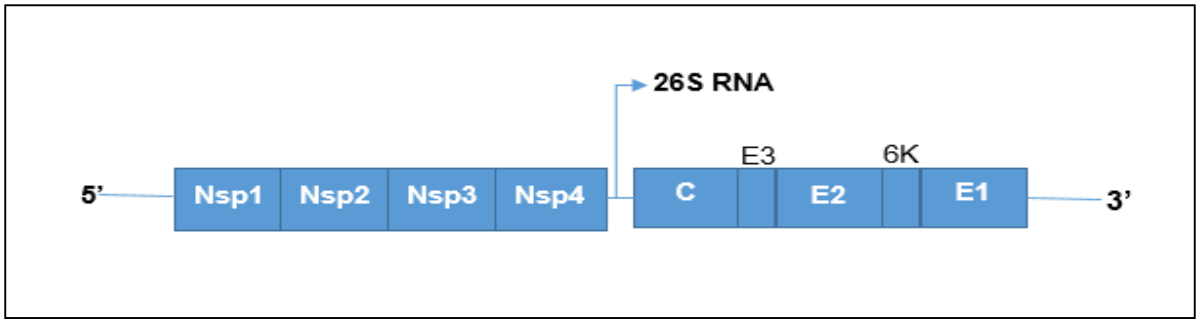


Figure 1.1: Alphavirus genome. The genome encodes two open reading frames. The N-terminal open reading frame which is translated from genomic RNA and encodes the nonstructural proteins (nsP1-nsP4) and the C-terminal open reading frame, which is translated from subgenomic 26S RNA and encodes for structural proteins, , E1, E2, E3, capsid and 6K proteins.

Viral RNA synthesis is accomplished via the activities of the viral nonstructural protein nsP1 to nsP4. The nsP1 is required for the synthesis of negative sensed RNA as it contains both the enzymes for methylation and capping of newly synthesized RNA (Jose et al., 2009). Previous studies on nsP1 show that nsP1 can regulate the activity of nsP2 protease as the presence of nsP1 reduces cleavage of nsP2 and nsP3. The nsP2 has multiple enzymatic activities, the N-terminal domain of the protein possesses a helicase enzyme and an RNA triphosphate enzyme, whereas the C-terminal possesses a cysteine protease required for proteolytic processing of non-structural polyprotein (Leung et al., 2011). The helicase enzyme functions in unwinding the RNA-RNA duplexes during replication and transcription. The N-terminal also functions in the folding of the non-structural proteins for the synthesis of subgenomic mRNA replication. The function of the nsP3 is not yet fully understood but plays a role in RNA synthesis (Rupp et al., 2015). The nsP3 consists of two domains, the first domain is highly conserved within alphaviruses, and the second domain is hypervariable (Foy et al., 2013). The nsP3 is a phosphoprotein, which is thought to play a role in the synthesis of a negative-sense RNA strand by mediating the association of the replication complex with the cytoplasmic membrane. The nsP4 protein functions as an RNA-dependent RNA-polymerase (RdRp) during the replication of positive and negative strands of virus-specific RNA during virus replication (Foy et al., 2013).

The subgenomic 26S RNA is translated into a single polyprotein that is cleaved into five structural proteins E1, E2, E3, C, and 6K. These proteins are required for viral encapsidation and budding (Thiberville et al., 2013). Glycoproteins E1 and E2 are

responsible for viral attachment and membrane fusion. The fusion of the viral membrane with the endosomal membrane is mediated by E1 glycoprotein, and E2 is responsible for receptor binding and receptor-mediated endocytosis (Leung et al., 2011). The E3 protein functions in the mediation of spike folding and spike activation for viral entry, the protein is also required for particle assembly (Jose et al., 2009). The E3 also has a function in pE2/E1 complex formation and transport of viral structural components to the site of budding (Jose et al., 2009). The smallest structural protein is the 6K protein that is incorporated into the virion in small amounts, it has been found to play a role in the budding process, and virion assembly (Jose et al., 2009).

1.4 Replication

Alphavirus replication takes place in the cytoplasm of infected cells and is mediated by nonstructural proteins, while the structural proteins are essential for viral encapsidation and budding (Thiberville et al., 2013). For replication to occur, the virus enters the plasma membrane via receptor-mediated endocytosis (Leung et al., 2011). Upon entry, the virus is disassembled and genomic RNA is released into the cytoplasm. After the release of viral RNA, two rounds of translation occur, positive-sense RNA is partially translated to produce nonstructural proteins. Downstream processing of the polyproteins enables the synthesis of genomic and negative-strand RNA. Replication of genomic RNA from negative-strand RNA allows the translation of structural proteins that are processed co-translationally and post-translationally (Thiberville et al., 2013). NsP4 and nsP123 precursors mediate negative sense RNA replication. Glycoproteins are translocated to the endoplasmic reticulum (ER) (Powers and Logue, 2007). Association of the glycoproteins with the nucleocapsid allows the newly synthesized virus particle to bud out from the host membrane completing the final assembly of the virion.

1.5 Pathogenesis

The pathogenesis of SINV and other arthritogenic alphaviruses is poorly understood, however previous studies indicate the involvement of different skin and migratory cells including dendritic cells (DC), monocytes and macrophages which might play a crucial role in virus pathology. Briefly, the virus is inoculated into the skin through a bite of an infected mosquito, for infection to occur, the blood meal must contain above a specific concentration of virus to enable infection of an individual (Assunção-miranda et al.,

2013). There is an incubation period of two to ten days, during which virus replication occurs before the infected individual becomes viremic and begins showing symptoms of infection (Rulli et al., 2007). Post-inoculation, the virus disseminates to the primary site where it replicates, including the liver, spleen, and lymph nodes. The virus then spreads to secondary sites, including the bones, muscles, and articular tissues resulting in local inflammation that accounts for the acute phase symptoms of the virus including fever, rash, headache, malaise, myalgia, and arthralgia, which may later result in chronic arthritis (Chen et al., 2015). The severity of SINV infection is determined by the viral load and the response of the infected individual's innate immune system via the release of cytokines and chemokines (Assunção-miranda et al., 2013).

Studies using mice models indicate articular tissue as one of the sites for viral replication. In a study done on adult mice infected with a virus from the Sindbis group, viral replication was detected in bone-associated connective tissue, and the infectious virus was isolated from bone and joint tissue (Assunção-miranda et al., 2010). In a study on RRV infection, severe inflammation within the joint and skeletal muscle tissues was observed in mice infected with RRV (Lidbury et al., 2000).

1.5.1 Role of the immune response

The host's innate immune response plays a vital role in the control as well as the pathogenesis of SINV infection. Macrophages, monocytes, natural killer (NK) cells, $CD4^{+,}$ and $CD8^{+}$ T lymphocytes are the main cells that result in the secretion of inflammatory mediators in animal models, indicating the involvement of these cells in the pathogenesis of arthritis induced by alphaviruses (Hirsch and Griffin, 1979). The cause of the persistence of symptoms may be associated with the intensity of the proinflammatory secretions, the extension of articular lesion, and the persistence of the virus in and around joint tissue (Assunção-miranda et al., 2013). *In vitro* and *in vivo* studies have also shown the importance of monocytes and macrophages, being among the major primary cells that result in pro-inflammatory cytokine secretion during infection.

The role of macrophages in rheumatoid arthritis (RA) is well understood, but their role in arthritis induced by alphaviruses is unclear. Macrophages are one of the major cell

groups found in articular tissue during SINV and other arthritogenic alphaviral infections, they are involved in the initiation of persistent inflammation where they secrete pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferons (IFNs), and different interleukins (IL) in response to infection (Assunção-miranda et al., 2010). Studies have shown that ,onocytes and macrophages play an important role in the pathogenesis of arthritogenic alphaviruses including SINV, CHIKV, RRV, and MAYV where they are a major source of pro-inflammatory cytokines (Morrison et al., 2006; Assunção-miranda et al., 2010; Her et al., 2010; Cavalheiro et al., 2016). *In vitro* infection of human macrophages with SINV promoted macrophage activation resulting in increased secretion of macrophage migration inhibitor factor (MIF), TNF-α, IL-6, and IL-1β similar to those secreted during RA infection (Assunção-miranda et al., 2010). CHIKV has been shown to infect monocytes and was undetectable in NK cells, $CD4^+$, and $CD8^+$ T cells (Her et al., 2010). Mouse model of RRV infection demonstrated that articular damage was due to macrophage infiltrates around joint tissue, resulting in secretion of proinflammatory cytokines (Morrison et al., 2006). Macrophage depletion before alphavirus infection results in less severe disease, this shows the importance of macrophages in the pathogenesis of arthritogenic alphavirus (Morrison et al., 2011; Stoermer et al., 2012). These studies show that monocytes and macrophages may play an important role in the immunopathogenesis of arthritogenic alphaviruses.

1.5.2 Effects of interferon during infection

IFNs are a group of proinflammatory cytokines that result in an antiviral state when produced by the cell in response to infection. There are three classes of IFNs, including type I, II, and III. Type I IFNs were discovered in 1957 and include IFN α, β, ε, κ, and ω, IFN-α, and IFN-β are the most important (Kalliolias and Ivashkiv, 2010). Type I IFNs are produced by most cells in the body directly in response to infection as part of the innate immune response (Mcnab et al., 2015). Type I IFNs are stimulated via the Janus kinase/ signal transducer and activator of transcription (JAK/STAT) pathway resulting in the stimulation of hundreds of interferon-stimulated genes (ISGs) resulting in an antiviral state (Ivashkiv and Donlin, 2014). Briefly, type I IFNs bind to a heterodimer interferon-alpha receptor (IFNAR), composed of IFNAR1 and IFNAR2 subunits. Binding of type I IFN to the receptors results in the activation of JAK1 and tyrosine kinase 2 (TYK2). Activated JAK1 and TYK2 phosphorylate STAT 1 and STAT 2

proteins, the two STAT proteins dimerize and translocate to the nucleus of the cell where they bind to IFN-stimulated response elements resulting in the transcription of ISGs. Figure 1.2 represents a simplified pathway for the induction of ISGs via the JAK/STAT pathway. Type II IFN comprises only of IFN-γ, which is also activated via the JAK/STAT pathway (Green et al., 2017). IFN-γ is produced by different cells, including NK cells, $CD4^+$, and $CD8^+$ T cells, to improve antigen recognition in antigen-presenting cells such as macrophages (Green et al., 2017). Type III IFNs consists of λ1-3, known as IL-29, IL-28A, and IL-28B, respectively (Kalliolias and Ivashkiv, 2010). Type III IFNs are also induced directly in response to infection using the same pathway as type I, IFN-α/β.

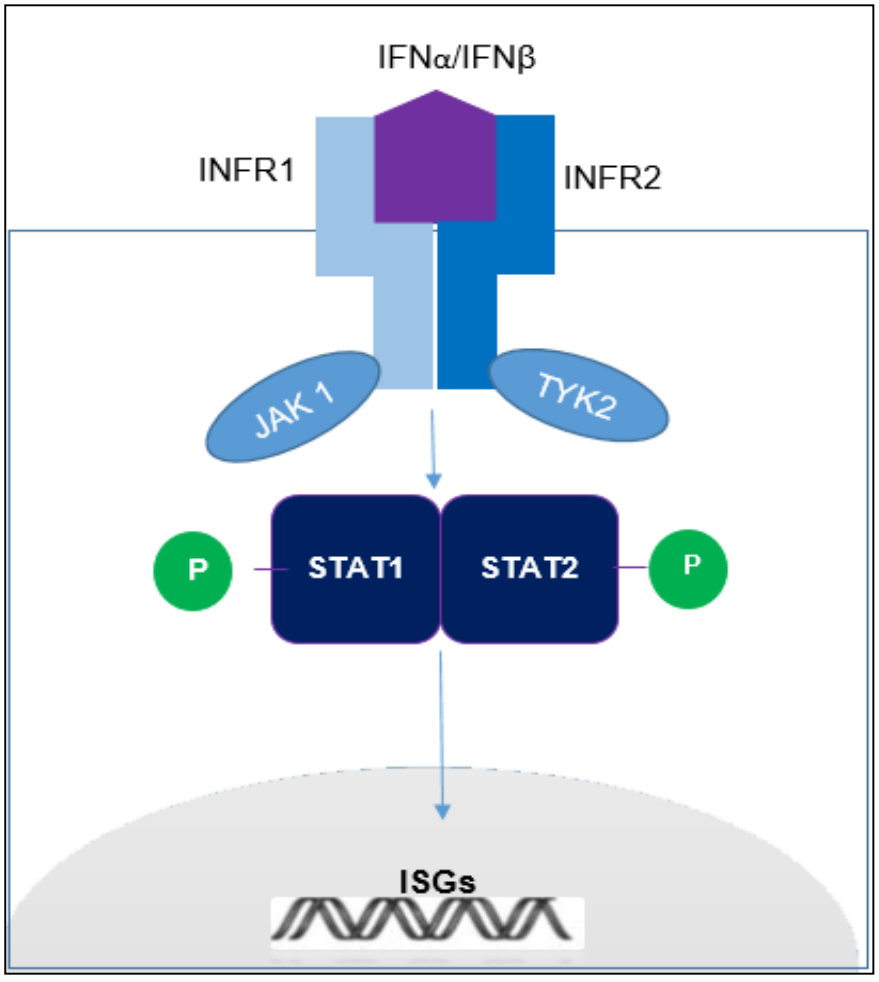


Figure 1.2: Type I interferon signaling pathway. Binding of IFNα/β to the receptors activates phosphorylation of Janus Kinase (JAK 1) and tyrosine kinase 2 (TYK2), which subsequently phosphorylate two STAT protein which dimerizes and translocate to the nucleus resulting in transcription of hundreds of interferon-stimulated genes resulting in an antiviral state (Modified from Ivashkiv & Donlin, 2014).

IFN is one of the critical signaling proteins that play a crucial role in the protection against viral infection by activation of macrophages, they function as the first line of defence in protecting cells that are essential for the development innate immunity in the absence of adaptive immunity (Thiberville et al., 2013). The importance of IFN has been shown in mice studies where IFN deficient mice result in a more severe disease in comparison to those with an active IFN response. Determination of the role of IFNα/β in protection against SINV in a mouse model demonstrated that mice lacking IFN receptors succumbed to more severe disease characterized by increased viral load and dissemination of virus to distal places. In mice with an active IFN response, viral replication and dissemination were restricted. Another study showed IFN-γ mediates clearance of SINV without any side effects (Ryman et al., 2000). These studies show that IFNs may play a role in the downregulation of virus replication and their importance as a form of defence against viral infection.

1.6 Transmission and epidemiology

1.6.1 Sindbis virus

SINV and other arthritis causing alphaviruses are maintained in the environment by a continuous cycle between the mosquito (vector) and the reservoir. The primary vectors for SINV are *Cx.* and *Culiseta* mosquitoes, *Aedes* (*Ae.*) mosquitoes can also serve as a vector for SINV transmission, and migratory birds and game birds serve as reservoirs for SINV (Chen et al., 2015). SINV has been isolated from the blood and tissues of birds, but there has not been any recorded evidence that it causes mortality or any symptoms in birds. The main amplifying hosts of SINV in Sweden include fieldfare, the redwing, and the song thrush bird, whereas, in Finland, game birds such as black grouse and capercaillie are the amplifying hosts(Lundström et al. 2001; Laine et al., 2004). Figure 1.3 illustrates the transmission cycle of SINV in nature, where it co-circulates between mosquito vectors and reservoir birds and humans being incidental hosts. Increased SINV activity in mosquito vectors and an increased abundance of mosquito vectors are markers that can be used for SINV outbreak (Lundström et al., 2019). In 2001,there was a low prevalence of SINV in mosquitoes in Europe, however, the prevalence increased in 2002 resulting in an outbreak, and a decline in SINV activity was further observed in 2003 post the outbreak (Lundström et al., 2019).

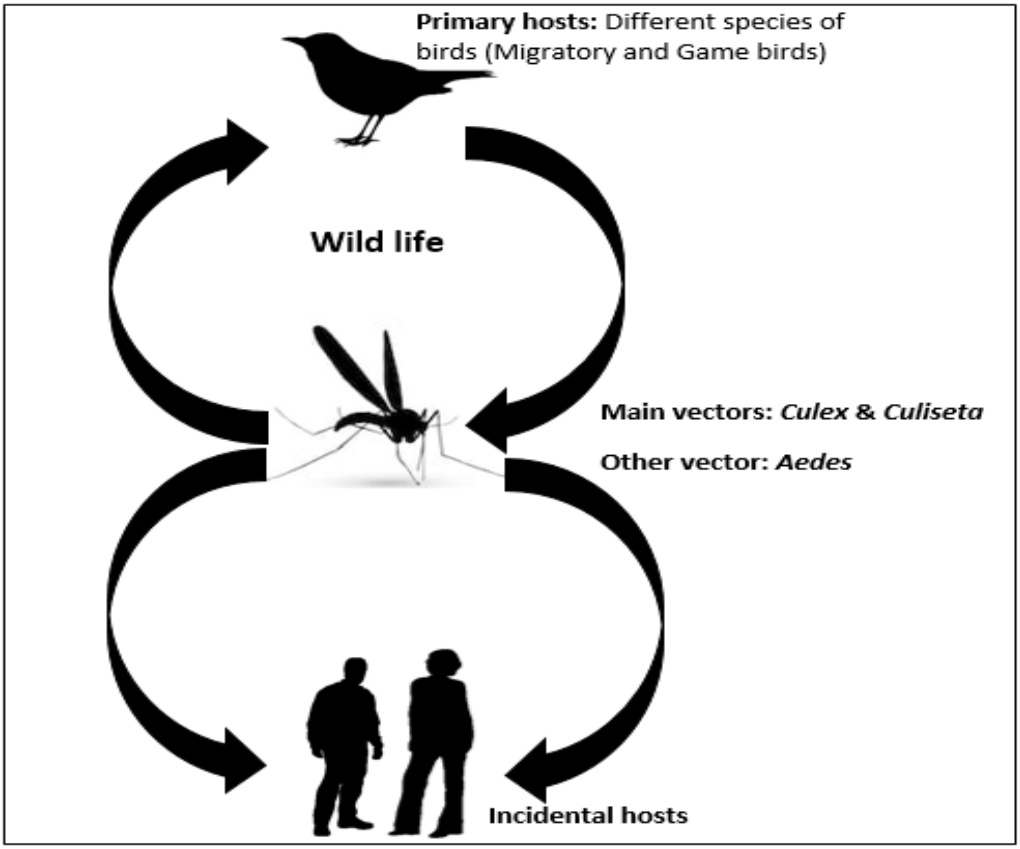


Figure 1.3: Schematic representation of alphavirus life cycle. Sindbis virus is maintained in the environment by a continuous cycle between different mosquito species and various species of birds that serve as reservoirs. Humans are usually incidental hosts. Humans are typically incidental hosts as the virus does not replicate at high enough titers to be transmitted to other humans (Modified from Napoleão-Pego et al., 2014).

SINV is the most widely distributed of the alphaviruses and comprises of different strains depending on geographical locations, previous studies on phylogenetic analysis show the northern European SINV strains as being closely related to those from South Africa (Kurkela et al., 2005). SINV strains isolated from Africa, the Middle East, and Europe share between 88.8% to 100% nucleotide identity (Norder et al., 1996). The close phylogenetic relatedness suggests that bird migration may be responsible for the dissemination of SINV (Kurkela et al., 2005). Previous studies have indicated that SINV was introduced to Europe by migratory birds from South Africa (Norder et al. 1996; Lundström et al., 2001). Recent studies on evolutionary history of SINV based on phylogenetic analysis suggest that SINV stains in Europe share a common ancestor with strains from central Africa, suggesting that all strains in Europe are from a single introduction of SINV in Sweden from central Africa which then spread to other parts of Europe (Ling et al., 2019).

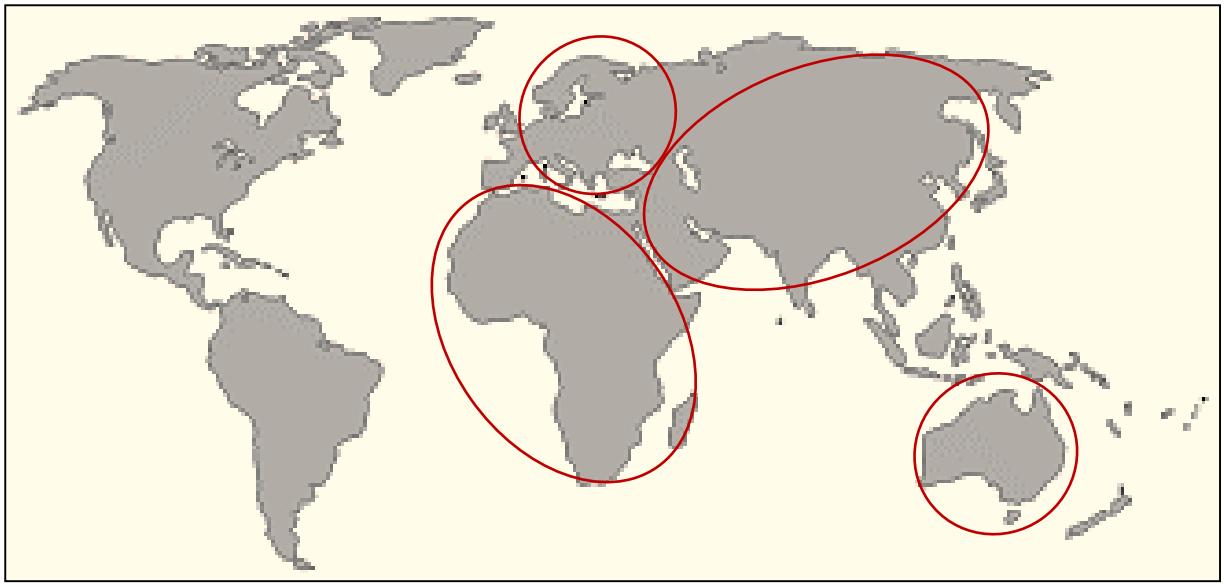


Figure 1.4: Global distribution of Sindbis virus. Sindbis virus has been isolated in different parts of the world, including Africa, Australia, Asia, Europe, and Russia (Modified from Kurkela et al., 2004).

SINV has a wide geographical distribution as it has been isolated in Africa, Asia, Europe, and Australia. Figure 1.4 represents the global distribution of SINV. Outbreaks of SINV have occurred in Sweden, Finland, and sporadic outbreaks have occurred in Uganda, South Africa, Zimbabwe, and Australia (McIntosh et al, 1976; Lundstrom et al., 1991; Brummer-Korvenkontoi et al., 2002; Kurkela et al., 2008; Storm et al., 2013). In South Africa, human infection with SINV occurs during the summer, especially after heavy rainfall favoring mosquito breeding. The majority of cases occur in the central plateau of the country, including the Free State, Northern Cape, and the Gauteng provinces. In South Africa, SINV was first isolated from skin lesions of an infected woman presenting with joint pain and severe headache in the Gauteng province in 1963 (Malherbe and Strickland-Cholmley, 1963). Since then, several outbreaks of the virus have been recorded in South Africa. The largest epidemic of SINV in South Africa was in 1974 in the Karoo, Northern Cape, with about 4000 human cases (McIntosh et al., 1976). The second largest outbreak occurred in the Pretoria region in the Gauteng province in 1984, resulting in hundreds of cases (Jupp et al., 1986). More recently SINV outbreak occurred in 2010 in South Africa, concurrently with Rift Valley fever virus outbreak (RVFV), in which 208 human samples tested positive for SINV IgM (Storm et al., 2014).

SINV was identified as the causative agent for Pogosta disease in the 1980s (Brummer-korvenkontio et al., 2002). The virus was identified in patients in Finland

and Sweden that presented with joint pain and rash. Cases occur during the summer months, and the first cases are usually detected towards the end of July, peak mid-August, and end towards the end of September. Since its first isolation in Finland, SINV epidemics occur every 7 years (in 1981, 1988, 1991, 1995, and 2002). Epidemiological studies show that there were over 2000 cases of Pogosta disease reported in Finland between 1981 and 1996, the majority of the cases occurred in Northen Karelia, Finland (Brummer-korvenkontio et al., 2002). Between August and September 2002, 597 cases of Pogosta disease were reported in Finland, the majority occurring in Northern Karelia. During the outbreak, the virus was isolated from the blood of one patient and skin lesions of four patients presenting with joint pain (Kurkela et al., 2004). In Sweden, Sindbis-related virus causing Okelbo disease was initially isolated from a pool of *Culex* and *Culiseta* mosquitoes and resident birds (Niklasson et al., 1984). Between 1981 and 1988, a total of 245 cases of Okelbo disease were confirmed in Sweden, the majority occurring in central Sweden (Lundström et al., 1991). In 2013 an outbreak occurred in the northern part of Sweden, an area thought to be nonendemic to SINV (Gylfe et al., 2018). From 18 of the confirmed 48 SINV cases, patients reported experiencing joint pain 6 to 8 months post-infection. IgM antibodies were still detected 6 months post-infection in some of the patients, which may be due to virus persistence (Gylfe et al., 2018). In Russia, Karelian fever was first identified in the early 1980s from a pool of *Ae.* mosquitoes (Lvov et al., 1984). Symptoms to Karelian fever are similar to those of Pogosta disease and Okelbo disease. Phylogenetic analysis show close phylogenetic relatedness of SINV isolates in Sweden, Finland, and Russia with only 0.1 to 1.4% nucleotide sequence difference and 0 to 2.1% amino acid difference in the nsP3 and nsP4 gene (Kurkela et al., 2004).

1.6.2 Arthritogenic alphaviruses

CHIKV is a member of the SINV group alphavirus that has recently re-emerged, causing major outbreaks from 2003 around the Indian Ocean islands and India (Rana and Lunia, 2015). The current spread of CHIKV to the Americas indicates how readily arboviruses can spread and become endemic in a new region. The first isolation of CHIKV was in Tanzania in 1953 from a febrile human and is primarily transmitted by *Ae.* mosquitoes (Ross, 1956). Since the first documented re-emergence in 2003, after decades where no outbreaks were documented, CHIKV has caused massive

outbreaks throughout the world (Porter et al., 2004; Laras et al., 2005; Rana and Lunia, 2015). In 2003 CHIKV was reported in Asian countries, causing outbreaks in Thailand, Indonesia, and southern regions of India. After the outbreak of 2004 in Kenya, the virus spread to the Comoros Islands, causing an epidemic in 2005 (Sergon et al., 2007). Shortly thereafter, the virus spread through the Indian Ocean, and cases were reported in Mayotte, Mauritius, and the French island of La Reunion. During the outbreaks in India between 2005 and 2006, the virus was also isolated in Europe and was believed to be the same strain that was causing outbreaks in India (Noridah et al., 2006). There were more than 1.3 million suspected cases of the virus alone in India in 2005 (Arankalle et al., 2007). Between 2006 and 2008, CHIKV cases were reported in Malaysia, and in 2007 CHIKV continued to spread to Europe (Noridah et al., 2006; Noridah et al., 2007; Amraoui and Failloux, 2016). In 2008 there were cases of CHIKV reported again in Asia. In 2009 another outbreak occurred in Thailand. In 2013 CHIKV was introduced to the Americas, causing an outbreak in the Caribbean (Morrison, 2014). Since 2013, CHIKV has been detected in 45 countries and territories, with over 2.6 million confirmed cases (Yactayo et al., 2016). Between 2016 to 2017, cases of CHIKV have also been reported in Dhaka and Pakistan (Kabir et al., 2017). In January 2019, cases of CHIKV were reported in the Republic of the Congo (Fritz et al., 2019). During these outbreaks, CHIKV was introduced to countries where it was previously not found including, Italy, France, Caledonia, Papua New Guinea, Bhutan, Yemen, and other countries.

Due to suitable climatic conditions such as increasing temperatures and increased rainfall, increase in mosquito's distribution, the ability of the virus to adapt to new mosquito species, and more species of mosquitoes being able to transmit the viruses, SINV, CHIKV, and other arboviruses will continue to spread as the conditions allow them to. The spread will further be aided by increased deforestation, travel, and urbanization. Because alphaviruses are arboviruses, environmental conditions such as urbanization, deforestation, and global warming have a significant influence on their spread. Individuals involved in outdoor activities and occupations have an increased risk of contracting the viruses.

1.7 Clinical manifestation and laboratory diagnosis

Arthritogenic SINV usually causes acute disease, with the symptoms occurring after an incubation period of two to ten days post-infection (Suhrbier and Gasque, 2012). Infection is characterized by fever, arthritis, myalgia, and rash. The viremic period is usually short, lasting for four to seven days. The duration of the disease varies between patients, in some individuals, the virus can be cleared without any development of clinical symptoms, whereas in others, the infection could lead to chronic arthritis.

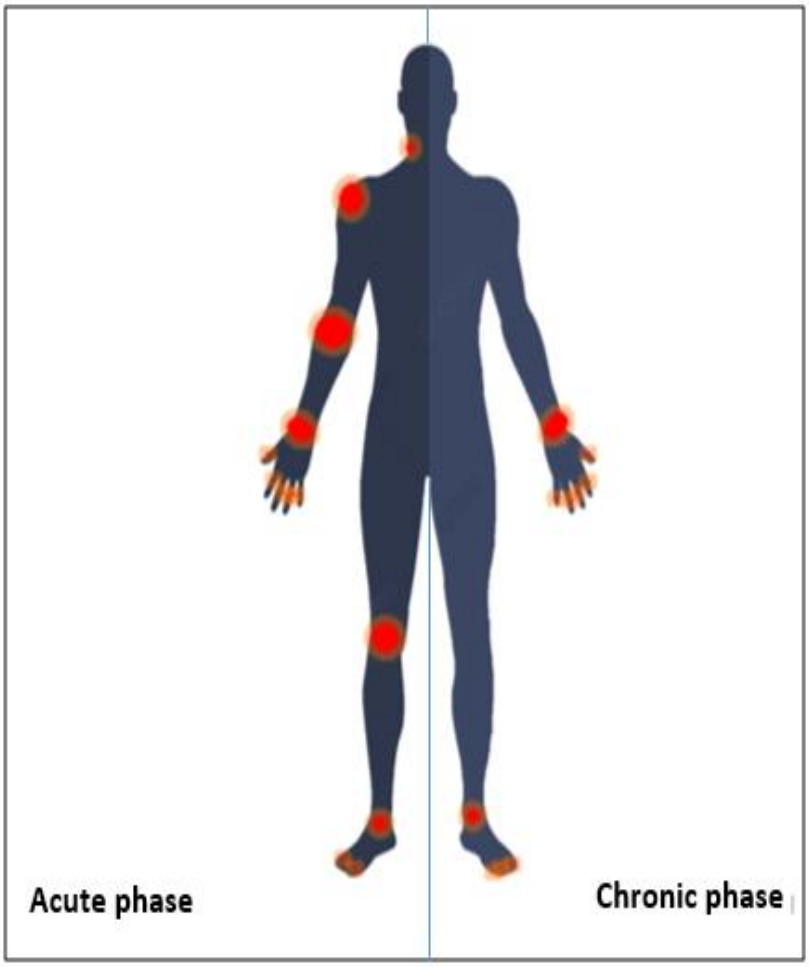


Figure 1.5: Clinical manifestation of Sindbis virus. The left represents joints affected during the acute phase of infection, including those of the neck, arms, knees, hands, and feet. The right represents joints affected during the chronic phase of infection, mainly the small joints, including those of the wrist, ankles, and fingers (Modified from Tanabe et al., 2018).

Diagnosis of arthritogenic alphaviruses in based on symptoms and patient history. Clinical symptoms for SINV disease include mild and moderate fever, fatigue, rash, and joint inflammation being the most prominent among the symptoms (Olivia et al.,

2015). Joint pain and inflammation mainly affect the small joints such as those from fingers and wrists and the large joints such as those from knees and shoulders, causing polyarthritis (Assunção-miranda et al., 2013). Other symptoms may include mild headache, fatigue, malaise, nausea, vomiting, and pharyngitis. In the chronic phase of infection, joint pain mainly affects only the small joints, including those of the wrists, ankles, fingers, and the larger joints are usually not affected. Figure 1.5 shows some of the joints affected during both the acute and the chronic phases of infection.

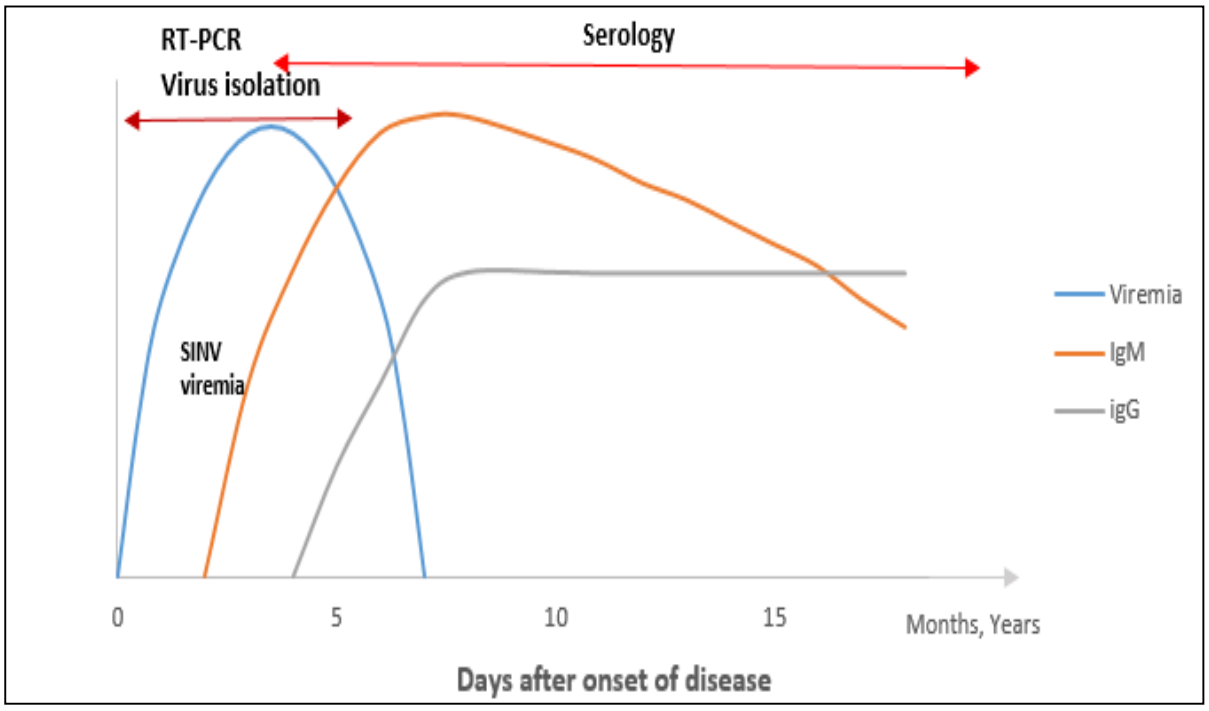


Figure 1.6: Biomarkers that can be used for the detection of the SINV infection. During the viremic phase, the virus can be isolated using cell culture techniques and viral RNA can be detected using different molecular techniques, and the virus can usually be detected for up to 5-7days. IgM can be detected from 2-8 days after the onset of symptoms and usually lasts 1-3 months. IgG can be detected from 4-10 days after the start of symptoms and is generally lifelong. Both IgM and IgG can be detected using serological methods (Modified from Barbara et al., 2016).

Clinical confirmation of arthritogenic alphavirus infection can be done using different methods, either by virus isolation, detection of viral nucleic acid, or serology. The most common sample used for clinical diagnosis is serum, other samples that be used for diagnosis of alphaviruses include synovial fluid, skin biopsies, plasma, and whole blood (Horling et al., 1993; Kurkela et al., 2004). Differential diagnosis has to be performed to rule out other viral infections that have similar symptoms to those of arthritogenic alphaviruses (Suhrbier and Gasque, 2012). Figure 1.6 represents the

course of SINV infection and the biomarkers that can be used to detect infection. During the acute phase of the disease, molecular techniques can be used for the detection of viral RNA or virus can be isolated using different methods. During the convalescent phase, serological techniques can be used to demonstrate infection. Because the viremic phase is very short majority of SINV diagnoses rely on serology.

The acute phase of infection corresponds with the period when the patient is still viremic, which lasts about a week from the beginning of symptoms. During this time, the virus can be isolated from the infected patient, and viral RNA and antigen can also be detected. Virus isolation requires growing the virus in suckling mice or cell culture. Alphaviruses are fast-growing viruses, and if kept under appropriate conditions, they can induce cytopathic effects (CPE) within the one to three days post-infection (Suhrbier and Gasque, 2012). Immunofluorescence assay (IFA) following virus isolation can be used as confirmation of the virus using specific antibodies directed against the virus antigen. Viral RNA can be detected using molecular techniques during the viremic phase of infection. SINV RNA can be isolated from blood and skin lesions of infected individuals by PCR (Horling et al., 1993). Molecular methods are much more rapid in comparison to virus isolation. Reverse transcription-polymerase chain reaction (RT-PCR) is used for the detection of viral RNA, PCR targets a specific region of the viral genome and can be used for rapid detection of the virus. Molecular techniques are advantageous in that they have high specificity and sensitivity and quick in diagnosis as they can detect the virus before the immune system responds to infection by production of antibodies.

Real-time PCR uses fluorescence signaling for the detection and quantification of targeted PCR products using hydrolysis probes such as TaqMan probe or SYBR Green (specific dye). The most commonly used hydrolysis probe is the TaqMan. The TaqMan probe is a short oligonucleotide that consists of a 5’ reporter and a 3’ quencher (Giulietti et al., 2001). The probe binds to a specific conserved region within the targeted sequence and cleavage of the probe by *Thermus aquaticus* (*Taq*) DNA polymerase during the elongation phase separates the reporter from the quencher resulting in emission of fluorescence, which is directly proportional to the amount of PCR product generated during cycling (Giulietti et al., 2001). The fluorescence is achieved by fluorescence resonance energy transfer (FRET), where energy is

transferred from the reporter to the quencher during the elongation phase (Agarwal et al., 2014). Quantification of the PCR product is then achieved by comparing the cycle threshold (Ct) of the unknown sample to that of the standard of known concentration. Real-time using SYBR Green is achieved by detection of fluorescence of double-stranded DNA. SYBR Green is an intercalating dye that binds specifically to double-stranded DNA (Santhosh et al., 2007). Binding of the dye to double-stranded DNA results in higher emission of fluorescence compared to unbound dye. The amount of fluorescence in the PCR reaction is directly proportional to the amount of double-stranded DNA in the reaction. Post PCR reaction using SYBR Green, melting curve analysis can be used for confirmation of amplification of the correct target as the dye binds to any double-stranded DNA, thus non-specific binding may occur (Smith & Osborn, 2009). Schematic representation of both assays can be seen in figure 1.7 and 1.8, respectively. The main advantage of real-time to conventional PCR is that it can quantify the amount of target DNA within a PCR reaction. The assay is highly specific and sensitive, and it more rapid, and the reaction can be seen in real-time (Mackay et al., 2002). Real-time PCR also does not require post-PCR reactions, including electrophoresis and post-staining.

Alphaviruses are positive-sense RNA virus, hence detection of negative-sense RNA in infected cells suggests virus replication is occurring. This can be achieved by strand-specific quantitative PCR, which allows detection and quantification of negative-strand RNA in infected cells. Quantification is done on the assumption that the cDNA measured during quantification reflects the amount of viral RNA reverse transcribed to cDNA. Previous studies have shown that negative-strand RNA is short-lived, only being detectable in the first few hours post-infection and the positive-sense being detectable later during infection (Sawicki & Sawicki, 1980). Strand-specific PCR has been developed for the detection of different viruses, including CHIKV and ONNV (Plaskon et al., 2009; Wei et al., 2013). Different strategies have been used to ensure accurate quantification and specificity including the use of tag-specific primers, reverse transcribing RNA to cDNA at higher temperatures, and using enzymes with better properties to minimize false priming (Vashist et al., 2012; Lim et al., 2013).

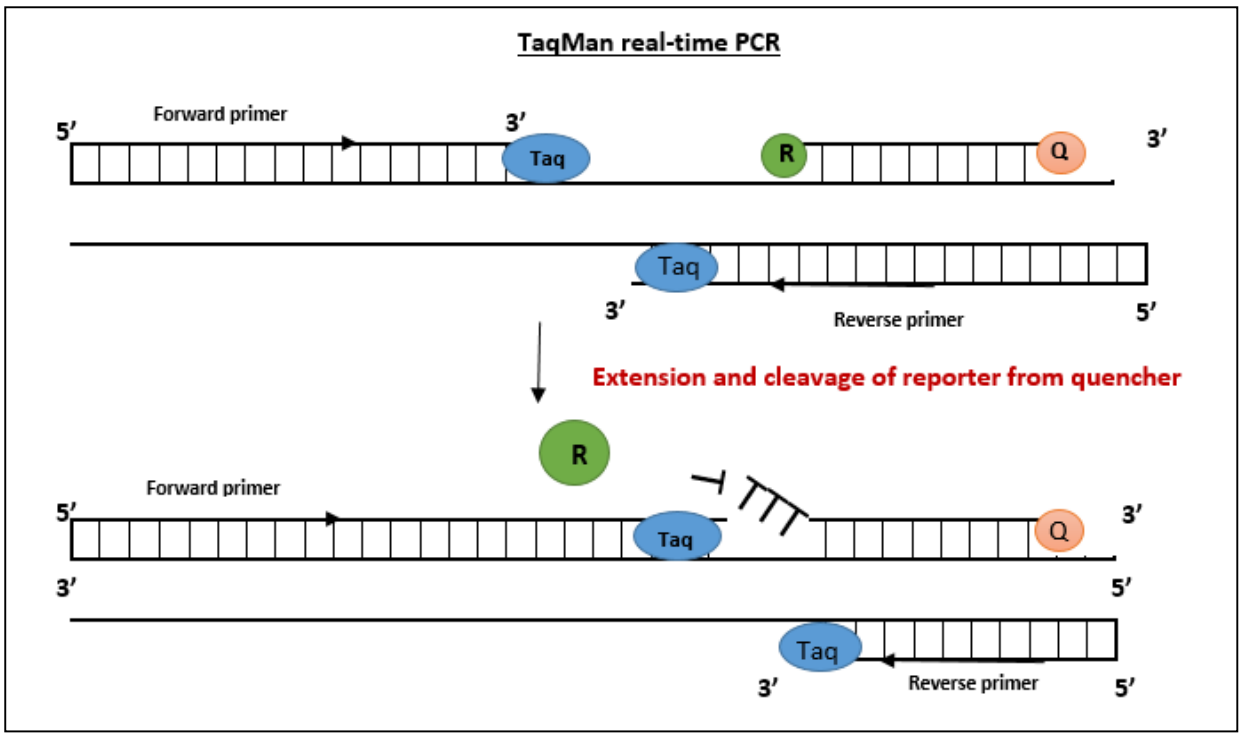


Figure 1.7: TaqMan real-time PCR. During the annealing phase, the probe binds to a conserved region within the target sequence. During elongation Taq polymerase cleaves the probe, separating the reporter (R) from the quencher resulting in fluorescence (Modified from Giulietti et al., 2001)

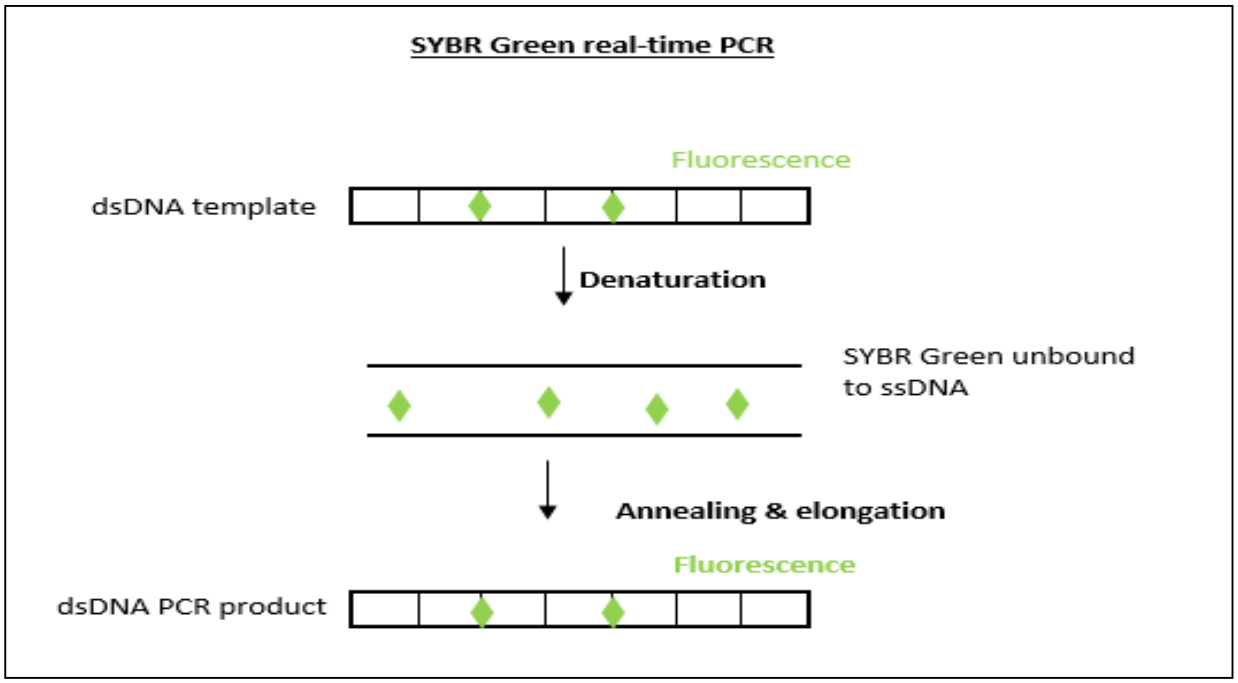


Figure 1.8: SYBR Green real-time PCR. SYBR Green binds to double-stranded (ds) DNA resulting in the emission of fluorescence. The dye does not bind to single-stranded DNA, unbound SYBR Green dye results in little to no fluorescence (Modified from Smith & Osborn, 2009).

Post the period of viremia, serological methods that detect viral antibodies against the virus, which is indicative of host immune response to infection, are used. These

include techniques such as enzyme-linked immunosorbent assay (ELISA), IFA, and haemagglutination inhibition (Barbara et al., 2016). Serological methods are mostly used for SINV diagnosis as the viremic period is short, and by the time the patient begins showing symptoms the virus is no longer detectable. Diagnosis of acute SINV infection is mainly done by detection of immunoglobulin (Ig) M antibodies or IgG seroconversion between paired samples taken two weeks apart showing a fourfold increase in titer using ELISA. Haemagglutination inhibition can also be used for the detection of antibodies. Haemagglutination inhibition relies on the ability of haemagglutinin to bind to erythrocytes to form agglutination. However, because HI cannot discriminate between IgM and IgG, a positive result can be an acute or previous infection. Studies show that IgM antibodies can be detected on the first day after the onset of symptoms (Calisher et al., 1985). Detection of IgM antibody is a marker for primary infection, however, sometimes it may not mean it is an acute infection as some patients still show IgM antibodies six months after initial infection, which may be related to the persistence of symptoms during prolonged joint pain. IgG antibodies can be detected soon after the appearance of IgM and remain detectable for life. Clinical diagnosis of alphaviruses using serology can sometimes be challenging as most alphaviruses present with the same clinical symptoms, the other primary concern is the serological cross-reactivity in regions where more than one alphavirus is endemic (Olivia et al., 2015). Therefore these techniques need to be validated to ensure their sensitivity and specificity are high. Previous studies show that IgM antibodies against SINV can cross-react with WEEV, CHIKV, MAYV, and RRV (Calisher et al., 1986). The most specific serological test is the plaque reduction neutralization test (PRNT) and is the gold standard for antibody detection.

1.8 Treatment, prevention, and control

No antivirals are available for treatment SINV infection. Treatment is symptomatic and supportive. Antihistamines can be used for itching rash, non-salicylate analgesics for joint pain, corticosteroids can be used for persisting joint symptoms. Nonsteroidal anti-inflammatory drugs (NSAID) are used for the treatment of arthritis (Suhrbier and Gasque, 2012).

Vaccines are one of the best strategies for preventing and controlling infections. Human vaccines against arthritogenic alphaviruses are currently not available, but due

to recent outbreaks, there has been an increase in vaccine developments. Several vaccine candidates have been evaluated for use as preventative measures against arthritogenic alphaviruses, especially CHIKV. They include live attenuated, inactivated, DNA vaccines, and subunit vaccines. These vaccines have been tested in animal models, and some are already in clinical trials. Phase I human trials of a formalin-inactivated and UV-inactivated RRV vaccine have been done by Baxter Bioscience (Her et al., 2010). India's Bharat Biotech is developing a virus-like particle CHIKV vaccine, and its testing has proven to be immunogenic in primates (Rana and Lunia, 2015). Recently, CHIKV live-attenuated vaccine was evaluated in nonhuman primates, the vaccine elicited a strong antibody response after a single dose of immunization (Roques et al., 2017). Upon animal challenge, 10 months post-vaccination, antibody levels were still high, preventing CHIKV replication (Roques et al., 2017). Another CHIKV live attenuated vaccine employs a picornavirus internal ribosome entry site (IRES), which also prevents infection in mosquito vectors (Plante et al., 2011). A single dose of the vaccine was shown to be highly immunogenic in mice with an active IFN response and IFN deficient mice (Plante et al., 2011). A phase II safety and immunogenicity study on a plaque-purified live CHIKV vaccine resulted in 98% of the vaccines developing neutralizing antibodies 28 days post-vaccination from which 85% still had detectable antibodies a year after vaccination (Edelman et al., 2014). However, 8% of the vaccinated individuals developed transient arthralgia. CHIKV-enveloped (pEnv) vaccine with nsP2 as an adjuvant was tested in mice models for immune protection. 90% of mice vaccinated with pEnv-nsP2 survived challenge compared to 70% that were vaccinated with pEnv vaccine without an adjuvant (Bao et al., 2013). Mice injected with pEnv-nsP2 vaccine also had delayed disease onset and increased antigen-specific neutralizing antibody response compared to those injected with pEnv alone (Bao et al., 2013). Thus the adjuvant enhances immune response. Phase I clinical trial with virus-like particle (VLP) CHIKV vaccine was performed in healthy adults, the participants were given three doses of either 10, 20 or 40 µg vaccine (Chang et al., 2014). Neutralizing antibodies were detectable after the second dose and significantly increased after the third. The vaccine was found to be safe and immunogenic (Chang et al., 2014).

Other approach to the control of viral epidemics is surveillance, which would allow for early identification of outbreaks. The population should also be advised to avoid

mosquito bites by wearing protective clothing, using insect repellents and mosquito nets with insect repellent (Suhrbier and Gasque, 2012). Elimination of mosquito breeding sites should be a priority, such as artificial sources of standing water in tins, tires, and any other water-holding containers in order to decrease the mosquito population. There should also be mosquito control activities using chemical or biochemical pesticide control and removal of larval habitats. Finally, there should be an increase in public awareness and health education where individuals are educated on arboviruses, especially those at risk on how to use personal protective measures to protect themselves from infection.

1.9 Problem Identification

Alphaviruses are arthritis causing viruses, and in recent years the number of outbreaks of these viruses has dramatically increased. However, the mechanism of how these viruses cause arthritis is poorly understood. Previous studies have associated chronic arthritis with the secretion of inflammatory mediators during infection, but the mechanism by which these viruses cause arthritis is not fully understood. Studies have shown that CHIKV can replicate in monocytes, infected monocytes have been detected in the synovial tissues of chronically CHIKV-infected patients, and these cells may be vehicles for virus dissemination, which may explain the persistence of joint symptoms despite the short duration of viremia. SINV has been shown to replicate in macrophages, these cells are the primary source of pro-inflammatory cytokines during infection. In a previous study performed in our laboratory, we were unable to confirm high levels of replication in macrophages from a volunteer infected *in vitro* with a South African strain of SINV virus, although a small increase in viral load suggested low-level replication had occurred. In the same study, it was shown that viral replication was reduced when HeLa cells were pre-treated with interferon prior to infection with the virus. However, there may be differences in susceptibility to infection, and even with low levels of replication, exposure to the virus may activate specific cytokines. Therefore the aim of the study was to investigate first if there are differences in susceptibility of macrophages isolated from different people and infected *in vitro* with a South African strain of SINV and then to further investigate the innate immune response *in vitro* after infection/exposure to SINV by profiling cytokines secreted and expressed by macrophages.

1.10 Aim and Objectives

1.10.1 Aim:

To investigate the immune response to infection with Sindbis virus.

1.10.2 Objectives:

1. To determine the difference in susceptibility of human macrophages to Sindbis virus infection *in vitro.*
2. To profile the cytokines secreted due to infection and or exposure/activation of macrophages *in vitro.*
3. To determine the role of interferon in Sindbis infected macrophages.

Chapter 2: *In vitro* replication of Sindbis virus in human macrophages

2.1 Introduction

The host's innate immunity plays an important role in viral infection prior to activation of the adaptive immune response. Monocytes, macrophages, DCs, and NK cells are the primary cells in innate immunity and are the targets for viral infection as they can assist in viral dissemination. Studies have identified monocytes and macrophages as the predominant cells found in circulation during infection and are primary targets of SINV and other arthritogenic alphaviruses (Her et al., 2010). Monocytes are precursors of macrophages, they are circulating peripheral blood mononuclear cells (PBMCs) that undergo differentiation and migrate to various tissues where they differentiate and become specialized innate immune cells, essential for recognition and elimination of pathogens. Macrophages also have a pro-inflammatory function, releasing a variety of cytokines in response to infection.

Infection with SINV results in subclinical symptoms, which are usually self-limiting, the symptoms are generally mild and resolve spontaneously, but some individuals develop arthritis. Arthritis in most individuals resolves within a few weeks, but in some cases, it can persist for months to years. Chronic arthritis due to SINV infection is characterized by joint damage affecting the extremities such as hands, ankles, and knees. As only a limited number of SINV infections result in severe chronic arthritis, it is essential to understand the difference in susceptibility between macrophages of different individuals as these cells are one of the main targets for SINV infection.

RA is an inflammatory autoimmune disease associated with chronic joint pain. The role of macrophages in RA is well established, mediating inflammation, and joint destruction in acute and chronic RA (Ma and Pope 2005, Bondeson et al., 2006). Macrophages have been implicated in the pathogenesis of RA, resulting in the secretion of proinflammatory and regulatory cytokines, including different interleukins, TNF-α, and granulocyte-macrophage CSF (GM-CSF) (Kinne et al., 2000). TNF-α and IL-6 are two of the main cytokines playing an essential role in the pathogenesis of RA (Kinne et al., 2000). IL-6 has also been used as a marker of the determination of prolonged arthritis in alphavirus infection and has been associated with the severity of

disease (Ng et al., 2009; Chaaitanya et al., 2011). Previous studies have confirmed the importance of macrophages in the pathogenesis of alphavirus arthritis (Lidbury et al., 2008; Assunco-Miranda et al., 2010). Macrophages are involved in initiating and prolonging inflammation at the site of infection. The mechanism by which infection with SINV and other arthritogenic alphaviruses cause chronic arthritis is poorly understood, however, the mechanism involves macrophages and their ability to regulate inflammation, and these inflammatory mediators have been implicated in the establishment of chronic arthritis.

Previous studies have shown the ability of alphaviruses to infect macrophages, these include a mouse model studies using macrophage depleted and undepleted mice, infection with RRV resulted in reduced signs of disease in macrophage depleted mice compared to mice with undepleted macrophages (Lidbury et al., 2008). *In vitro* studies show that alphaviruses such as CHIKV and SINV are able to replicate in macrophages resulting in the secretion of pro-inflammatory cytokines, which play an important role in the pathogenesis of these viruses (Assunção-miranda et al., 2010). A study involving MAYV in which the authors wanted to determine if MAYV had tropism for macrophages showed that the virus could replicate in different macrophage cell lines including RAW 264.7 and J774, resulting in an increase in viral titers over time. This showed that MAYV and SINV have similar replication profiles with viral titers peaking at about the same time post-infection (Cvalheiro et al., 2016). In another study, CHIKV infected patients in an acute phase of infection presented with high levels of monocyte chemoattractant protein (MCP-1) similar to that measured in joint tissue of CHIKV infected mice, however treatment of mice with MCP-1 inhibitor prior to infection with CHIKV lead to decreased recruitment of macrophages to the site of infection, resulting in reduced inflammation in synovial tissue and skeletal muscle of infected mice, in comparison to untreated mice, therefore resulting in milder disease (Rulli et al., 2011). Macrophages have also been shown to play a prominent role in the persistence of the virus during infection, CHIKV has been found three months post-infection in macrophages of infected macaques, indicating that these cells may be a reservoir of persistent CHIKV infection (Labadie et al., 2010). These studies show that macrophages are one of the targets of alphavirus replication and highlight the importance of macrophages in alphavirus infection and alphavirus induced arthritis.

There are different methods available to detect virus in infected cells, including indirect IFA, which uses antibodies directed against the virus for detection in which fluorescence of the cells would be indicative of a positive infection. Observation of CPE with viruses that cause cytopathic changes within infected cells can also be used as a detection of a positive infection. Conventional and real-time PCR can be used for the detection of a region of the genome to show infection. However, real-time PCR is more advantageous over conventional PCR and other methods as it is more rapid, has higher sensitivity and specificity, reproducibility, and also able to quantify the amount of virus within the infected cells.

SINV is a positive-sense RNA virus, and replication occurs via negative-sense RNA intermediate, which acts as a template for positive-sense genomic RNA. The negative strand is only synthesized during the early stages of infection, thus can be used as a target for determining viral replication. In order to determine whether viral replication has occurred, it is important to quantify the negative-sense RNA of the viral genome, and this can be done using strand-specific PCR targeting the negative-sense RNA. Strand-specific PCR has been developed for the detection and quantification of different alphaviruses (Plaskon et al., 2009; Wei et al., 2013). In this study, strand-specific PCR was used for the detection of negative-sense RNA of SINV, targeting a region of the nsP2 gene.

Some individuals can survive SINV infection without developing chronic arthritis, while others develop severe joint pain lasting for months or years. Because previous studies have shown that SINV and other associated arthritogenic alphaviruses can replicate in macrophages, and other studies have shown that its persistence may result in the secretion of pro-inflammatory cytokines, which are secreted for weeks to months post-infection, it is crucial to study them and understand if there is a difference in susceptibility of macrophages from different individuals (Labadie, et al. 2010; Her et al., 2010; Santiago et al., 2015). This chapter focuses on examining the susceptibility of macrophages from different individuals with no previous exposure to alphavirus infection by performing an *in vitro* study and infecting macrophages with SINV to determine the different viral kinetics between macrophages of different individuals, in terms of infectivity and viral replication, using a two-step qRT-PCR. A qRT-PCR that targets negative-strand RNA was used to determine if the virus was replicating in the

macrophages. As a positive-strand RNA virus, an intermediate negative-strand RNA is produced during replication. Hence the reverse transcription step is performed using the forward sense primer only to transcribe negative-sense cDNA and the RT-PCR assay performed in two steps.

2.2 Methods and materials

2.2.1 Preparation of viral stocks and determination of $TCID_{50}$

2.2.1.1 Preparation of virus stocks

A stock of SINV S.A.AR86 was prepared by infecting African green monkey kidney epithelial cells, Vero 76 (ATCC® CCL-81 TM), and harvesting the supernatant. The virus was kindly donated by Professor Paweska from the National Institute of Communicable Diseases (NICD). Briefly, Vero cells were seeded at an optimal density of $9.6x10^5$ in a T25 vented cell culture flask in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Life Technologies, California, USA), 0.1mg/ml penicillin/ streptomycin (pen/strep) (Lonza, Basel, Switzerland) and 2mM L-glutamine (L-glut) (Life Technologies, California, USA). When they reached 80-90% confluency, culture media was carefully aspirated from the flask. A volume of 5 µl of SINV was added to the cells in DMEM supplemented with 2% FBS, 0.1mg/ml pen/strep, and 2mM L-glut maintenance media. Infected cells were incubated at 37˚C in a humidified CO_2 incubator for 48 hours and monitored for CPE. SINV S.A.AR86 virus stock from infected cells was harvested at the first sign of CPE, aliquoted, and stored at -80°C, SINV S.A.AR86 virus stock was later used for infection of macrophages.

2.2.1.2 Indirect immunofluorescence assay (IFA)

IFA was performed on SINV infected Vero cells for confirmation of infection with SINV prior to the determination of tissue culture infectious dose ($TCID_{50}$). Briefly, Vero cells infected with SINV were scraped off the T25 flask and fixed onto a microscope slide with acetone at -20 °C for 20 minutes. The cells were blocked using a blocking buffer consisting of 10% sucrose, 0.5% Triton X-100 and phosphate-buffered saline (PBS) (pH 7.4) and incubated for 20 minutes at room temperature. Following blocking, cells were incubated with anti-SINV immunoglobulin G (IgG) from a convalescent patient, as primary antibody diluted 1:100 in blocking buffer, at 37 °C for 30 minutes. For removal unbound antibodies, the slide was washed three times with PBS containing 1% Tween 20 (1% PBST). After washing, the cells were incubated with secondary antibody, goat anti-human IgG conjugated to fluorescein isothiocyanate (FITC) (KPL,

Missouri, USA) diluted 1:20 in Evans blue (Sigma-Aldrich, USA), at 37 °C for 30 minutes. Post-incubation, the slides were washed three times with 1% PBST for 60 seconds with shaking. The slides were air-dried and mounted using glycerol mounting media (1 part PBS and 9 parts glycerol, pH 8.5) and a coverslip. The slides were examined under a fluorescent microscope (Nikon Eclipse N*i*) for fluorescence of infected cells.

2.2.1.3 Calculating the tissue culture infectious dose

$TCID_{50}$ is a method used to measure virus titer, the method measures the amount of virus needed to produce 50% CPE in infected cells. The method is used to determine how much infectious virus is in the stock preparation. The $TCID_{50}$ was calculated by performing a tenfold serial dilution of the virus stock, on a confluent layer of Vero cells in flat-bottomed 96 well plates, from 10^{-1} to 10^{-7}, in replicates of six. Briefly, a 96 well-plate was seeded with Vero cells at a seeding density of $8X10^5$ cells/well. The cells were infected with SINV S.A.AR86 stock diluted 1:10 in maintenance media, DMEM supplemented with 2% FBS, 1% L-glut and 1% pen/strep, and monitored for CPE over a period of three days. The $TCID_{50}$ was calculated using the Reed and Muench method (Ramakrishnan, 2016) as follows:

$$-\text{Log of endpoint titer} = -\left(\frac{sum\ of\ \%\ of\ each\ dilution}{100} - 0.5\right) * \log of\ dilution$$

2.2.2 Preparation of standard curve

A region of the nsP2 was amplified from viral RNA extracted from SINV infected Vero cells, and the RT-PCR product (DNA) was used to construct a standard curve for quantification of viral RNA titers of SINV infected macrophages using a quantitative real-time assay.

2.2.2.1 RNA extraction

Viral RNA was extracted from the supernatant of Vero cells infected with SINV using QIAamp viral RNA mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Briefly, a 140 µl aliquot of SINV virus stock was added to 560 µl AVL buffer (supplied in the kit) containing 1% carrier RNA (supplied in the kit) and incubated at room temperature for 10 minutes to allows lysis of the cells and

inactivation of RNases, ensuring isolation of intact viral RNA. A 560 µl aliquot of absolute ethanol was added to the sample post-incubation and mixed by pulse-vortex. A 630 µl aliquot of the solution was applied to the QIAamp mini-column (supplied in the kit) and centrifuged at 6000 g for 1 minute, this step was repeated with the remaining solution. The column was washed with 500 µl of AW1 buffer (supplied in the kit) at 6000 g for 1 minute and 500 µl of AW2 buffer at 12000 g for 3 minutes, respectively. For removal of residual AW2 buffer, the column was centrifuged at 12000 g for 1 minute, and the RNA was eluted using 60 µl of AVE buffer at 6000 g for 1 minute. RNA was stored at -80 °C until used.

2.2.2.2 Synthesis of complementary deoxyribonucleic acid (cDNA)

Synthesis of cDNA was performed using SuperScript III reverse transcriptase (Invitrogen, California, USA) in a final volume of 20 µl. The reaction consisting of 1 µl nsP2F forward primer (2pmol), sequence of the primer can be seen in Table 1.1, 1 µl dNTP (10mM), 1 µl RNA template and 10 µl nuclease-free water (NFW), the reaction was incubated at 65°C for 5 minutes in a pre-heated thermocycler (Proflex PCR system, Applied biosystem, New York, USA) followed by incubation at 4°C for 2 minute. At 1 minute post-incubation at 4°C the thermocycler was paused and 4 µl 5X SuperScript III RT buffer, 1 µl 100 mM DTT, 0.5 µl RNase inhibitor, 1 µl SuperScript III Reverse Transcriptase (200U/ µl) and 0.5 µl NFW were added. The reaction mixture was incubated at 50°C for 60 minutes and finally at 85°C for 5 minutes.

2.2.2.3 Conventional PCR

A conventional PCR using GoTaq G2 Hot Start polymerase (Promega, Madison, USA) was performed to amplify SINV cDNA using primers targeting the 180bp region of nsP2, sequences of the primers used can be seen in Table 2.1. PCR reaction was prepared as shown in Table 2.2 and carried out using the Proflex PCR system with the following cycling conditions: initial denaturation at 95 °C for two minutes for one cycle, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 48 °C for 30 seconds and elongation at 72 °C for one minute, final extension was at 72 °C for five minutes for one cycle, and the samples were held at 4 °C indefinitely. The amplified PCR product was separated using gel electrophoresis, the band of interest was excised from the gel and purified using Wizard SV Gel PCR kit (Promega,

Wisconsin, USA) according to the manufacturer's instructions and sequenced to determine the nucleotide sequence. The concentration of the PCR product was measured using Qubit fluorometer (Invitrogen, California, USA) and was used to generate a standard curve for qRT-PCR.

Table 2.1: Primer pair targeting the nsP2 region of SINV

Name	Position (5'-3')	Primer sequence (5' to 3' direction)	T_m (^{0}C)
nsP2F	65-86	5'-GACAGTATATCGTTGTCTCGCC-3'	50
nsP2R	245-225	5'-GAATTCTGGCCATGGTACGGC-3'	51

The sequences are relative to the nsP2 region of SINV S.A.AR86. The nsP2F is the forward primer and the nsP2R, the reverse primer.

Table 2.2: Reagents for conventional GoTaq™ DNA PCR

Reaction components	Reaction volume (µl)	Final concentration
5X Green GoTaq reaction buffer (Promega, Wisconsin, USA)	10	1X
PCR nucleotide mix (10 mM)	1	2 mM each dNTP
nsP2F (20 pmol)	1	0.4 µM
nsP2R (20 pmol)	1	0.4 µM
GoTaq G2 Hot Start polymerase (5U/µl)	0.25	1.2 5U
SINV template cDNA	5	-
NFW	27.75	-
$MgCl_2$ (25mM)	4	2 mM
Total reaction	50	

2.2.2.4 PCR product confirmation and purification

Electrophoresis of a 20 µl aliquot of the PCR product was performed using a 2.5% agarose gel prepared in 1 X Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) buffer (pH 8). Lonza SimplyLoad™ DNA ladder (Lonza, Basel, Switzerland), compromising of fragments between 20 bp to 500 bp, was used to predict the expected fragment length of the PCR amplicon. Gel electrophoresis was performed using the BioRad PowerPac Basic system (BioRad, California, USA) at 100V for 50 minutes. The gel

was post-stained using 0.02% GelRed nucleic acid gel stain (Biotium, California, USA) for 45 minutes with gentle shaking and visualized using GelDoc™ under UV transilluminator (BioRad, California, USA).

The PCR amplicon was purified with Wizard® SV Gel PCR Clean-Up System (Promega, Wisconsin, USA) according to the manufacturer's instructions. The purification is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts, allowing removal of excess nucleotides and primers. Briefly, following gel electrophoresis equal volume of membrane binding solution (Supplied in the kit) was added to the excised band, vortexed, and incubated at 55 °C until the gel was fully dissolved. Post-incubation dissolved gel was transferred to the SV minicolumn (supplied in the kit) and centrifuged (Labnet International, Edison, USA) at 14000 g for 1 minute. The PCR amplicon was washed twice using 700 µl and 500 µl of membrane wash solution (Supplied in the kit) at 14000 g for 1 minute and 5 minutes respectively to remove excess nucleotides. Purified DNA was eluted with 30 µl of NFW. The concentration of the DNA was measured using Qubit fluorometer (Invitrogen, California, USA). The purified PCR product was serially diluted by tenfold from neat to 10^{-7} to construct a standard curve. The standard curve was used to quantify viral loads from SINV infected macrophages.

2.2.2.5 Sequencing

Sanger sequencing was performed to determine the nucleotide sequence of the amplicon. Sequencing was performed using Big Dye™ Terminator Sequencing Ready Reaction kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The sequencing reaction components can be seen in Table 2.3. The reactions were cycled using Proflex PCR system thermocycler with the following conditions: initial denaturation at 96 °C for 1 minute for 1 cycle, followed by 30 cycles of denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds and extension at 60 °C for 4 minutes. Finally, the samples were held at 4 °C indefinitely. The nsP2F and nsP2R primers, flanking the gene of interest, were used for the sequencing reactions. The primers were designed in a previous study in our laboratory, and their sequences can be seen in Table 2.1.

Table 2.3: Sequencing reaction components

Reaction components	Reaction volume (µl)
Terminator Ready Reaction	2
Sequencing primers: nsP2F/nsP2R (0.8 pmol/µl)	4
Sequencing buffer	2
DNA template	0.5
NFW	1.5
Total reaction	10

For post-reaction clean-up, EDTA/ethanol precipitation was performed. Briefly, the sequencing reaction volume was adjusted to 20 µl with NFW. The 20 µl mixture was transferred to a tube containing 5 µl 125mM EDTA and 60 µl absolute ethanol. The tubes were vortexed for 5 seconds to mix. The reactions were allowed to precipitate at room temperature for 15 minutes. Following precipitation, the samples were centrifuged (Hermle Labortechnik, Wehingen, Germany) at 14 000 x g for 2 minutes at 4 °C and the supernatant was aspirated. A 500 µl aliquot of 70% ethanol was added to the tubes and centrifuged at 14 000 x g for 10 minutes at 4 °C, and the supernatant was aspirated. The pellet was dried by incubating at 37 °C for 2 hours. The samples were submitted to the Department of Microbiology, Biochemistry and Food Biotechnology, Faculty of Natural and Agricultural Sciences, University of the Free State, Bloemfontein, South Africa for electrophoresis.

2.2.3 Isolation of peripheral blood mononuclear cells (PBMCs) and their differentiation to macrophages

2.2.3.1 Ethics approval

Ten SINV antibody naïve individuals were enrolled in the study, and for each participant, a total volume of 20 ml of blood was drawn, and PBMCs were isolated. The study was approved by the University of the Free State Health Science Research Ethics Committee (Appendix A), and all participants signed an informed consent document. The study was also approved by the Biosafety and Environmental Research Ethics Committee (UFS-ESD2018/0002) of the University of the Free State to work with SINV S.A.AR86 in biosafety level 2 (BSL 2) laboratory and a Section 20

permit for research obtained from the Department of Agriculture, Forestry and Fisheries. These can be found in Appendices B and C, respectively.

2.2.3.2 Isolation of PBMCs

A total volume of 20 ml of blood was collected in ACD-A 8.5 ml tubes (BD, California, USA) from designated participants 1 to 10 enrolled in the study. PBMCs were isolated from whole blood using Ficoll-Paque^{TM} PLUS density gradient media (GE Healthcare, Illinois, USA) according to the manufacturer's instructions. Briefly, the total volume of blood was diluted with Roswell Park Memorial Institute (RPMI) 1640 growth media (Lonza, Basel, Switzerland) supplemented with 10% FBS, penicillin 100u/ml, streptomycin 0.1mg/ml (pen/strep) and 2mM L-glut to a final volume of 50 ml. A 25 ml aliquot of the diluted blood was layered onto 20 ml of Ficoll-Paque media solution and separated by low-speed centrifugation at 1800 g for 20 minutes at zero deceleration. Differential migration of cells during centrifugation resulted in the formation of layers containing different cell types. The buffy coat was separated from the media and washed twice with RPMI media at 1200 g for 10 minutes and at 1000 g for 8 minutes respectively. After washing, the supernatant was removed, and the pellet was resuspended in 5 ml of RPMI media.

A 10 µl aliquot of the cell suspension was diluted with 10 µl of 0.4% trypan blue (Thermo Fisher, Massachusetts, USA). A 10 µl aliquot of cell/trypan blue suspension was added to a counting chamber, and cells were counted using Countess II FL (Invitrogen, California, USA). Trypan blue stains dead cells blue because the cell wall is no longer intact, in comparison to the live cells which are not stained. The cell counter determines the number of live cells, which is used to calculate the total number of cells needed to be seeded for infection work. The cells were seeded at the seeding rate of 8x10^5 cells/well in a 24 well plate for differentiation. The seeding rate was calculated with the following formula: $\text{seeding rate } (\mu l) = \frac{8 \text{ x } 10{*}5 \text{ cells}/cm^2}{\text{number of live cells (ml)}} \text{ x } 1000$

2.2.3.3 *In vitro* differentiation of monocytes to macrophages

Monocytes were differentiated using different culture conditions to determine optimal conditions for macrophage differentiation. The cells were seeded at an optimal seeding rate of 8x10^5 cells/well in a 24 well plate. The macrophage population was cultured for six days at 37 ˚C in a humidified CO_2 incubator in differential media

supplemented with 25 µg of human macrophage colony-stimulating factor (HM-CSF) (Life Technologies, California, USA). HM-CSF is a cytokine that controls the production, differentiation, and function of macrophages allowing differentiation of monocytes over several days. HM-CSF supplemented media was prepared by adding 25 µg HM-CSF in RPMI media with 10% FBS and 1% pen/strep. Different concentrations of HM-CSF was used to determine the optimal concentration, including 10 µg, 25 µg, and 50 µg. Using 10 µg of HM-CSF resulted in a lower percentage of cells differentiating whereas 25 µg, and 50 µg resulted in an equal percentage of differentiation of about 70 to 75 percent, therefore 25 µg was used. Macrophage morphology was analyzed under a light microscope following differentiation under the different culture conditions.

2.2.4 Infection of macrophages with SINV S.A.AR86 and determination of viral load by two-step qRT-PCR

Macrophages were infected with SINV S.A.AR86, and viral loads were determined from a standard curve generated using serial dilutions of SINV DNA. The real-time qRT-PCR assay was used to amplify the intermediate negative-strand of SINV RNA. The negative strand is usually evident 2 to 4 hours after infection, with the positive-strand RNA level increasing later in replication. Because SINV is a positive-sense RNA virus, it produces an intermediate negative-strand RNA when it replicates (Sawicki & Sawicki, 1980). Therefore detection of negative-strand viral RNA during infection is indicative of SINV replication (Plaskon et al., 2009). The forward primer was used to reverse transcribe negative-sense cDNA. Superscript III was used for the synthesis of cDNA, the assay functions at high temperatures, which minimizes false priming of the opposite strand, thus increasing the specificity of the reverse transcription assay. After reverse transcription, the cDNA was used as a template for qPCR for quantification of viral load.

2.2.4.1 *In vitro* infection of macrophages with SINV

Macrophages were infected *in vitro* with SINV S.A.AR86 virus stock at a multiplicity of infection (MOI) of 0.1. MOI refers to the number of infectious viral particles used to infect a single cell. The MOI is related to plaque-forming units (PFU) and was calculated using the following formula: MOI= PFU of SINV/ number of cells seeded

per well. PBMCs were seeded at a seeding rate of 8 x10^5/well in a 24 well plate and incubated at 37°C in a humidified 5% CO_2 incubator for six days and allowed to differentiate into macrophages before infection. Briefly, post-incubation, RPMI media was removed from the cells, and SINV virus stock was added to the cells and incubated for 60 minutes at 37°C in a humidified CO_2 incubator, to allow absorption of the virus into cells. Post incubation, the virus stock was removed, and macrophage cells were washed twice with sterile PBS (Life Technologies, California, USA), and 2 ml fresh maintenance (RPMI) media was added to the cells. SINV replication in human macrophages was assessed by titration of infectious viral particles in culture supernatants collected at different times post-infection from baseline (time = 0), 6, 12, and 24 hours by using two-step qRT-PCR

2.2.4.2 Viral RNA extraction and quantification by two-step qRT-PCR

Briefly, RNA was extracted from culture supernatant using QIAamp viral RNA mini kit according to the method described in section 2.2.2.1 and used as a template for cDNA synthesis. RNA was reverse transcribed to cDNA using Superscript III reverse transcriptase according to the method described in section 2.2.2.2.

To determine if the virus was replicating in infected macrophages from the different participants and to measure the viral load, cDNA was used as a template for quantitative qPCR with virus primers nsP2F and nsP2R together with the Taqman probe. Sequences for both primers and the probe can be seen in Table 2.1 and 2.4 respectively. The cDNA was quantified using Lightcycler TaqMan master mix, and viral loads were determined from a standard curve generated using tenfold serial dilutions of DNA prepared from Vero cells infected with SINV. PCR was carried out using Lightcycler 2.0 thermocycler with software version 4.1 (Roche, Basel, Switzerland). The master mix was prepared, as shown in Table 2.5, the reaction was run according to the cycling conditions shown in Table 2.6.

Table 2.4: Real-time PCR probe targeting the nsP2 region of Sindbis virus

Name	Position (5'-3')	Probe sequence (5' to 3' direction)
TaqMan probe	148 – 174	5'-ATCATAACGCACTCCGGAAGATCAGGA-3'

The sequence of the probe is relative to the nsP2 region of SINV S.A.AR86

Table 2.5: Reagents for the amplification cDNA in macrophages infected with SINV using LightCycler® 480 Probes Master PCR kit (Roche)

Reagents	**Volume (µl)**
nsP2 probe (2 pmol/µl)	1
nsP2F (10 pmol/µl)	2
nsP2R (10 pmol/µl)	2
Master mix	4
cDNA template	1
NFW	10
Total reaction	**20**

Table 2.6: Real-time PCR cycling conditions for SINV cDNA amplification

Programs				
Program name	**Cycles**		**Analysis Mode**	
Initial Denaturation	1		None	
Amplification	45		Quantification	
Cooling	1		None	
Temperature targets				
	Target [^{0}C]	**Hold [hh:mm:ss]**	**Ramp Rate [^{0}C/s]**	**Acquisition mode [per ^{0}C]**
Initial Denaturation	95	00:10:00	20	None
Amplification	95	00:00:10	20	None
	50	00:00:30	20	None
	72	00:00:01	20	Single
Cooling	40	00:00:30	20	None

2.3 Results

2.3.1 Confirmation of infection of Vero cells with SINV by IFA

After a virus has been propagated, it is important to determine the virus infectivity titer. This can be done *in vitro* using increasing dilutions of the virus to inoculate a susceptible cell line in which the virus is known to produce CPE. Vero cells are highly susceptible to SINV and were used for the preparation of virus stocks and to determine the virus titer. Vero cells were infected with SINV S.A.AR86 and analyzed 48 hours post-infection by indirect IFA. The cells were incubated with human anti-SINV IgG primary antibody, and fluorescence was detected using FITC-conjugated goat anti-human IgG secondary antibody. Figure 2.1 below shows the fluorescence of Vero cells, which confirms infection and replication of the virus in Vero cells.

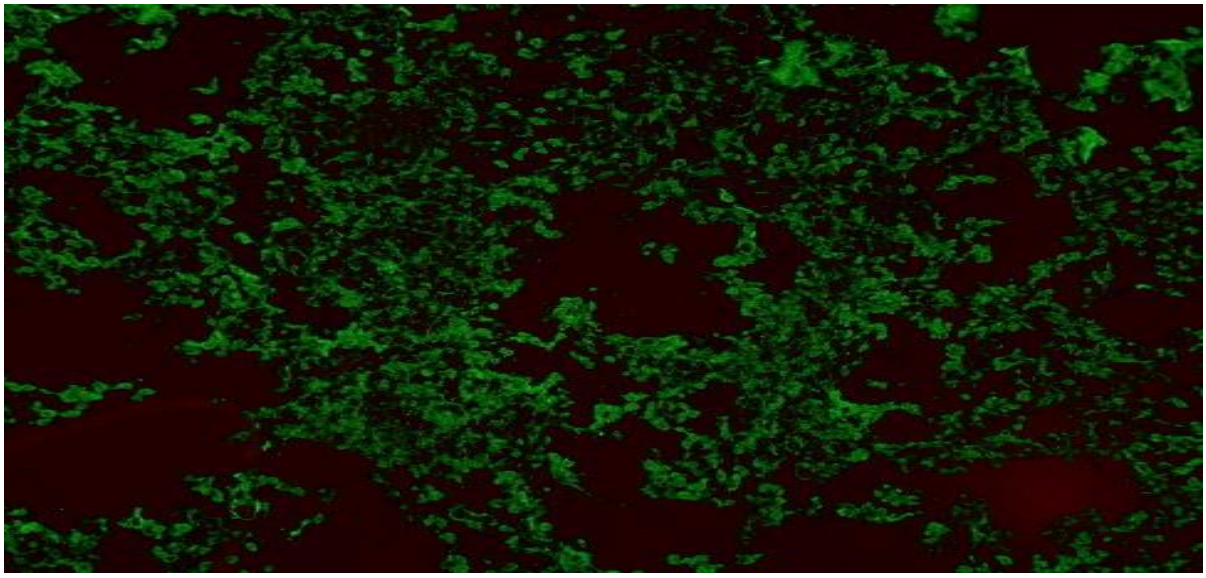


Figure 2.1: Immunofluorescent staining with anti-SINV antibody. Vero cells were infected with Sindbis virus S.A.AR86 for preparation of virus stocks and analyzed 48 hours post-infection by indirect immunofluorescence assay for confirmation of infection. The cells were incubated with anti-SINV IgG primary antibody, and fluorescence was detected using FITC-conjugated goat anti-human IgG secondary antibody. Fluorescence confirms infection and replication of the virus in the cells. Cells were viewed under Nikon eclipse N_i (3F Scientic, Centurion, South Africa) at 10X magnification.

2.3.2 Calculation of SINV $TCID_{50}$ in Vero cells using the Reed and Muench method

The $TCID_{50}$ was calculated for virus stock prepared using Vero cells. The technique is based on determining the highest dilution of virus that produces CPE in 50% of

infected cell culture. The virus stock was diluted tenfold, and Vero cells were infected with each dilution, in replicates of six. The cells were monitored daily for the appearance of CPE. The $TCID_{50}$ was calculated as $10^{5.7}$ $TCID_{50}$/ml, and the calculation can be seen in section 2.3.2.1 below. Table 2.7 shows the percentage of CPE observed on day three post-infection.

Table 2.7: Calculation of virus titer in Vero cells using the Reed and Muench method

Virus dilution	CPE Ratio[1]	CPE		Cumulative total			
		>50%	<50%	>50%	<50%	Ratio[2]	%
10^{-1}	6/6	6	0	31	0	31/31	100
10^{-2}	6/6	6	0	25	0	25/25	100
10^{-3}	6/6	6	0	19	0	19/19	100
10^{-4}	6/6	6	0	13	0	13/13	100
10^{-5}	6/6	6	0	7	0	7/7	100
10^{-6}	1/6	1	5	1	5	1/6	16.7
10^{-7}	0/6	0	6	0	11	0/11	0

CPE- Cytopathic effects; >50%- CPE above 50%; <50%- CPE below 50%

CPE ratio[1]: Ratio of inoculated wells showing >50% CPE of the total inoculated wells

Cumulative ratio[2]: The cumulative ratio represents the accumulated number of wells with more than 50% CPE over the cumulated total number of inoculated wells.

2.3.2.1 TCID50 calculation

$$-\text{Log of endpoint titer} = -\left(\frac{sum\ of\ \%\ of\ each\ dilution}{100} - 0.5\right) * \log of\ dilution$$

$$= -1 - [\left(\frac{100 + 100 + 100 + 100 + 100 + 16.7}{100} - 0.5\right) * \log 10]$$

$$= \text{-5.66}$$

$$TCID_{50} = 10^{5.7}$$

2.3.3 Generation of a standard curve for determination of viral loads

A standard curve was constructed using DNA amplified from RNA extracted from SINV infected Vero cells. The PCR amplicon was separated using a 2.5% agarose gel, and the PCR product of approximately 180bp according to the molecular weight marker, which is consistent with the expected size, is shown in figure 2.2. The nucleotide sequence of the amplicon was determined using Sanger sequencing. Nucleotide

sequence data was edited using ChromasPro Version 2.1.8 (www.technelysium.com.au/ChromasPro/html). SINV S.A.AR68 was confirmed by comparison with nucleotide sequence data retrieved from GenBank by Basic Local Alignment Search Tool (BLAST) analysis. Sequence data showed identity to SINV S.A.AR86.

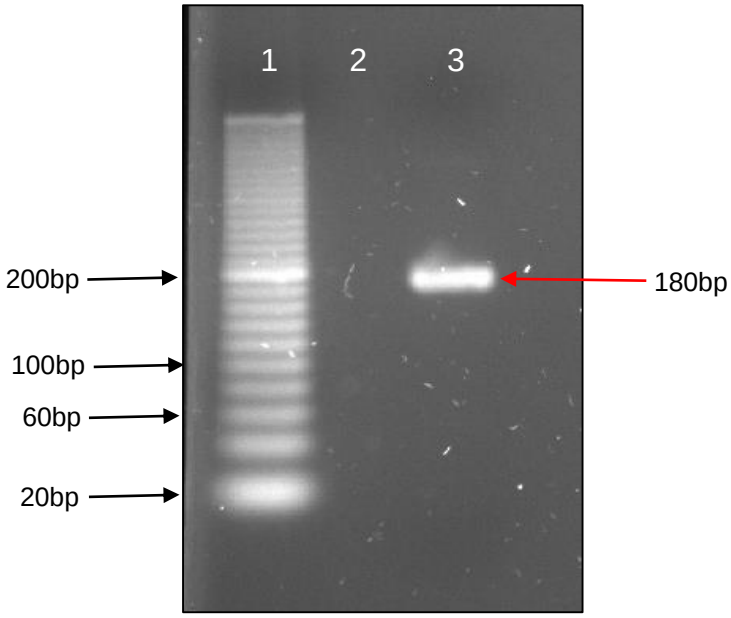


Figure 2.2: Agarose gel electrophoresis analysis of SINV nsP2 on a 2.5% agarose gel. Lane 1: Lonza SimplyLoad™ 20bp DNA ladder. Lane 2: Negative control. Lane 3: nsP2 180 bp.

A standard curve was constructed using RT-PCR product (DNA) amplified from the RNA template extracted from SINV infected Vero cells. The number of copies of DNA was determined, and the DNA diluted tenfold and used as a template for qPCR to construct a standard curve. The standard curve was used as a reference to determine the viral load, represented as copy number of cDNA reverse transcribed from RNA, at each time point from infected human macrophages.

To prepare the standard curve, the concentration of DNA was determined to be 21ng/µl using the Qubit (Invitrogen, California, USA) and the viral copy number was determined using copy number calculator (https://cels.uri.edu), the calculation requires the amount of template (ng) and the length of the template (bp) and uses the following formula: number of copies= (amount *6.022x10^{23}) / (length * 1x10^{9} * 650). A 1 µl aliquot DNA was used to determine the copy number. The tenfold serial dilutions

of DNA was used in constructing the standard curve, which ranged from $1x10^{-1}$ to $1x10^{-7}$, equivalent to $1.07x10^{11}$ to $1.07x10^{5}$ copies of DNA/µl.

The Cp values were plotted against the log concentration of the DNA, and a linear correlation was determined. The amplification curves are shown in figure 2.3, and the standard curve is shown in figure 2.4. The qPCR lightcycler 2.0 instrument also generated the error and efficiency rate of the standard curve, which were determined to be 0.00350 and 2.000, respectively. The error value reflects how well all data fit the regression line and the efficiency value, for which the highest amplification efficiency is 2, which means the number of targeted DNA doubled with each cycle.

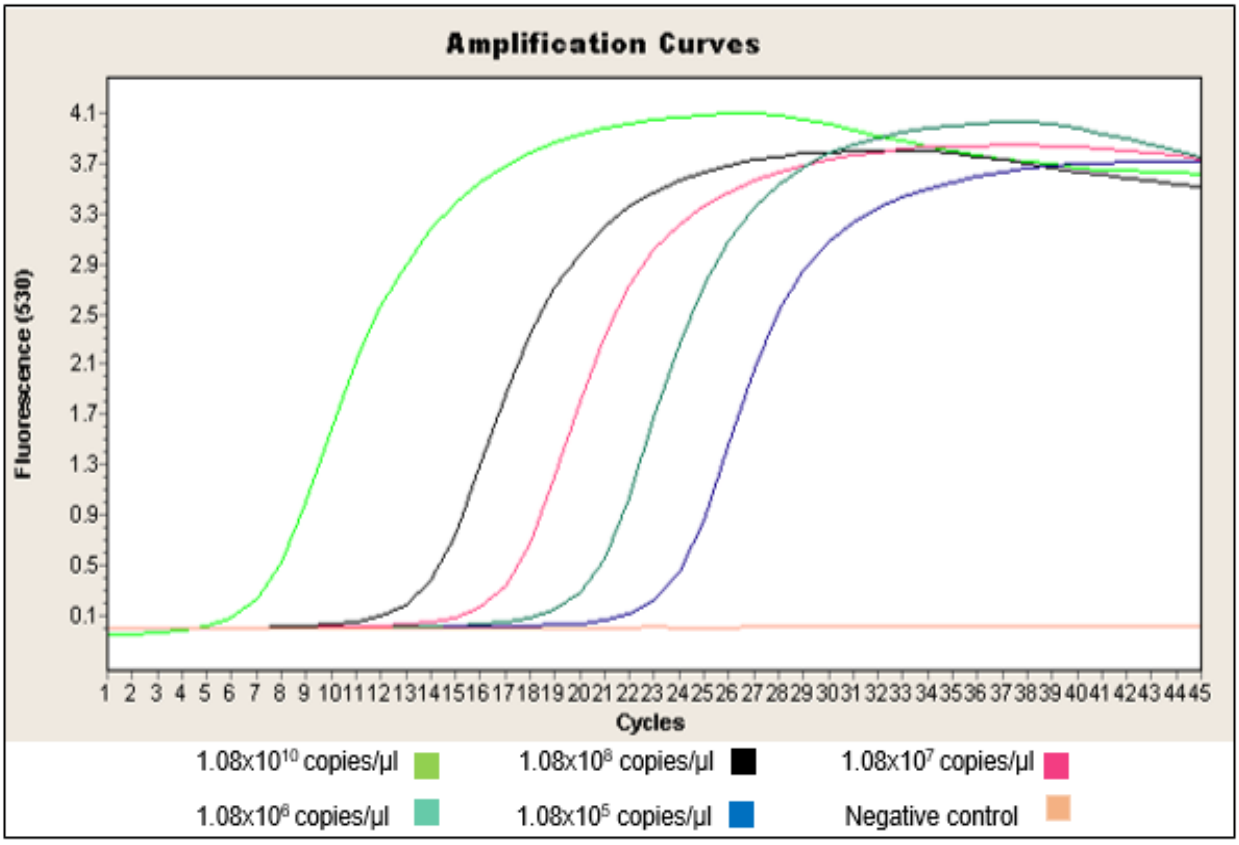


Figure 2.3: qPCR for SINV. SINV DNA was diluted tenfold from $1x10^{-1}$ to $1x10^{-7}$ ($1.08 x10^{11}$ to $1.08 x10^{5}$ copies/µl), and the dilutions were used to construct a standard curve. Negative control was included in the run. The graph shows the number of PCR cycles plotted against increasing fluorescence. The lightcycler software used 5/7 resulting curves shown to construct a standard curve.

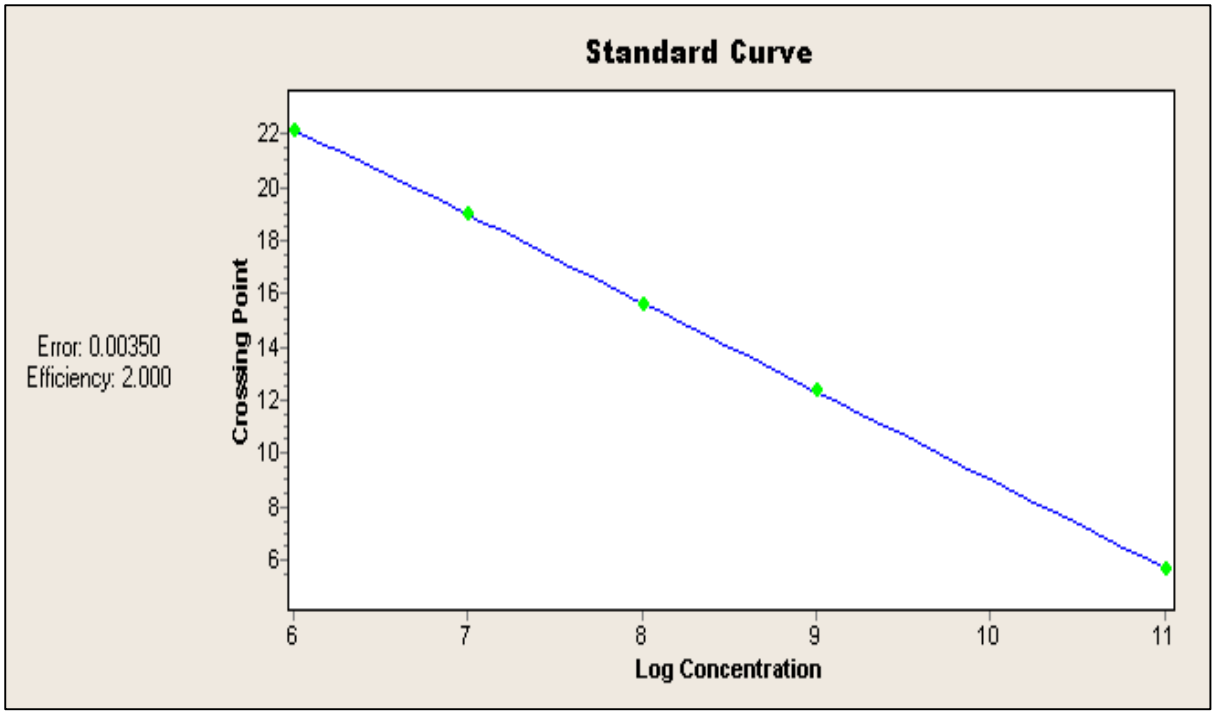


Figure 2.4: Standard curve for determination of viral loads in SINV infected macrophages. A standard curve was constructed using a tenfold serial dilution of PCR product obtained from cDNA reverse transcribed from RNA extracted from SINV supernatant of infected Vero cells, the dilutions ranged from $1x10^{-1}$ to $1x10^{-7}$ equivalent to $1.08x10^{11}$ to $1.08x10^{5}$ copy number/µl, respectively. Diluted samples were amplified by qPCR, and the crossing point (C_P) value was plotted against log concentration. The lightcycler software used 5/7 resulting curves shown to construct a standard curve.

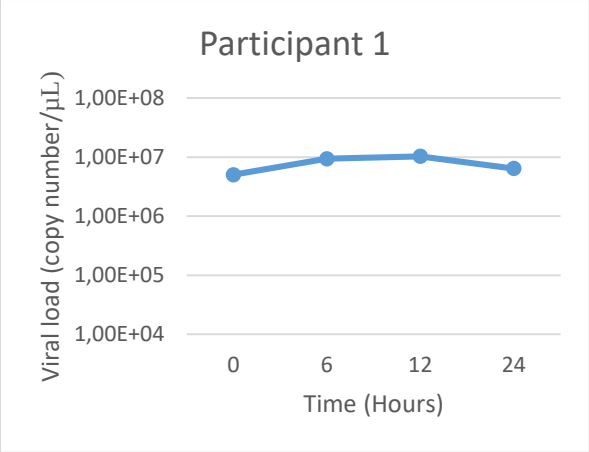
2.3.4 *In vitro* infection of human macrophages with Sindbis virus

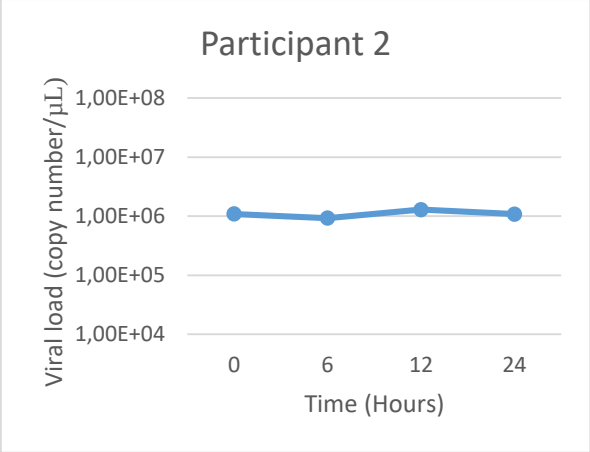
Previous studies have identified macrophages as one of the targets for viral replication during an alphavirus infection (Lidbury and Mahalingam, 2000; Assunco-Miranda et al., 2010). For this objective, ten participants with no previous exposure to SINV were recruited for the study. To evaluate if there was a difference in susceptibility to SINV infection of macrophages from different individuals, monocyte-derived macrophages were prepared from PBMCs of ten healthy participants. Macrophages from the participants were infected with SINV S.A.AR86 at an MOI of 0.1. Cell infectivity and viral replication were assessed by two-step qRT-PCR, measuring the viral load at different times post-infection for 24 hours, using a primer pair targeting a region of the SINV nsP2 gene. Each run included a standard of known concentration and a negative control to allow the determination of viral load and confirmation of no contamination, respectively. The viral loads determined are shown in Appendix D with final results represented as mean ±standard deviation. The qRT-PCR assay was used to amplify the intermediate negative strand of the viral RNA. The negative strand is

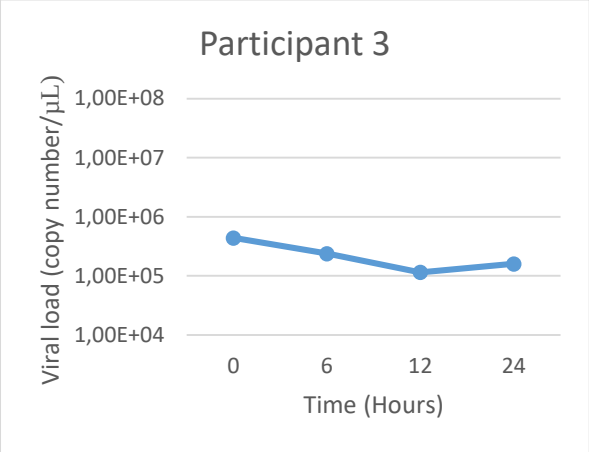
usually evident 4 to 6 hours after infection, with the positive-strand RNA level increasing later during the replication cycle. The qPCR reactions were performed in triplicate for each participant, and the mean viral load was plotted against time after inoculation of the cells. The viral loads determined at different intervals from time 0 to 24 hours after infection for each of the ten participants can be seen in figure 2.5 below.

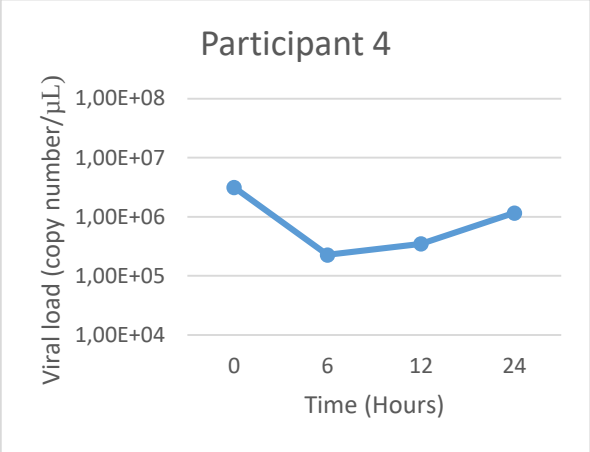
As expected, SINV was detected in macrophages of all participants at time 0 post-infection. However, an increase in viral load was only observed in macrophages from 5/10 participants (designated participants 1, 2, 6, 8, and 9) with viral load increases of between twofold to twenty-six fold. The viral load increased and peaked at either 12 or 24 hours post-infection. At 24 hours post-infection, the viral load appeared to decline in macrophages of most participants. This decline in viral load may be due to the increased death of macrophages. During the 24 hours of infection, the viral load determined for infected macrophages varied for different participants ranging from twofold for participant 1, fourfold for participant 9, fivefold for participant 2, and sixfold for participant 6. A twenty-eightfold increase was observed for participant 8. Figure 2.5 shows the viral load for each participant at each time interval after infection. The results are shown as a mean for three repeats at each time point. Biological replicates were not possible due to a limited number of cells available for *in vitro* infection experiments, therefore for each time interval, the qRT-PCR was repeated thrice per RNA extraction.

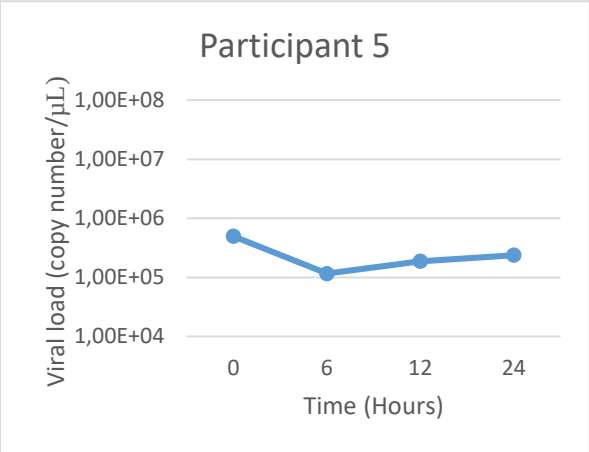
Little to no viral replication in macrophages from the five participants, 3, 4, 5, 7, and 10, was observed, with viral loads decreasing from baseline at subsequent time points. The decrease in viral loads differed between macrophages of participants that demonstrated little to no virus replication. The was a +/- fourfold decrease in macrophages of participant 3, a thirteen-fold decrease in macrophages of participant 4, fourfold decrease in macrophages of participant 5, sevenfold in macrophages of participant 7 and the highest decrease of seventeen-fold in macrophages of participant 10.

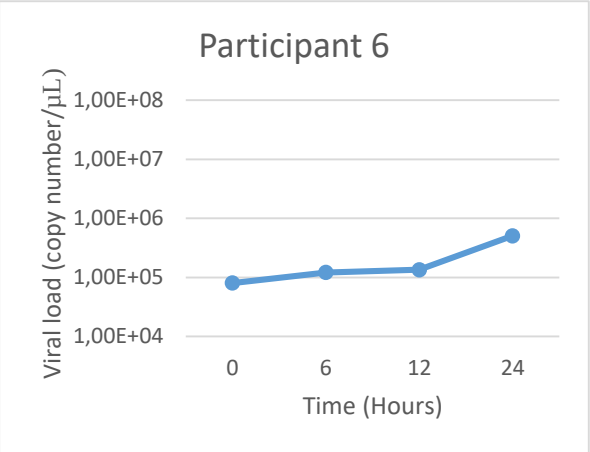

Participant 1
Viral load (copy number/µL)
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
0
6
12
24
Time (Hours)


Participant 2
Viral load (copy number/µL)
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
0
6
12
24
Time (Hours)


Participant 3
Viral load (copy number/µL)
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
0
6
12
24
Time (Hours)


Participant 4
Viral load (copy number/µL)
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
0
6
12
24
Time (Hours)


Participant 5
Viral load (copy number/µL)
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
0
6
12
24
Time (Hours)


Participant 6
Viral load (copy number/µL)
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
0
6
12
24
Time (Hours)

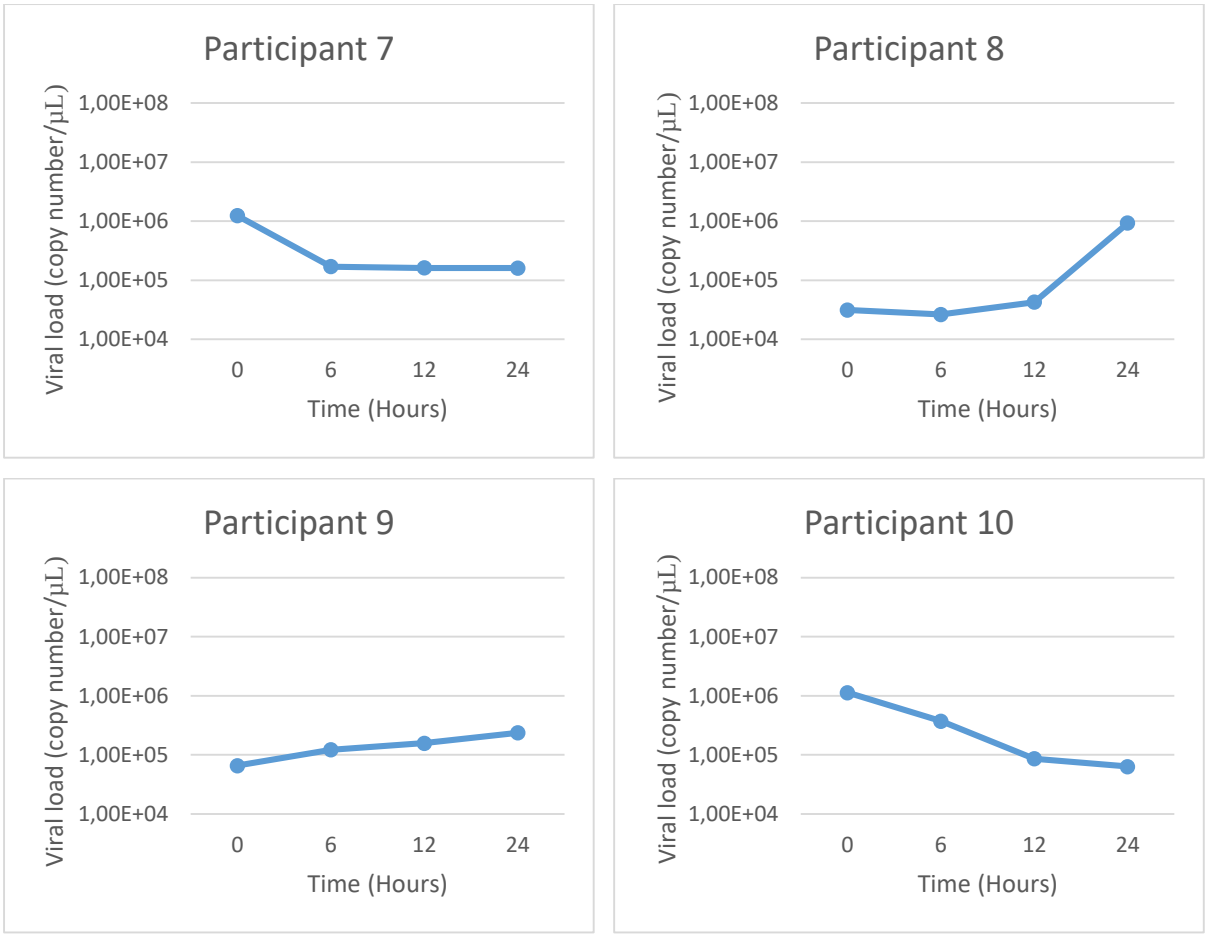
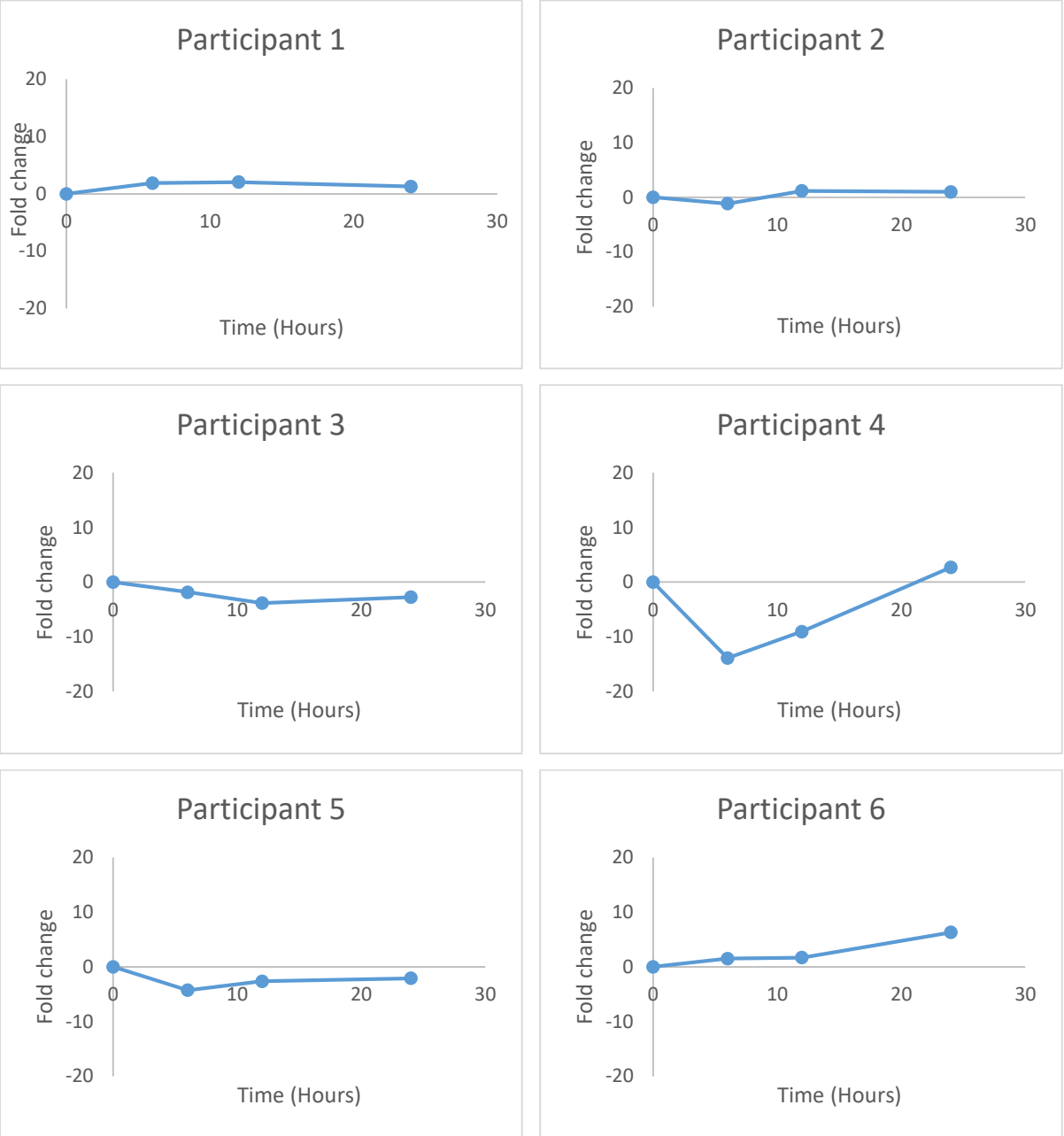


Figure 2.5: **Viral load curves for SINV infected macrophages**. Monocyte-derived macrophages from 10 participants were infected *in vitro* with SINV S.A.AR 86 at an MOI of 0.1. Cell culture supernatant was collected at different time points, from baseline (Time= 0 hours), 6, 12, 24 hours post-infection. Data is represented as arithmetic mean of three repeats from each time point.

The goal was to determine if there was a difference in susceptibility between macrophages isolated from participants. The same number of monocytes were seeded for differentiation for each participant. However, the number of monocytes differentiated to macrophages likely differed for each participant and the number of differentiated macrophages per well could not be accurately determined, hence we considered an increase or decrease in viral load over time relative to time 0 for each participant rather than comparing viral loads between participants. The results suggested that macrophages from different individuals do differ in susceptibility to SINV as infected macrophages from some participants resulted in an increase in viral load, as determined by an increase in negative-strand RNA, suggesting replication. However, for some participants, there appeared to be a decrease in viral load. In order to normalize the data for comparison of participants, fold change difference in viral

load during the investigated time was determined and plotted against the time interval at which the sample was collected. Figure 2.6 shows the fold change in viral load at intervals post-infection relative to the viral load obtained at time 0. The results suggest that there is a difference in viral kinetics in macrophages isolated from different participants suggesting a possibility of difference in susceptibility to SINV replication.



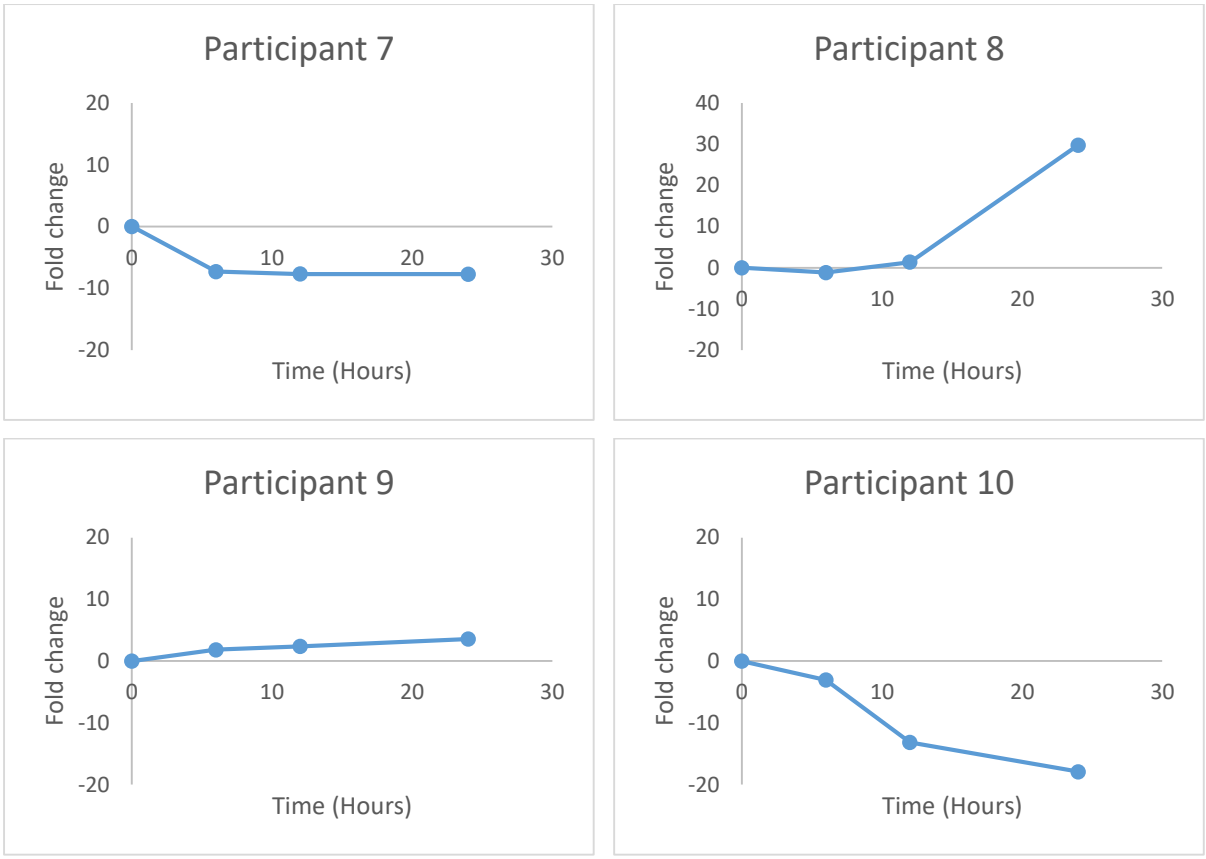


Figure 2.6: Fold change difference in viral loads post-infection with SINV relative to viral load at time 0.

2.4 Summary

SINV is an arthritis causing virus but the mechanism through which it causes chronic/ prolonged joint pain is not yet fully understood. Monocytes and macrophages are known cell targets in arthritogenic alphaviral infections. Disease outcome differs between individuals, with some individuals developing self-limiting symptoms while others develop arthritis, which sometimes may last for months or years. In this study a difference in susceptibility between macrophages from different individuals in terms of viral load as a representation of virus replication post-infection with SINV was investigated. Ten participants with no previous exposure to SINV were enrolled in the study, blood was collected from each participant, and PBMCs were isolated using Ficoll-paque low-speed centrifugation method. The monocytes were stimulated with HM-CSF and allowed to differentiate into macrophages over six days prior to infection. SINV S.A.AR86 stock was used to infect macrophages, and virus replication was assessed at different times post-infection using a quantitative two-step qRT-PCR, targeting the negative-sense RNA strand produced if there is replicating virus.

SINV S.A.AR86 grown in Vero cells for the preparation of virus stock was confirmed using indirect IFA and conventional RT-PCR prior to the calculation of the $TCID_{50}$. Briefly, a confluent monolayer of Vero cells in a vented T25 cell culture flask was infected with an aliquot of SINV stock, and the supernatant was collected at the first indications of CPE. The supernatant fluid was clarified, aliquoted, and virus stocks were stored at -80 °C. To determine the $TCID_{50}$ of the virus stock, Vero cells were infected with tenfold serial dilutions of SINV, in replicates of 6, in 96 well plates, from $1x10^{-1}$ to $1x10^{-7}$. The infected cells were incubated and observed daily for CPE for three days. The$TCID_{50}$ was determined to be $10^{5.7}$ $TCID_{50}$/ml.

For the preparation of a control for use in the development of molecular assays, RNA was extracted from an aliquot of virus stock. A forward and reverse primer pair that amplifies a 180 bp region of the nsP2 gene, designed in a previous study was used in a conventional two-step RT-PCR. The PCR amplicon was purified and sequenced for confirmation that the amplicon was the targeted region of SINV. Nucleotide sequence data was edited using Chromas Pro, aligned using Clustal Version X1.8 and SINV confirmed by comparison with nucleotide sequence data retrieved from GenBank and by BLAST analysis. The concentration and DNA copy number of the purified PCR

product was measured using Qubit and copy number calculator, respectively. Tenfold serial dilutions of the DNA were prepared and used to construct a standard curve for the two-step qRT-PCR.

In vitro studies have shown that several alphaviruses replicate in macrophages. PBMCs were isolated from 10 participants and differentiated into macrophages over six days in media supplemented with HM-CSF. Cells were infected with SINV at an MOI of 0.1 and viral load determined at different time points post-infection for 24 hours using a two-step qRT-PCR targeting the intermediate negative RNA strand. For each participant, the change in viral load was used as an indicator of virus replication within the cells. Viral loads increased in the macrophages of 5/10 participants and decreased in 5/10 participants.

There are limitations from this approach and from the number of cells that were available for the study. Amplification of a reference gene would have been an alternative approach. Results are represented as mean ± standard deviation of three repeats at each time point (Appendix D). Biological repeats were not possible due to a limited number of macrophages available, and limited draws of blood from participants as requested by the Ethics Committee. Hence technical repeats of qRT-PCR were performed in triplicate from extracted RNA. The viability of the cells was determined based on observation of attached, differentiated cells. Stimulation of the cells with a known immunogen such as poly IC would have provided a useful control to ensure that the cells were reactive to immunogens. However, the number of cells available also limited what experiments could be performed. However, the fold changes in viral load over time provide evidence suggesting differences in the replication of the virus between macrophages isolated from different participants warranting further investigation.

Chapter 3: Pro-inflammatory cytokine secretion post-infection with Sindbis virus

3.1 Introduction

There is increasing evidence that long term joint pain/ chronic arthritis due to arthritogenic alphaviruses is linked to the immune response, which results in the secretion of pro-inflammatory cytokines in and around joints during infection. Pro-inflammatory cytokines can be secreted months to years post alphavirus infection, and some of these cytokines have been linked to the severity of chronic joint pain (Ng et al., 2009; Chow et al., 2011). To better understand the immune factors during acute SINV infection and other associated arthritogenic alphaviruses that contribute to chronic arthritis pathogenesis, it is important to know which pro-inflammatory cytokines are secreted post-infection. Cytokines are inflammatory mediators that play a role in both the acute and chronic phases of alphavirus arthritis but they can also have a protective role during viral infection. Infection with alphaviruses results in a strong innate immune response, resulting in the production and secretion of pro-inflammatory cytokines and chemokines, some of which may play an important role in prolonged viral arthritis. Macrophages are one of the important sources of pro-inflammatory cytokines during infection and previous studies have demonstrated that several alphaviruses are able to replicate in macrophages, including SINV, RRV, and MAYV (Way et al., 2002; Cavalheiro et al., 2016; Assunco-Miranda et al., 2010). Infection with arthritogenic alphaviruses results in the recruitment of macrophages to the site of infection, triggering the secretion of pro-inflammatory cytokines. Previous studies have suggested that chronic arthritis may be due to increased secretion of various cytokines and chemokines during infection.

Viral replication within joint tissues is thought to contribute towards the development of arthritis, with the host immune response playing an important role in the pathogenesis through increased secretion of pro-inflammatory cytokines. Multiple studies have characterized pro-inflammatory cytokine and chemokine responses during the acute and chronic phases of arthritogenic alphavirus infection in human cohort studies. It has been shown that increased levels of IL-1β and IL-6, and decreased level of RANTES resulted in a more severe CHIKV disease (Ng et al., 2009). Similarly, increased levels of IL-6 and GM-CSF were suggested as possible

markers of severity of disease of CHIKV (Chow et al., 2011). Secretion of pro-inflammatory cytokines have also been shown to differ between individuals with high viral loads in comparison to those with low viral loads. Inflammatory mediators, including IFN-α, IL-6, MCP-1, and IL-12, were secreted at higher levels in patients with high viral loads compared to those with low viral loads (Chow et al., 2011). Levels of IL-6, MCP1 and TNF-α, were shown to be secreted at higher levels in CHIKV infected patients than in an uninfected control group. These cytokines have also been associated with increased severity of RA (Chirathaworn et al., 2013). A long duration of upregulated cytokine responses results in systemic inflammatory response syndrome (SIRS), where the pro-inflammatory response can no longer be regulated, resulting in cytokines becoming destructive to the host, rather than protective. The persistence of the virus in the macrophages together with SIRS may contribute to the development of arthritis. Infection of the mice deficient in IFN α/β receptors ($IFN^{-/-}$) resulted in elevated cytokine levels demonstrating SIRS whereas those with active IFN-α/β signalling were protected against SINV suggesting an important role for IFN response during viral infection (Ryman et al., 2000). In contrast, a robust cytokine response including the secretion of TNF-α, IL-12, and IL-1β, during infection has been shown to contribute towards decreased joint pain and these cytokines were necessary for viral clearance in CHIKV infected patients (Chang et al., 2018).

Studies have shown that CHIKV infected patients produce high levels of IFN-α, and similar results have been demonstrated in animal models (Hoarau et al., 2010). Furthermore, arthritogenic alphavirus infection is usually more fatal in murine models deficient in IFN-α/β. Previous studies using a mouse model deficient in IFN-α/β signalling resulted in more severe disease, indicating that the innate immune system is important in the primary protection against alphavirus infection by preventing dissemination and replication of the virus in primary cells (Zhang et al., 2007). In a study using a mouse model in which the authors wanted to investigate the mechanism of protection against acute ONNV infection, mice deficient in T and B cell response, IFN-γ receptors, and IFN-α/β receptors were infected with ONNV. Mice deficient of IFN-α/β receptors developed a fatal disease, suggesting an intact IFN-α/β response is important in controlling disease severity (Seymour et al., 2013). Thus a strong type I IFN response appears to be associated with less severe disease.

Cytokines and chemokines are some of the key inflammatory mediators released by macrophages during viral infection. Therefore their quantification is important in studying inflammation and immune responses. Cytokine profiling assays are designed to measure cytokines and chemokines released from chosen target cells. Real-time PCR and enzyme-linked immunosorbent assay (ELISA) platforms are two of the most widely used techniques for quantification of cytokines. To investigate the effect of SINV on cytokine secretion from infected macrophages, cytokine levels were determined in response to *in vitro* infection of human macrophages. IFNs are a group of cytokines that are released by host cells as a primary defence against viral infections as part of the innate immune response. There are three types of IFNs, type I, II, and III, type I being the most important. During a viral infection secreted type I IFN-α/β bind to IFN receptor (IFNRA1/IFNRA2) subunits which result in the transduction of a signal to the nucleus by JAK/STAT complex resulting in the upregulation of hundreds of ISGs resulting in the synthesis of many antiviral proteins and the establishment of an antiviral state. To further determine the effect of interferon on virus replication, viral loads were monitored in macrophages treated with and without a JAK1/2 inhibitor, ruxolitinib. Macrophages from selected patients were treated with ruxolitinib, which results in the inhibition of type I IFN response via the JAK/STAT pathway, prior to infection with SINV to investigate the influence of inhibiting JAK1/2 on the kinetics of SINV replication. A two-step quantitative RT-PCR was used to determine viral loads in SINV infected macrophages post-treatment with ruxolitinib.

3.2 Methods and materials

3.2.1 Determining the role of interferon in SINV infection

To investigate the importance of type I IFN in protection against SINV, macrophages were pre-treated with an interferon inhibitor, ruxolitinib, prior to infection with SINV S.A.AR86 and viral kinetics were assessed in terms of virus infectivity and replication.

3.2.1.1 Pre-treatment of macrophages with ruxolitinib and infection with SINV

PBMCs were isolated from the blood collected from each of the ten participants and differentiated into macrophages as previously described in Chapter 2, Section 2.2.3.2. Ruxolitinib (Abcam, Cambridge, UK), an IFN inhibitor that inhibits type I IFN via the JAK/STAT pathway was used to pre-treat macrophages prior to infection with SINV. Ruxolitinib was prepared as a 16mM stock in dimethyl sulfoxide (DMSO). To determine a suitable concentration of inhibitor that was tolerated by the cells, macrophages from a single participant were treated with different final concentrations of ruxolitinib, including 2 µM, 4 µM, 6 µM, and 10 µM, to determine the highest concentration of ruxolitinib that did not result in macrophage cell death over 24 hours. Cells were incubated for 24 hours with different concentrations of ruxolitinib in maintenance media and monitored for cell death. A concentration of 4 µM was selected as it resulted in minimal cell death and it was in the range of concentrations that previous studies had used for the pre-treatment of mammalian cell lines to inhibit the JAK/STAT pathway (Silva et al., 2016; Febvre-james et al., 2018). Subsequently, PBMCs from each patient were seeded in a 24 well plate ($8x10^5$ cells/well) and incubated for six days in RPMI 1640 maintenance media supplemented with 25 µg of HM-CSF to allow differentiation into macrophages. At day six, maintenance media was removed, and cells were pre-treated with a final concentration of 4 µM ruxolitinib in RPMI 1640 media and incubated at 37 ˚C for 2 hours, prior to infection. Similarly, some cells were prepared and treated with ruxolitinib immediately prior to infection (time= 0). The cells were then infected with SINV S.A.AR86 virus stock at an MOI of 0.1 and incubated for 60 minutes at 37 ˚C in a humidified CO_2 incubator to allow absorption of the virus into the cells. After 60 minutes of incubation, the inoculum was removed, and cells were washed twice with PBS, and fresh maintenance media with 4 µM of ruxolitinib was added to the cells. RNA was extracted from aliquots of cell supernatant

collected at 0, 6, 12, and 24 hours post-infection and viral load determined using a two-step qRT-PCR targeting negative strand RNA.

3.2.1.2 Quantification of viral RNA by two-step qRT-PCR

Viral replication was determined using a two-step quantitative RT-PCR. RNA was extracted from virus supernatant using a QIAamp viral RNA extraction kit according to the manufacturer's instructions, as described in Chapter 2 section 2.2.4.2. RNA was reverse transcribed into cDNA using Superscript III according to the manufacturer's instructions, and the cDNA was used as a template for two-step qRT-PCR, targeting the nsP2 region of SINV using the primer pair, nsP2F and nsPR, together with a Taqman probe. Sequences of both primers and probe can be seen in Table 2.1 and 2.4 in Chapter 2, respectively. Detailed methodology of the techniques are found in Chapter 2 methods and materials Section 2.2.2.1, 2.2.2.2, and 2.2.4.2. A standard curve was generated from the PCR product amplified to quantify viral loads in infected macrophages. Briefly, the DNA was serially diluted tenfold and used as a template for qPCR. The C_p values determined for each standard were plotted against the concentration, and the standard curve was used as a reference to determine the viral loads in SINV infected macrophages.

3.2.2 Profiling cytokine secretion from macrophages infected with SINV

Pro-inflammatory cytokines are the key modulators in inflammation that have been shown to play a role both in the acute and chronic phases of arthritogenic alphavirus infection. Macrophages from selected participants were infected with SINV S.A.AR86 *in vitro,* and the levels of six cytokines, known to be induced in response to alphavirus infection, were determined using a commercial assay. Macrophages were prepared from five participants based on the virus replication kinetics determined in Chapter 2. Briefly, two participants (designated 1 and 2) in which virus was shown to replicate in infected macrophages, two participants (designated 3 and 10) in which SINV infection resulted in little to no replication, and one participant (designated 7) that showed viral replication only when the macrophages were pre-treated with ruxolitinib to inhibit IFN response prior to SINV infection were selected for cytokine assay.

3.2.2.1 Primary culture of human macrophage

PBMCs were isolated from whole blood using Ficoll-Paque protocol according to the manufacturer's instructions. Mononuclear cells were plated in a 24-well plate ($8x10^5$ cells/well) in maintenance media (RPMI 1640 growth media supplemented with 10% FBS, 1% pen/strep, and 1% L-glut) supplemented with 25 µg HM-CSF. The cells were incubated in a humidified CO_2 incubator at 37°C and allowed to differentiate into macrophages. A detailed protocol can be seen in Chapter 2 section 2.2.3.2 methods and materials

3.2.2.2 Infection of macrophages with SINV

Post-differentiation, macrophages were infected using an aliquot of SINV S.A.AR86 virus stock prepared in Chapter 2, Section 2.2.1.1, at an MOI of 0.1. Mock infections using heat-inactivated SINV (HI SINV) were performed in parallel for macrophages from each participant. SINV S.A.AR86 was heat-inactivated (HI) by incubating at 56°C for 60 minutes. Macrophages were inoculated with SINV or inactivated virus and incubated for 60 minutes at 37 °C in a humidified CO_2 incubator. Control cells were prepared with no inoculum. Post-incubation, the inoculum was removed, and infected macrophages were washed twice with PBS to remove residual inocula and maintenance media was added to the cells. A 1 ml aliquot of cell culture media (supernatant) was collected at different time intervals, at baseline (time 0), 6, 12, and 24 hours post-infection, baseline is an hour after incubation with SINV at 37 °C. The cytokine levels for six pro-inflammatory cytokines known to be secreted by macrophages were determined using commercially available ELISA kits.

3.2.2.3 Quantification of expressed cytokines using ELISA

Cytokine levels in cell-free supernatant collected at different times post-infection were determined using Elabscience ELISA kits (Elabscience, Texas, USA) (Table 3.1). The layout of the ELISA plate can be seen in figure 3.1. Briefly, a standard curve was generated for each cytokine using twofold serial dilutions of reference standards (supplied in each kit) tested in duplicate. The mean optical density (OD) value at 450nm for each dilution was used to generate a standard curve for each cytokine.

For quantification of each cytokine, ELISA plates were prepared, as shown in figure 3.1 and all reagents were supplied in the kit. Briefly, 100 µl aliquots of known standards (in duplicate) and samples were tested. The plates were incubated for 90 minutes at 37 ˚C. Post-incubation, the samples (and standards) were removed from each well and 100 µl aliquot of biotinylated detection antibody specific for each human cytokine that was tested (i.e. IFN-α) was added and incubated for 60 minutes at 37 ˚C. Post-incubation, the detection antibody was removed, and the plate was washed three times with 350 µl of wash buffer. The antibody was reacted with 100 µl aliquot of avidin-horseradish peroxidase (HRP) conjugated antibody and the plates incubated at 37 ˚C. The conjugated antibody was aspirated from each well and the plate was washed five times with 350 µl of wash buffer. A 90 µl aliquot of substrate reagent was added to each well and incubated for 15 minutes at 37 ˚C, and the reaction stopped with a 50 µl aliquot of stop solution. The OD values at 450 nm were determined after 15 minutes using a microplate reader (Bio-Tek ELx800, BioTek, Vermont, USA).

Table 3.1: List of investigated cytokines and their functions (Turner et al., 2014)

	Abbreviation	**Function**
Interferon-alpha	(IFN-α)	Anti-viral, prevents infection of neighbouring cells and reduces viral replication in infected cells.
Tumour Necrosis Factor-alpha	(TNF-α)	Induction of cytokine production, activation of adhesion molecules and growth stimulation.
Interleukin 1 beta	(IL-1β)	Promote differentiation into the T helper cell and mediates inflammatory signalling responses.
Interleukin 6	(IL-6)	Involved in the final maturation of B-cells into antibody-producing B cells
Interleukin 8	(IL-8)	Recruitment of neutrophils and activation of monocytes and lymphocytes at the site of inflammation.
Interleukin 12	(IL-12)	Stimulation of cell proliferation to fight off infection.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St	St	P1 SINV	P1 SINV	P1 SINV	P1 SINV	P1 SINV	P1 Mock	P1 Mock	P1 Mock	P1 Mock	P1 Mock
B	St	St	P1 Control	P1 Control	P1 Control	P1 Control	P1 Control	P2 SINV	P2 SINV	P2 SINV	P2 SINV	P2 SINV
C	St	St	P2 Mock	P2 Mock	P2 Mock	P2 Mock	P2 Mock	P2 Control	P2 Control	P2 Control	P2 Control	P2 Control
D	St	St	P3 SINV	P3 SINV	P3 SINV	P3 SINV	P3 SINV	P3 Mock	P3 Mock	P3 Mock	P3 Mock	P3 Mock
E	St	St	P3 Control	P3 Control	P3 Control	P3 Control	P3 Control	P10 SINV	P10 SINV	P10 SINV	P10 SINV	P10 SINV
F	St	St	P10 Mock	P10 Mock	P10 Mock	P10 Mock	P10 Mock	P10 Control	P10 Control	P10 Control	P10 Control	P10 Control
G	St	St	P7 SINV	P7 SINV	P7 SINV	P7 SINV	P7 SINV	P7 Mock	P7 Mock	P7 Mock	P7 Mock	P7 Mock
H	St	St	P7 Control	P7 Control	P7 Control	P7 Control	P7 Control					

Figure 3.1: Plate layout for each cytokine ELISA

St= twofold dilution of each standard in duplicate

P1, P2, P3, P7, P10= macrophages prepared from selected participants

SINV= supernatant collected from macrophages infected with SINV

Mock= supernatant collected from macrophages exposed to HI SINV

Control= Media from uninfected macrophage cell

3.3 Results

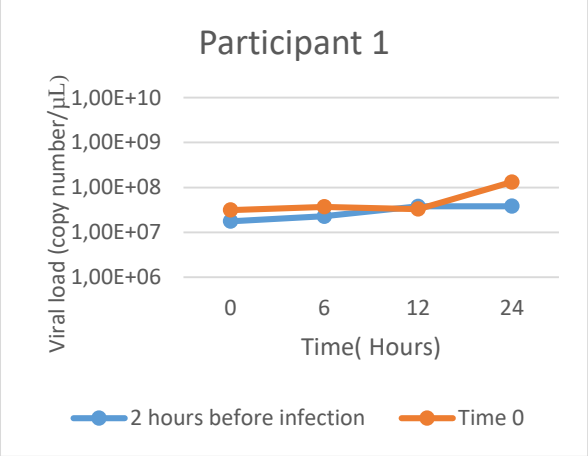
3.3.1 Inhibition of interferon responses prior to infection of macrophages with SINV

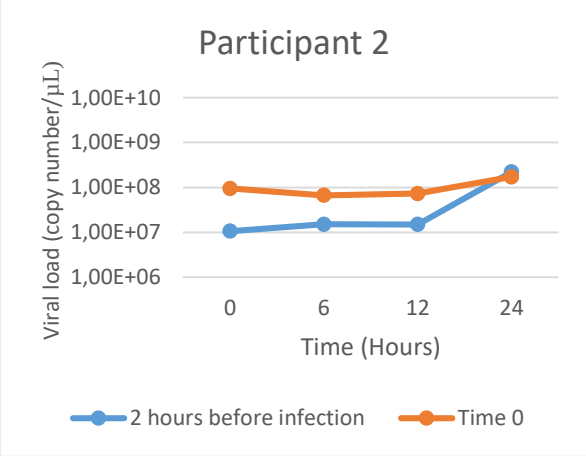
Type I IFN functions as part of the first-line defence in innate immunity controlling virus replication and dissemination. Therefore, inhibition of IFN-α/β may result in increased viral replication in human macrophages. To determine if type I IFN plays a role in inhibiting SINV replication, ruxolitinib, a drug that belongs to the Jak kinase inhibitors class, that suppresses type I IFN signally pathway was used for pre-treatment of macrophages prior to *in vitro* infection with SINV. Macrophages were pretreated with 4 µM of ruxolitinib either 2 hours before infection or immediately before infection (at time 0). The optimal concentration was determined using published recommendations and a preliminary study indicating limited cell death observed at 24 post-treatment. Macrophages were infected with SINV at an MOI of 0.1, and RNA was extracted from cell supernatant at times 0, 6, 12, and 24 hours post-infection and used for evaluation of virus infectivity and replication by measuring viral loads using a two-step qRT-PCR. Replicates of cDNA were reverse transcribed from RNA extracted at different times post-infection. Results are represented as arithmetic mean ± standard deviation of three repeats from each time point post-infection (Appendix D). There was considerable variability between viral loads measured for technical repeats. These samples were frozen and thawed which may have contributed to the variability, and the standard control was also frozen and thawed. Hence the results were analysed based on trends rather than absolute numbers due the significant variability within data. The limited number of blood draws was a significant limitation as biological repeats are actually essential to determine inter-experiment variability not relying on frozen and thawed samples.

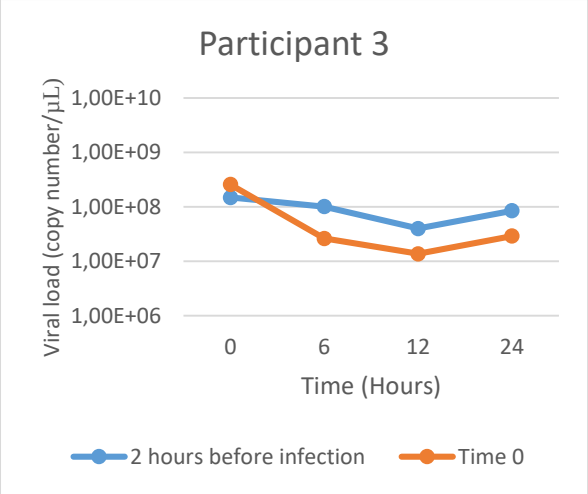
Treatment of macrophages with ruxolitinib prior to infection with SINV was found to increase viral load in infected macrophages. Inhibition of IFN by treating macrophages with ruxolitinib immediately prior to infection with SINV resulted in viral replication in macrophages from 7/10 participants, including participants 1, 2, 4, 5, 7, 8, and 9 (figure 3.2). The amount of replication was variable and in some instances, there was a delay before replicating virus was detectable. This was more evident when considering fold

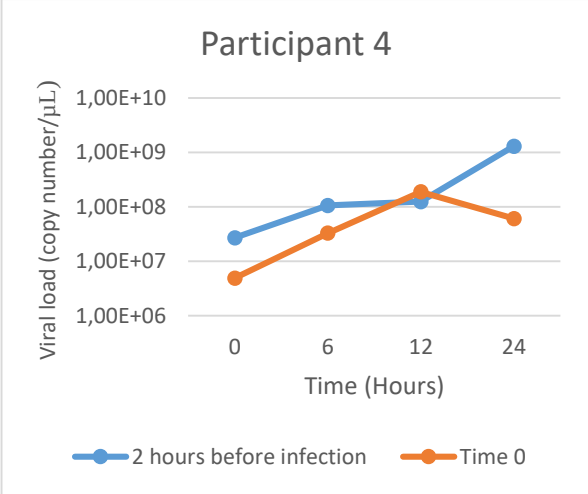
changes in viral load compared with Time 0 (figure 3.3). Similarly, an increase in viral load was detected in the same participants, plus participant 6, when pre-treated two hours prior to infection, viral loads can be seen in figure 3.2. Viral loads for both macrophages treated at time 0 of infection and those treated 2 hours before infection can be seen in Appendix D, where final results are represented as mean ±standard deviation.

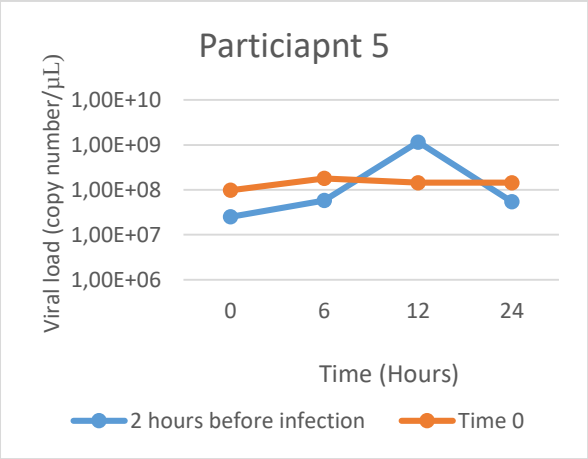
Treatment of macrophages with ruxolitinib immediately before infection resulted in a twofold increase in the viral load from macrophages of participants 8 and 5, threefold from macrophages of participant 9, and a ≥ fourfold increase in viral load from macrophages of participants 1 and 7. The highest increase in viral load (+/- 38 fold) was observed in macrophages of participant 4. Viral load in macrophages of participant 10 increased by eleven-fold in the first 6 hours of infection however, declined thereafter. There was delayed viral replication in macrophages of two participants, viral load in macrophages of participants 2 and 6 decreased initially and then increased by 24 and 12 hours post-infection, respectively. Viral load results for participant 3 suggested no evidence of viral replication as viral loads decreased by +/- 18 fold in the investigated time. For most macrophages cultures, the addition of ruxolitinib prior to infection and subsequent inhibition of Jak1 may have resulted in the downregulation of phosphorylated STAT1, therefore decreasing the production of IFN, resulting in higher viral replication in the macrophages, with exception to infected macrophages that showed a decrease in viral load, such as those of participant 3.

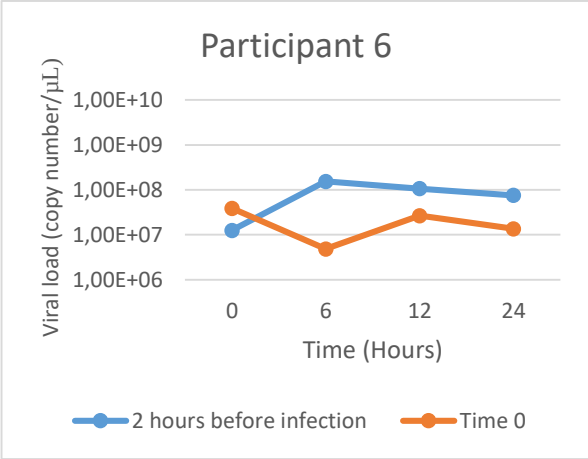


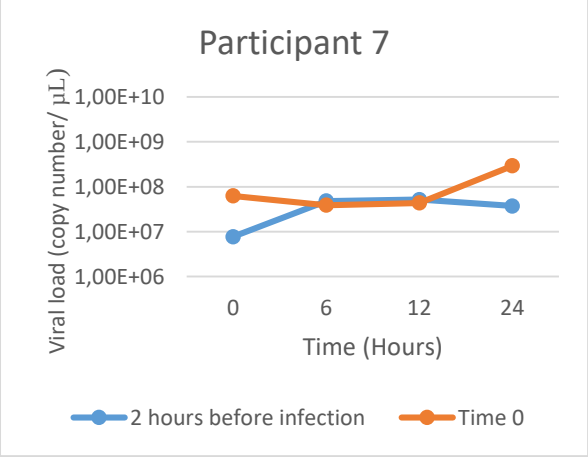


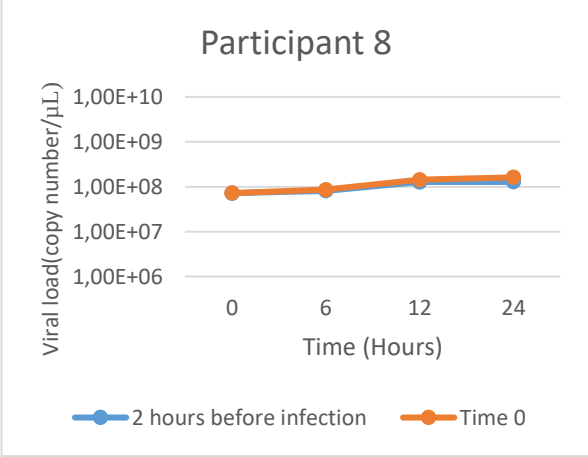

Participant 3
Viral load (copy number/µL)
1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
0
6
12
24
Time (Hours)
2 hours before infection
Time 0


Participant 4
Viral load (copy number/µL)
1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
0
6
12
24
Time (Hours)
2 hours before infection
Time 0


Particiapnt 5
Viral load (copy number/µL)
1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
0
6
12
24
Time (Hours)
2 hours before infection
Time 0


Participant 6
Viral load (copy number/µL)
1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
0
6
12
24
Time (Hours)
2 hours before infection
Time 0


Participant 7
Viral load (copy number/ µL)
1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
0
6
12
24
Time (Hours)
2 hours before infection
Time 0


Participant 8
Viral load(copy number/µL)
1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
0
6
12
24
Time (Hours)
2 hours before infection
Time 0

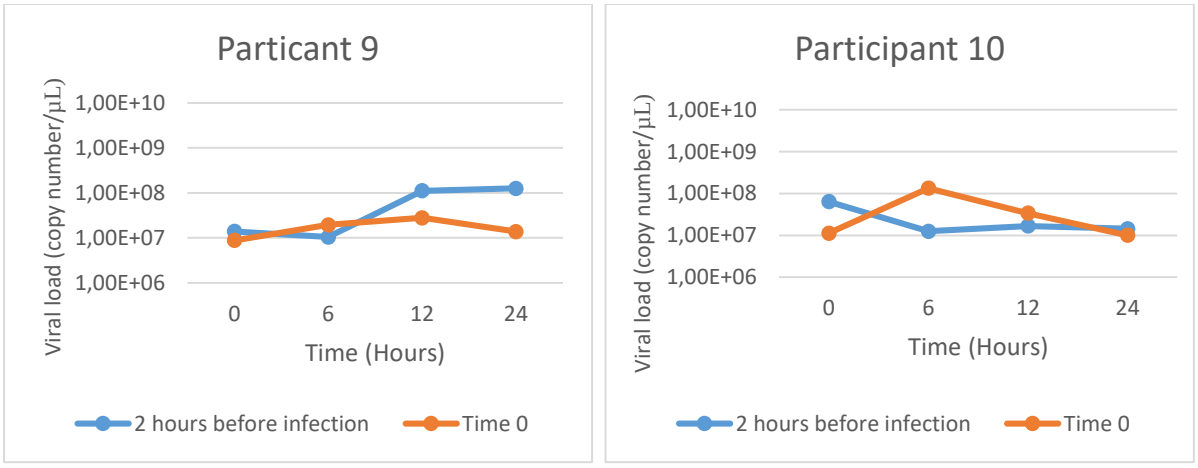
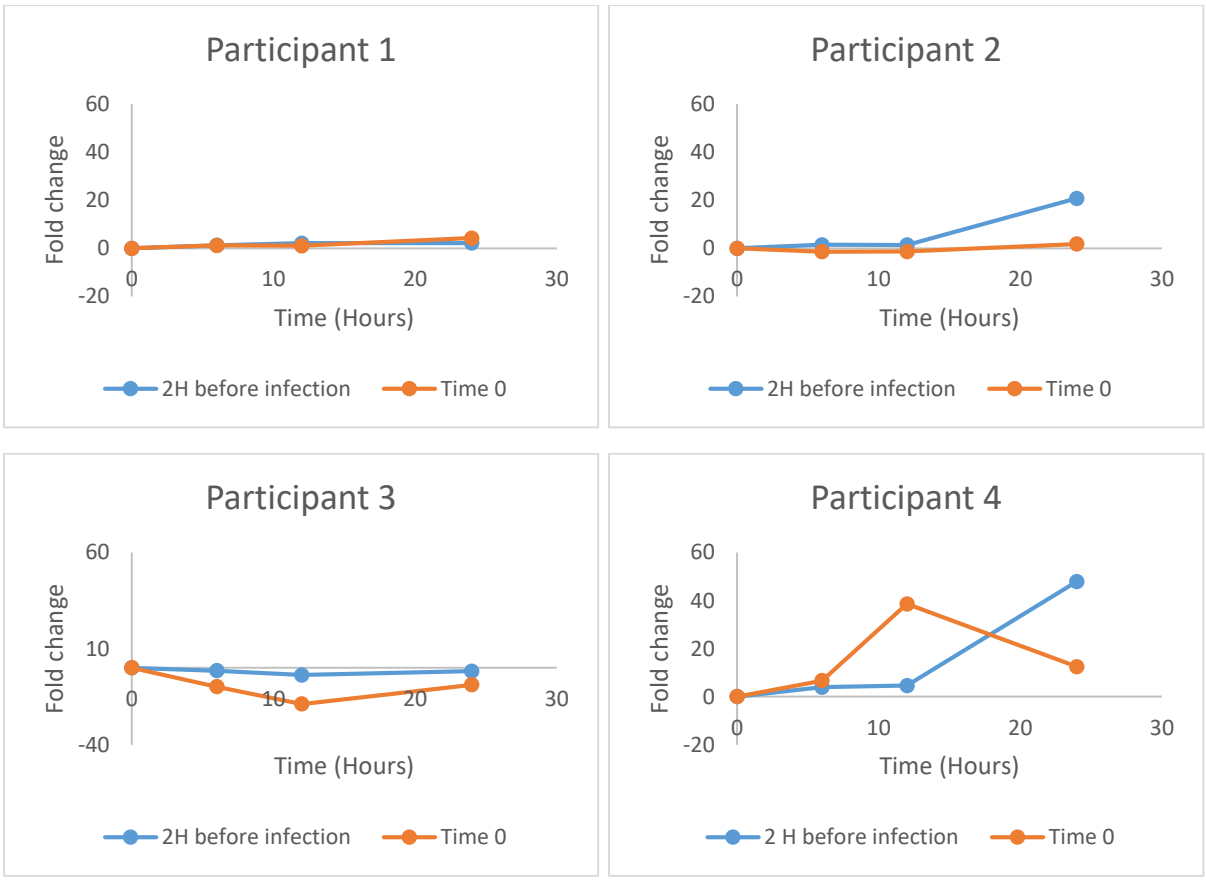


Figure 3.2: Effects of JAK inhibitor on viral replication: macrophages were pre-treated with 4µM of ruxolitinib 2 hours before infection with SINV. Cell supernatant was collected at times 0, 6, 12, and 24 hours post-infection with SINV, and viral replication was determined using two-step qRT-PCR.



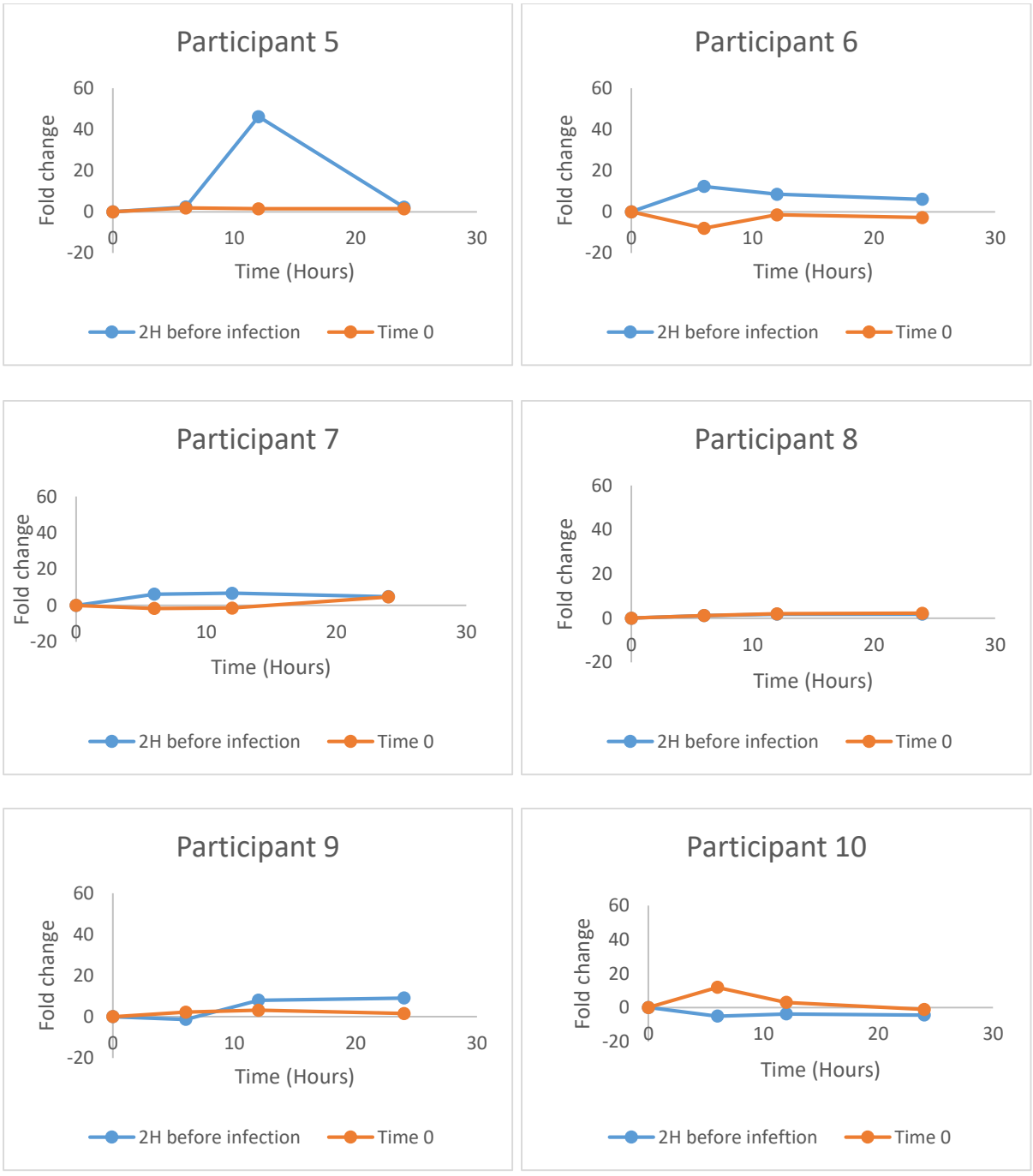


Figure 3.3: Fold change in viral loads of participants macrophages treated with ruxolitinib 2 hours before infection and at time 0 of infection.

Table 3.2: Comparison of fold changes in viral loads from macrophages infected with SINV when untreated, treated with ruxolitinib immediately prior to infection and 2 hours prior to infection

	Fold change post-infection with SINV		
Participant	**Untreated**	**Ruxolitinib treated at time 0**	**Ruxolitinib treated 2H prior to infection**
Participant 1	2 fold increase	4 fold increase	2 fold increase
Participant 2	2 fold increase	2 fold increase	20 fold increase
Participant 3	3 fold decrease	18 fold decrease	3 fold decrease
Participant 4	14 fold decrease	38 fold increase	47 fold increase
Participant 5	4 fold decrease	2 fold increase	46 fold increase
Participant 6	6 fold increase	8 fold decrease	12 fold increase
Participant 7	8 fold decrease	4 fold increase	8 fold increase
Participant 8	29 fold increase	2 fold increase	2 fold increase
Participant 9	3 fold increase	3 fold increase	9 fold increase
Participant 10	17 fold decrease	12 fold increase	5 fold decrease

Treatment of macrophages with ruxolitinib 2 hours prior to infection generally resulted in higher viral loads in comparison to those treated at time 0 of infection, except for macrophages isolated form participants 1 and 10. Viral loads were unchanged for participant 8. This can be seen in figure 3.3 where the data is presented at fold change difference in viral loads relative to the viral load observed at the initial time of infection. There was variability in the increase in viral load when cells were treated with ruxolitinib, with a twenty-fold change for participant 2, forty-seven fold change for participant 4, forty-six fold for participant 5 and twelve-fold for participant 6. Comparison of macrophages infected with SINV without treatment with ruxolitinib to those treated with ruxolitinib before infection (Table 3.2) showed an increase in viral replication, represented by viral load, in treated macrophages. An exception was participant 8 in which untreated macrophages had a fold change increase of twenty-eight fold compared to twofold when treated with ruxolitinib. However as there were no biological repeats this could have been the result of other factors such as cell viability and the result should be interpreted with caution.

3.3.2 Profiling of cytokine secretion by human macrophages post-infection with SINV

Cytokines are important immune mediators that control infection however, many of these cytokines may also play a role in the immunopathogenesis of SINV infection and other associated arthritogenic alphaviruses. To evaluate the secretion of cytokines from SINV infected macrophages, human macrophages were infected *in vitro* with SINV at an MOI 0.1. Virus supernatant was harvested at times 0, 6, 12, and 24 hours post-infection and analysed for levels of secreted IFN-α, TNF-α, IL-1β, IL-6, IL-8 and IL-12 using sandwich ELISA. Uninfected cells and mock-infected cells (infected with HI SINV) were used to differentiate non-specific cytokine secretion to that secreted due to SINV infection and replication. Macrophages from five participants including two showing viral replication (participants 1 and 2), two with little to no virus replication (participants 3 and 10) and one participant in which viral replication in macrophages was only evident when pre-treated with ruxolitinib (participant 7), were used for the cytokine assays. A standard curve (Appendix F) was constructed for each cytokine by preparing a twofold serial dilution of the reference standard, in duplicates. The OD values of TNF-α, IFN-α, IL-1β, and IL-8 tested within the range of values obtained in the standard curve, secreted levels of these cytokines at different time points for each participant can be seen in Tables 3.3, 3.4, 3.5, and 3.7, respectively. The values for some samples tested were out of the detectable range for IL-6 (7.81 to 500 pg/mL) and IL-12 (<15.63pg/mL) as specified by the manufacturer (Table 3.6 and 3.8). Concentrations of secreted cytokines were extrapolated from respective standard curves and constructed using reference standards of known concentrations. The standard curves for each cytokine and the concentration levels can be found in Appendices F and G, respectively. The fold change in cytokine concentrations of SINV and HI SINV infected macrophages relative to secretion levels of uninfected macrophages at different times post-infection was determined (figure 3.4 to 3.9).

Overall, the cytokine levels did fluctuate within experiments and the lack of biological repeats were taken into consideration when interpreting the data. Macrophage preparations in which fold changes greater than 2 were considered to be associated with infection rather than experimental fluctuation.

Limited replication was detected in macrophages of participant 1, and little change was noted for cytokines levels compared to the control groups. Similarly, infection of untreated macrophages from participant 2 resulted in limited virus replication, however subsequent to treatment with ruxolitinib the viral load increased +/- 20 fold. After infection of the macrophages, high levels of IFN-α, IL-1β, IL-6, and IL-8 were detected compared with cells infected with HI virus. No viral replication was detected for participant 3 and results showed little influence of infection on cytokine profiles. A robust stimulation of cytokine secretion, increasing with time, was observed in macrophages from participant 7, particularly IFN-α, IL-6, and IL-8. Levels of IFN-α, IL-8 and IL-12 were increased at some points post-infection for participant 10. For all participants, during the initial 24 hours after infection, TNF-α levels fluctuated but there was no consistent increase and no discernible trend (Appendix G).

Table 3.3: Concentration of TNF-α in cell culture supernatant collected from SINV infected, mock-infected and uninfected macrophages

		Time (hours after infection)			
		0	**6**	**12**	**24**
Participant 1	**SINV**	46,54	94,10	79,46	101,17
	HI SINV	122,88	75,32	75,56	166,78
	Uninfected	71,41	86,05	64,59	83,85
Participant 2	**SINV**	98,24	88,73	85,32	94,83
	HI SINV	72,63	95,07	78,24	90,20
	Uninfected	115,80	117,75	102,39	165,32
Participant 3	**SINV**	57,51	139,71	85,56	121,17
	HI SINV	93,61	75,80	104,83	104,34
	Uninfected	71,90	79,71	54,10	78,98
Participant 10	**SINV**	57,76	52,88	75,07	98,73
	HI SINV	70,93	103,37	67,51	86,05
	Uninfected	85,32	115,80	109,7	100,93
Participant 7	**SINV**	119,71	90,68	93,61	101,66
	HI SINV	95,31	106,29	114,59	114,83
	Uninfected	89,71	106,54	106,54	87,02

SINV-Macrophages infected with Sindbis virus

HI SINV- macrophages infected with heat-inactivated Sindbis virus

Uninfected- Uninfected macrophages

All concentrations were measured as pg/mL

Table 3.4: Concentration of IFN-α in cell culture supernatant collected from SINV infected, mock-infected and uninfected macrophages

		Time			
		0	6	12	24
Participant 1	SINV	105,84	165,84	207,95	121,63
	HI SINV	205,32	203,21	134,26	261,63
	Uninfected	82,16	88,47	120,58	141,11
Participant 2	SINV	243,74	764,79	974,79	729,00
	HI SINV	76,89	352,16	975,84	81,11
	Uninfected	114,79	182,68	184,26	167,42
Participant 3	SINV	62,16	66,89	75,31	80,05
	HI SINV	109,00	114,79	79,52	121,11
	Uninfected	369,53	596,37	710,58	536,37
Participant 10	SINV	82,16	457,42	540,05	147,42
	HI SINV	102,68	875,84	312,16	519,00
	Uninfected	111,11	289,00	302,16	148,47
Participant 7	SINV	289,53	605,32	1098,47	1342,16
	HI SINV	84,26	113,21	191,11	244,79
	Uninfected	101,63	157,95	261,63	216,89

Table 3.5: Concentration of IL-1β in cell culture supernatant collected from SINV infected, mock-infected and uninfected macrophages

		Time (Hours)			
		0	6	12	24
Participant 1	SINV	9,19	15,36	22,64	26,47
	HI SINV	20,54	34,25	28,44	28,07
	Uninfected	16,96	10,17	9,80	8,19
Participant 2	SINV	21,90	13,13	23,38	34,74
	HI SINV	11,77	17,08	4,37	4,62
	Uninfected	16,96	15,36	10,79	24,12
Participant 3	SINV	1,53	2,27	5,73	13,14
	HI SINV	12,27	26,71	15,11	14.00
	Uninfected	23,63	16,49	13,75	10,54
Participant 10	SINV	18,07	20,42	15,85	9,30
	HI SINV	23,51	11,78	22,40	6,72
	Uninfected	17,46	25,23	14,49	0,54
	SINV	23,63	33,14	22,77	15,23

Participant 7	**HI SINV**	26,45	24,86	20,91	21,28
	Uninfected	4,99	36,35	29,93	14,25

Table 3.6: Concentration of IL-6 in cell culture supernatant collected from SINV infected, mock-infected and uninfected macrophages

		Time (hours)			
		0	**6**	**12**	**24**
Participant 1	**SINV**	<7,81	<7,81	33,96	<7,81
	HI SINV	<7,81	<7,81	<7,81	<7,81
	Uninfected	<7,81	12,76	<7,81	40,22
Participant 2	**SINV**	91,57	228,88	271,57	219,63
	HI SINV	13,36	107,09	345,45	132,76
	Uninfected	17,24	13,06	14,1	23,21
Participant 3	**SINV**	<7,81	<7,81	<7,81	<7,81
	HI SINV	<7,81	<7,81	25,74	26,79
	Uninfected	214,25	278,28	311,57	249,78
Participant 10	**SINV**	83,21	327,09	327,09	179,63
	HI SINV	21,42	272,76	285,59	234,25
	Uninfected	10,67	139,18	140,82	96,19
Participant 7	**SINV**	264,04	377,09	>500	>500
	HI SINV	54,55	41,71	103,66	119,32
	Uninfected	45,29	128,13	149,03	202,46

Secreted levels of IL-6 were compared between macrophages of five participants. The detection range of the assay was between 7.81 to 500 pg/mL. Levels of secreted IL-6 were below the detection range in macrophages of participants 1 and 3 at some investigated time points, whereas IL-6 concentrations for participant 7 SINV infected macrophages were above the detectable rage of 500pg/mL at times 12 and 24 hours post-infection.

Table 3.7: Concentration of IL-8 in cell culture supernatant collected from SINV infected, mock-infected and uninfected macrophages

		Time			
		0	**6**	**12**	**24**
Participant 1	**SINV**	6,68	35,63	69,32	22,47
	HI SINV	21,42	22,47	58,79	103,52
	Uninfected	5,63	14,56	80,89	209,32
Participant 2	**SINV**	433,52	923,00	1161,42	1137,21
	HI SINV	61,95	507,21	127,74	796,68

	Uninfected	98,79	158,79	204,05	126,16
Participant 3	**SINV**	60,89	41,94	58,26	23,53
	HI SINV	43,53	52,47	71,94	104,58
	Uninfected	399,84	591,42	681,95	528,80
Participant 10	**SINV**	113,00	686,16	750,37	231,42
	HI SINV	47,74	476,68	561,42	387,74
	Uninfected	57,74	190,37	237,21	204,05
Participant 7	**SINV**	327,74	658,26	1336,16	1396,16
	HI SINV	101,42	179,32	204,58	385,63
	Uninfected	38,26	167,74	238,79	270,37

Table 3.8: Concentration of IL-12 in cell culture supernatant collected from SINV infected, mock-infected and uninfected macrophages

		Time			
		0	**6**	**12**	**24**
Participant 1	**SINV**	<15,63	110	61,15	79,62
	HI SINV	<15,63	62,3	92,69	57,31
	Uninfected	57,69	50,77	83,84	50
Participant 2	**SINV**	<15,63	55,38	46,54	120,77
	HI SINV	<15,63	46,54	<15,63	34,23
	Uninfected	<15,63	100,77	<15,63	93,08
Participant 3	**SINV**	<15,63	<15,63	<15,63	<15,63
	HI SINV	<15,63	24,62	39,23	108,46
	Uninfected	72,69	<15,63	<15,63	<15,63
Participant 10	**SINV**	37,69	75	22,7	25,38
	HI SINV	114,62	175,38	<15,38	116,15
	Uninfected	121,92	56,92	82,31	11,54
Participant 7	**SINV**	144,23	62,69	87,69	163,85
	HI SINV	163,46	108,85	42,31	15,63
	Uninfected	127,69	<15,63	160,77	132,69

Secreted levels of IL-12 were compared between macrophages of five participants. The detection range of the assay was between 15.63 to 1000 pg/mL. Concentration levels of IL-12 were below the detectable range in macrophages of the five participants at some of the investigated times.

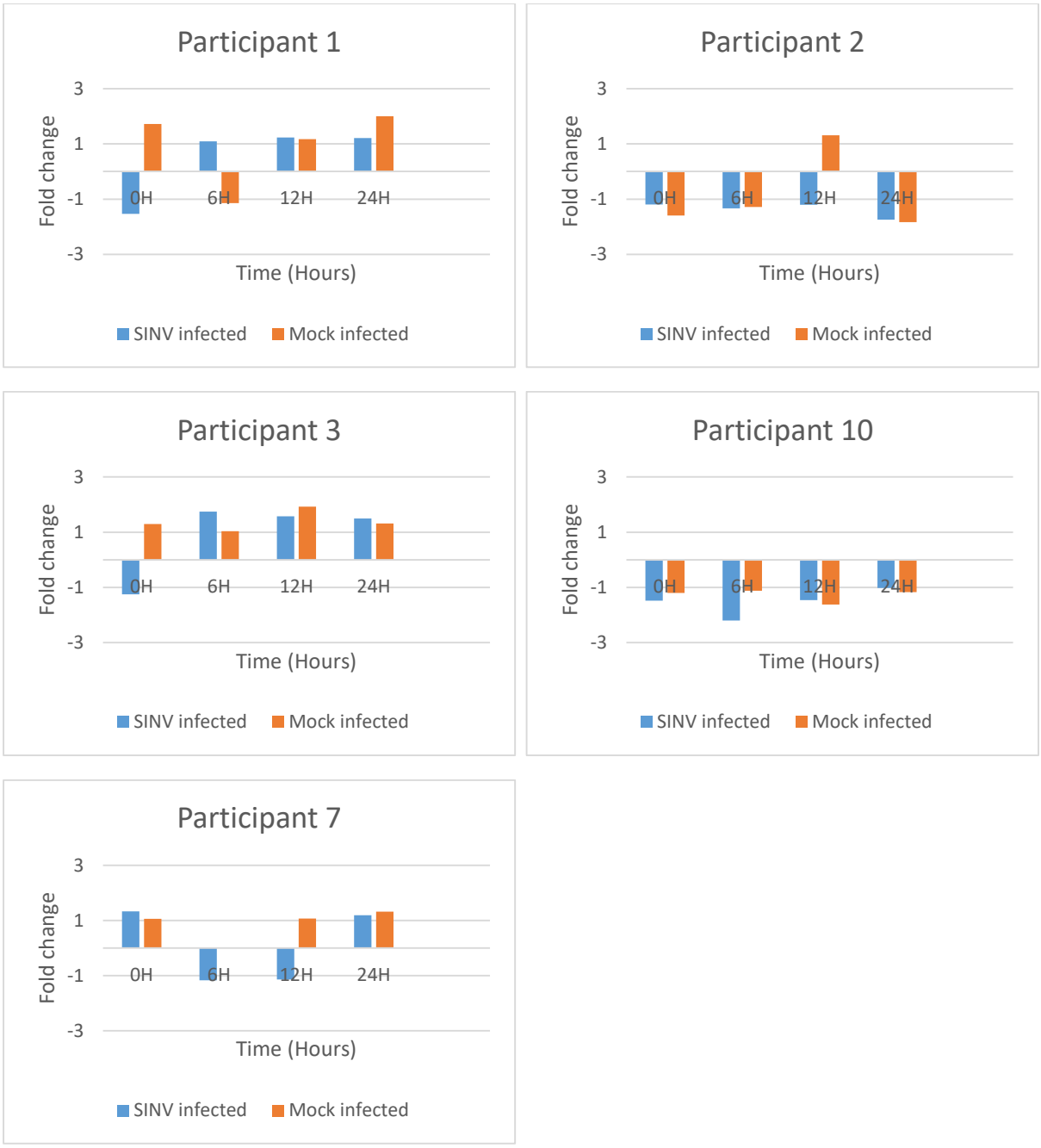


Figure 3.4: Fold change in concentration levels of TNF-α in macrophages of five participants infected with SINV and HI SINV relative to uninfected macrophages.

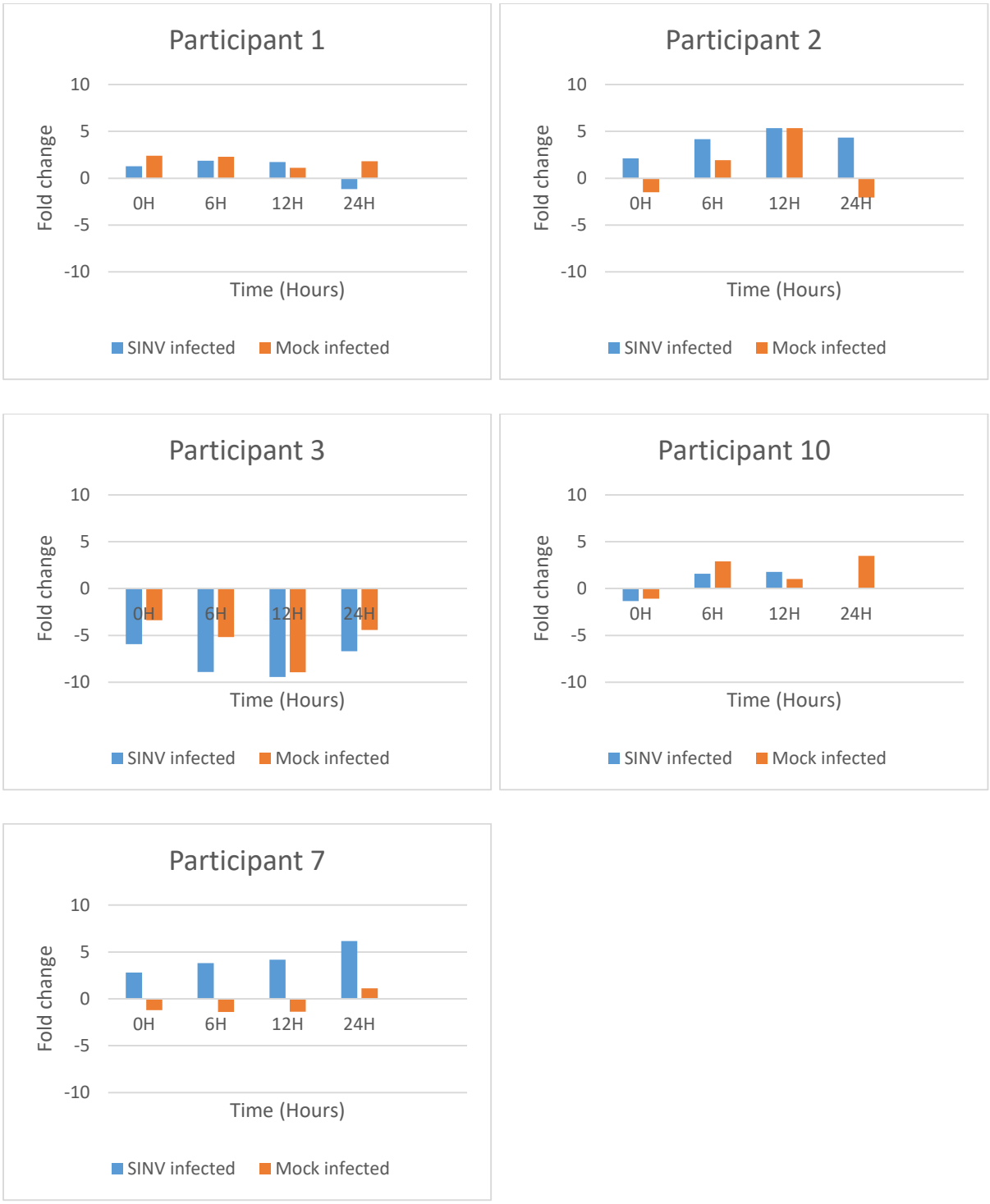


Figure 3.5: Fold change in concentration levels of IFN-α in macrophages of five participants infected with SINV and HI SINV relative to uninfected macrophages.

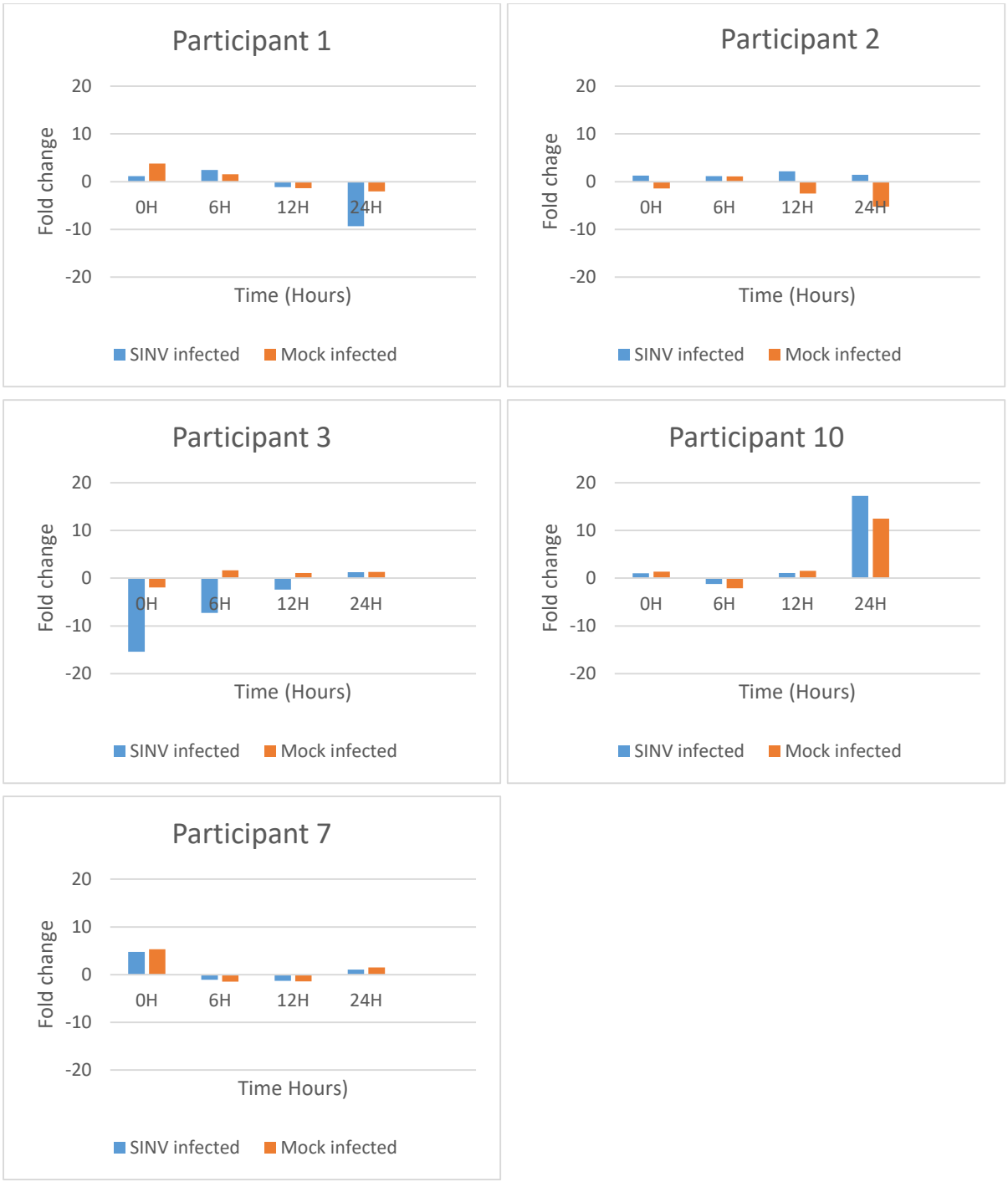


Figure 3.6: Fold change in concentration levels of IL-1β in macrophages of five participants infected with SINV and HI SINV relative to uninfected macrophages.

Figure 3.7: Fold change in concentration levels of IL-6 in macrophages of five participants infected with SINV and HI SINV relative to uninfected macrophages.

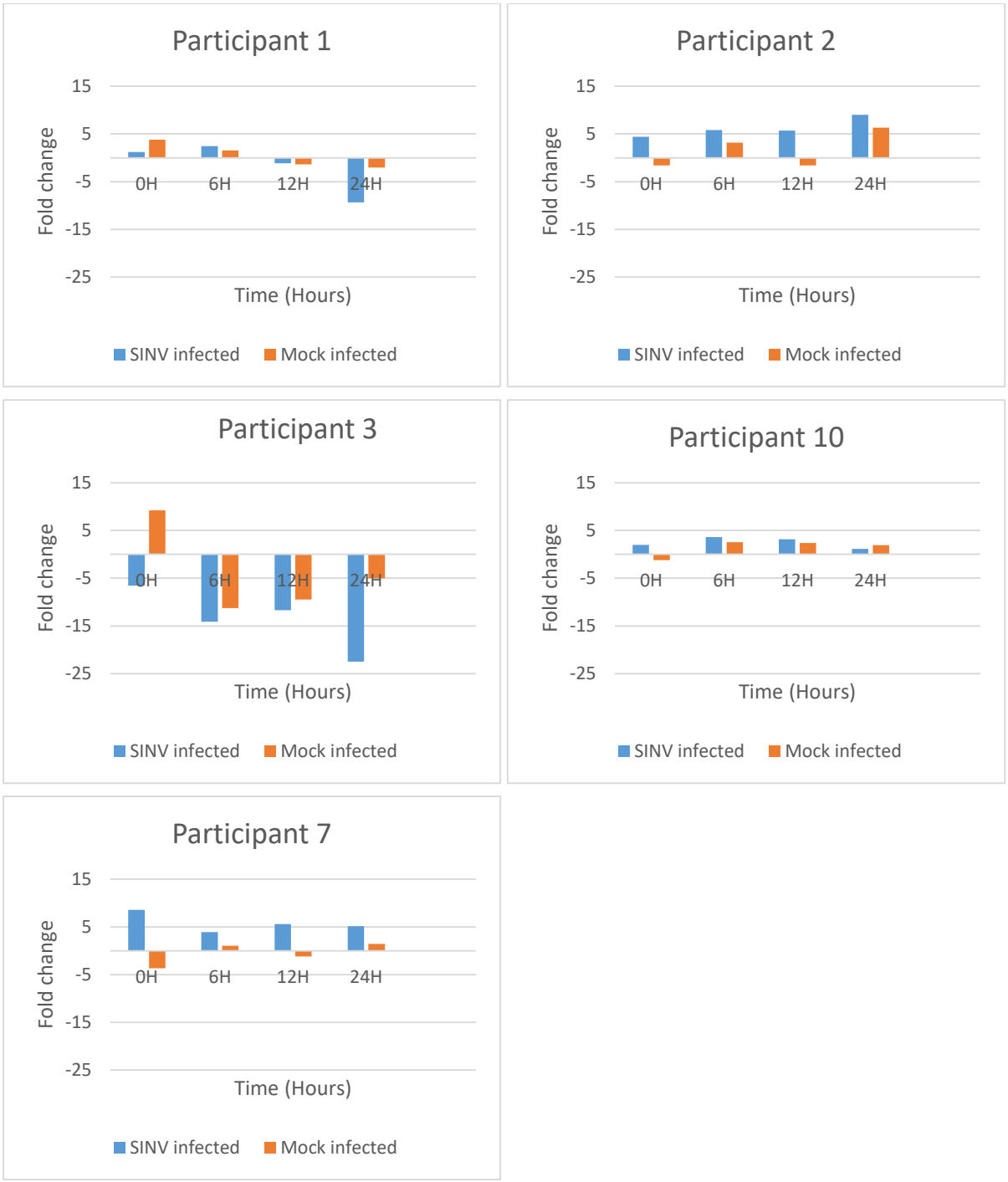


Figure 3.8: Fold change in concentration levels of IL-8 in macrophages of five participants infected with SINV and HI SINV relative to uninfected macrophages.

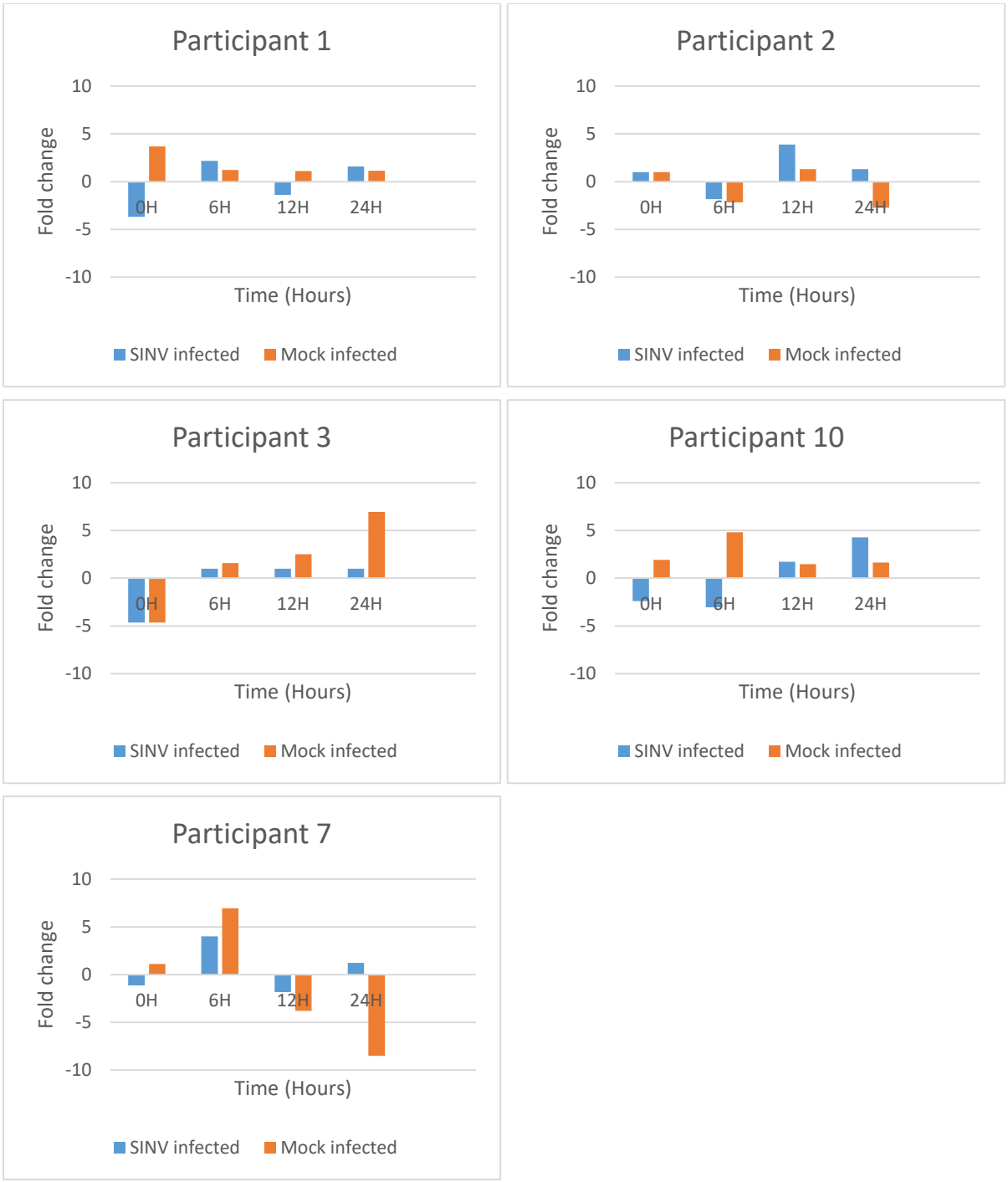


Figure 3.9: Fold change in concentration levels of IL-12 in macrophages of five participants infected with SINV and HI SINV relative to uninfected macrophages.

3.4 Summary

Studies have shown the importance of type I IFN in the protection against arthritogenic alphaviruses. Studies using mouse models have demonstrated that mice lacking IFN receptors succumb to more severe disease in comparison to those with an intact IFN response. Therefore to determine the importance of IFN in protecting against SINV, macrophages were infected with SINV post-treatment with ruxolitinib, either 2 hours prior to infection or at time 0. Ruxolitinib is a molecule that belongs to the Janus kinase group that results in the inhibition of type I IFN response. Viral kinetics were assessed using two-step qRT-PCR targeting negative strand RNA to determine the viral load at different times post-infection for 24 hours. A standard curve constructed from DNA (PCR product) amplified from RNA, extracted from Vero cells infected with SINV, was used for estimation of viral loads. Results show that pre-treatment of macrophages with ruxolitinib prior to infection resulted in a more than a tenfold increase of viral load at baseline for both macrophages pre-treated at 2 hours and those treated immediately prior to infection when compared to viral loads of macrophages infected with SINV without treatment with ruxolitinib in Chapter 2. In addition, pre-treatment of macrophages with ruxolitinib resulted in macrophages of 7/10 and 8/10 participants being susceptible to virus replication when treated immediately before infection and 2 hours before infection, respectively in comparison to the 5/10 that resulted in replication when macrophages were not pre-treated. Thus pre-treatment of macrophages with ruxolitinib, which targets the Jak component of type I IFN induction, resulted in higher viral loads of SINV in infected macrophages. Additionally, pre-treatment of macrophages at 2 hours prior to infection resulted in a greater fold change in viral load over the investigated time when compared to macrophages that were treated immediately before infection.

Previous studies have correlated pro-inflammatory cytokine secretion with disease severity during arthritogenic alphaviral infection. Macrophages from five participants with different virus replication kinetics were assessed for secretion of six proinflammatory cytokines known to be secreted by macrophages using cell-free virus supernatant from cultures of infected macrophages and control groups including HI SINV and uninfected macrophages. Cytokine profiles were measured using Elabscience sandwich-ELISA kits with plates already pre-coated with the specific pro-inflammatory cytokine, according to the manufacturer's instructions. SINV induced

responses were compared with levels observed using both HI SINV and uninfected macrophages. A standard curve was prepared for each cytokine, and concentration levels of the tested cytokines were determined using the standards. Macrophages from five participants which had different outcomes post-infection with SINV in objective 1 were selected, two showed viral replication, two had no viral replication, and one showed viral replication only when pre-treated with ruxolitinib.

Analysis of cytokine secretion in SINV infected macrophages showed that infection resulted in secretion of different cytokines between macrophages of different participants. Macrophages from participants 1 and 3 showed limited activation of cytokine secretion. In contrast, cytokine secretion from macrophages 2, 7 and 10 were activated by virus infection. Overall, the cytokine profiling of selected pro-inflammatory cytokines suggests that the *in vitro* infection of macrophages from participants 2, 7 and 10, stimulated secretion of IFN-α , IL-6 and IL-8 and increased levels of IL-1β were detected from infected macrophages from participant 2. These are key pro-inflammatory cytokines that have been shown to be secreted by macrophages during the early phase of the immune response to infection. Secretion of these cytokines mounts a strong innate immune response which may result in the inhibition of virus replication or control/limit replicating virus. In contrast, macrophages from participant 3 showed no evidence of viral replication, no activation of pro-inflammatory cytokines in infected cells however pro-inflammatory cytokines were demonstrated in uninfected cells. Some of these cytokines also function in normal cell development, and mediate apoptosis inhibition, hence the differentiation of these macrophages from monocytes and/or other physiological activation may have induced a response which stimulated the release of cytokines and also inhibited viral replication. Pro-inflammatory cytokines secreted at high levels are some of the key mediators secreted in the early phase of infection to control virus replication and dissemination.

Chapter 4: Discussion

Several large outbreaks of SINV have been recorded in South Africa (McIntosh et al., 1976; Jupp et al., 1986; Storm et al., 2014) and sporadic cases of SINV infection are known to occur annually. Infection with SINV is associated with a mild self-limiting febrile illness characterized by fever, rash, headache, and fatigue. However, infection can also result in severe prolonged joint pain that may persist for months to years. Previous studies have shown that infection with arthritogenic alphaviruses results in the production of neutralizing antibodies. However, these alone cannot protect against prolonged joint pain as levels of these antibodies have been shown to be similar in patients that develop chronic arthritis and in those that recover without the develop prolonged disease (Santiago et al., 2015). Therefore, other factors either than neutralizing antibodies are important in protection against arthritogenic alphavirus infection. The mechanism on how arthritogenic alphaviruses cause prolonged arthritis is not yet fully understood. Studies have implicated primary cells such as monocytes and macrophages as targets for the viruses, because these cells are found in circulation where they aid in the dissemination of the virus to different targets where they also function as inflammatory mediators, resulting in the secretion of cytokines that may play an essential role in the immunopathogenesis of arthritogenic alphaviruses.

To contribute to the understanding of the immunopathogenesis of arthritis induced by arthritogenic alphaviruses, the study focused on understanding the difference in replication of the virus *in vitro*, in macrophages obtained from different participants, and to determine the difference in cytokine secretion between these macrophages depending on viral kinetics. Additionally, the study also investigated the replication kinetics of SINV infection in macrophages when type I IFN responses were inhibited prior to infection with SINV, using ruxolitinib.

The innate immune response is known to play an essential role in protection against infection before the adaptive immune response is mounted. Monocytes and macrophages are innate immune cells, and previous studies have shown that they are target cells and their involvement in the pathogenesis of arthritogenic alphavirus, both in *in vitro* studies and using animal models. A two-step qRT-PCR was developed, targeting a conserved nsP2 region encoding the nsP2 protein. A standard curve was

generated using a PCR product of known concentration. When using PCR for quantification, amplification efficiency is very important. The error and efficiency rates of the standard curve were generated and were determined to be 0.00350 and 2.000, respectively. The error rate below 0.167 indicates that the assay is in good precision, with the 0.00350 obtained being within the acceptable range. The error value reflects how well all data fit the regression line. The efficiency of the assay was determined to be 2, meaning the number of targeted cDNA doubled with each cycle. For infection with SINV, macrophages from ten participants were differentiated from PBMCs stimulated with HM-CSF. Differentiated macrophages were infected with SINV at an MOI of 0.1, and viral loads were determined at different time intervals post-infection for 24 hours. The reverse transcription step was performed using the forward primer to target negative-strand RNA as an indication of replicating virus. SINV replication was suggested in macrophages from 5/10 participants, based on an increase in viral load over time (fold change). Using fold change also accommodated the presence of negative-strand RNA in the inoculum. Viral loads appeared to increase and peak at 24 hours post-infection. Previous studies have shown that viral titers increase and peak between 12 and 24 hours post-infection (Assunção-miranda et al., 2010; Her et al., 2010), similar to the replication kinetics observed in this study. For some participants (participants 2 and 8), there was a delay in viral replication with viral loads beginning to increase at 12 hours post-infection. For the remaining, there was little to no increase in viral loads or a decrease. The results suggest a variation in replication kinetics may occur during infection *in vitro*. Differences in susceptibility to virus replication may be due to different factors including whether an active IFN response is induced. Therefore to further investigate susceptibility, macrophages from the different participants were pre-treated with ruxolitinib prior to infection, which inhibited type I IFN pathway.

Type I IFNs are an important part of the innate immune system, and previous studies involving different viruses have demonstrated the importance of type I IFN system during the acute phase of viral infection (Her et al., 2010). Other studies have also shown that pre-treatment of cells with IFN prior to infection inhibits viral replication of different alphaviruses. Infection of CSM14.1 cell line with SINV post-treatment with IFN-γ protected cells from virus-induced cell death, caused reduced virus replication and resulted in an antiviral state (Burdeinick-kerr & Griffin, 2005). Most importantly $IFN^{-/-}$ knockout mice have been used to study the importance of IFN during the acute

phase of viral infection, these studies have shown that defective IFN response results in a more severe form of disease in mice compared to mice with an active IFN response. Adult mice deficient of either IFN-α or IFN-β (IFN-α/$\beta^{-/+}$) developed mild disease whereas those deficient of both receptors (IFN-α/$\beta^{-/-}$) developed a more severe disease when infected with CHIKV compared to wild type adult mice with active IFN receptors being resistant to CHIKV infection (Couderc et al., 2008). Assessment of IFN-α/β in protection against SINV replication and dissemination demonstrated that IFN-α/β deficient mice had increased viral replication in comparison to wild type mice which resulted in low-level replication and viral clearance within hours (Ryman et al., 2000). Therefore to study if type I IFN plays an important role in protecting against SINV replication during infection, macrophages from participants were pretreated *in vitro* with ruxolitinib to inhibit the JAK/STAT pathway for IFN production. If type I IFN plays a role in protection against SINV, its inhibition would result in increased virus replication in infected macrophages. Monocyte-derived macrophages from 10 participants were infected with SINV at an MOI of 0.1 after treatment with ruxolitinib, incubated for an hour to allow absorption of virus and interferon treatment, and viral replication was assessed at different time intervals post-infection by qRT-PCR. Pre-treatment resulted in viral loads being more than tenfold higher in macrophages of all participants at baseline in comparison to when the macrophages were infected with SINV without treatment with ruxolitinib prior to infection. These results suggest that because type I IFN was inhibited, thus more macrophages may have easily been infected with SINV, resulting in higher viral loads an hour after incubation with SINV. Higher viral loads were detected in macrophages from 7/10 and 8/10 participants when treated at time 0 of infection and 2 hours prior to infection, respectively. Suggesting that if interferon induction was inhibited the virus was able to replicate and that pretreatment with ruxolitinib further increased susceptibility resulting in higher viral loads for the majority of infected macrophages compared to those treated immediately prior to infection. The difference in fold change in viral loads was higher in ruxolitinib treated macrophages compared to untreated macrophages. These results suggest that IFN may plays an essential role in protection against SINV. Only macrophages from two participants resulted in little to no viral replication post-treatment with ruxolitinib at 2 hours prior to infection suggesting that there are other factors apart from type I IFN involved in protection against virus replication. Ruxolitinib treatment

rendered more macrophages susceptible to viral replication, this corresponds with several studies that illustrated that a strong IFN response protects the host from severe disease, resulting in decreased viral loads compared to high viral loads associated with the downregulation/ inhibition of IFN-α responses (Ryman et al., 2000; Couderc et al., 2008). Results also suggest that a strong IFN response during SINV infection could be important in the control of infection and viral loads as IFN inhibition resulted in increased viral loads in infected macrophages. Therefore, macrophages of participants 4, 5, and 7 may have had a strong IFN response, which resulted in a decrease in viral loads when they were not treated with ruxolitinib, suppressing virus replication. Collectively these results suggest that type I IFN is one of the important immune factors that protect against SINV replication because in the absence of IFN due to inhibition with ruxolitinib, infected macrophages resulted in higher viral loads. This has also been shown in other previous studies with different arthritogenic alphaviruses (Ryman et al., 2000; Gardner et al., 2010; Poddar et al., 2016). This study, in support of other previous studies, shows that during SINV infection robust type I IFN response is essential in limiting disease severity.

The gold standard for virus quantification and replication is plaque assay. Even though plaque assay has been the gold standard for virus detection and quantification, there are some disadvantages to the method, including that it is time-consuming, has a long incubation period, resulting in long turnaround time. In this study, we opted to screen using qRT-PCR because it is rapid, sensitive and can also be used for quantification. In addition, there were limited volumes of macrophage supernatant for analysis and hence viral loads. Further studies could benefit from confirmation using a plaque assay.

Studies using mouse models as well as *in vitro* studies using human and other mammalian macrophages and monocytes have shown the critical role of the host's pro-inflammatory response during alphavirus infection and its role in the damage of joints and musculoskeletal system during infection (Chaaitanya et al., 2011; Lidbury et al., 2008). Previous studies have shown that pro-inflammatory cytokines may play an important role in the development of chronic arthritis. One study showed that increased levels of IL-6, IL-1β, and decreased levels of RANTES may be used as markers for the severity of the disease and virus persistence (Ng et al., 2009). In this

study, cytokine profiles for six pro-inflammatory cytokines were evaluated at different time intervals for 24 hours post-infection from macrophages isolated from five participants selected based on different outcomes post-SINV infection. Levels of IL-1β at higher levels than the controls, were only recorded in macrophages from participant 2. IL-1β has been shown to be a marker for disease severity, suggesting that the cytokine would only be secreted in macrophages that show virus replication. IL-6 plays an essential role in the progression of inflammation and has been shown to play a role in virus persistence (Ng et al., 2009) and is a marker for disease severity. IL-6 was upregulated in macrophages from participants 2 and 7, which demonstrated limited replication during initial experiments. Pro-inflammatory cytokine IL-8 was also rapidly produced in macrophages of participant 2 and 7 throughout the investigated time and at some points in macrophages of participant 10. IL-12 is a pro-inflammatory cytokine that functions in the activation of NK cells resulting in an increase in antiviral activity, IL-12 was produced in macrophages of participants 1, 2, and there was poor secretion in macrophages of participant 10. Infection of macrophages with SINV had little influence on levels of TNF-α. TNF-α has been shown to play a role in the development of joint pain during human infection and has been shown to be secreted at high levels in the later stages of infection (Cavalheiro et al., 2016), which may explain why no significant difference between SINV infected and control groups were observed in this study. Robust production of IFN-α was seen in macrophages of participants 7 and 2. The robust IFN-α response may explain the quick decline in viral load in macrophages of participant 7 and the limited increase in viral load in macrophages of participant 2. This concurs with the increase in viral replication detected in participant 7 when pretreated with inhibitor. The most important role of IFN-α is the prevention of viral replication and controlling the severity of viral infection. The importance of IFN-α against alphavirus infection is also supported by the fact that viruses have developed mechanisms of inhibiting IFN responses (Göertz et al., 2018). The results from IFN cytokine assay suggest that a strong IFN response may be essential in the control of SINV replication, as a strong IFN response that resulted in macrophages of participant 7 may have prevented viral replication. These results show that infection with SINV trigged secretion of pro-inflammatory cytokines that may aid in the control of SINV infection. However the limited activation of the interferon system in participants 1 and 3 demonstrated when measuring cytokine levels and the low viral loads suggest alternative mechanisms for inhibiting viral replication. Similarly, inhibition of the

interferon system via the JAK/STAT pathway with limited increase in viral load also support the suggestion that multiple factors and alternative pathways could contribute to the innate response to infection.

This study has several limitations that need to be considered. Although the number of monocytes seeded per participant was the same, it was difficult to determine the number of differentiated macrophages. Differentiation was determined by visual observation, and hence there may have been some variation. Hence it was not accurate to compare viral loads between patients. The use of reference genes may have permitted between participant comparisons. However, it was selected to look at within participant variation and to normalize the data, virus replication was represented as fold change over time to determine an increase or decrease of viral load relative to time 0 for each experiment. Another limitation of the study was the lack of biological repeats. To ensure experimental reproducibility and to confirm the validity of the results, it is important that biological repeats are performed for each participant. However, due to the conditions of Ethics Committee approval, limited blood draws limited some of the studies. Therefore for the determination of viral kinetics, technical repeats were performed in triplicate for quantification. There was variation in technical repeats that was likely due to freezing and thawing of samples and as there were insufficient cells for repeating, the results were interpreted based on trends observed. Despite the variability there were distinct trends. In addition, although biological repeats for each experiment were not performed, viral load profiles and trends for each participant in the initial experiment (described in chapter 2) concurred with results shown for IFN inhibition (chapter 3) using differentiated monocytes from a second draw of blood. Finally, stimulation of the macrophages with a known immunogen could also have confirmed the responsiveness of the macrophages.

The mechanism by which arthritogenic alphaviruses cause chronic infection implicates monocytes and macrophages, in which infection results in the secretion of various cytokines involved in the immunopathogenesis. For future studies, it would be interesting to evaluate some of the alphavirus non-structural proteins to determine the immunological response of monocytes or macrophages when exposed to them and to compare those responses to those induced by virus infection in order to understand their importance in virus pathogenesis, looking at secretion of some of the important

cytokines and chemokines released due to infection to understand their role in chronic arthritis. It would also be interesting to compare these responses among different viruses such as SINV, CHIKV and RRV. Understanding the role of the common inflammatory mediators during alphavirus infection and the role of some of the non-structural proteins would help increase understanding of the pathogenesis of these viruses.

Currently, there are no specific vaccines or therapeutics for the prevention or treatment of arthritis induced by alphaviruses, treatment is only symptomatic. SINV induced arthralgia can be disabling and long-lasting, promoting social and economic impacts. Therefore, better understanding the replication kinetics of arthritogenic alphaviruses and pro-inflammatory responses post-infection can help contribute to the understanding of the pathogenesis of these viruses, bringing us closer to developing novel therapeutics and vaccines. Taken together, this study suggests that infection with SINV results in a strong pro-inflammatory response. Increased secretion of cytokine response seemed to control viral load, resulting in a decrease or slow increase over time. This indicates that high cytokine secretion may play a role in combating the viral load, but these cytokines have been implicated in severe and prolonged joint pain. The study also shows that there may be a difference in susceptibility between macrophages of different participants in terms of viral replication with an increase in viral load only observed in macrophages of 5/10 participants. Whether this is due to innate responses limiting the replication or possibly occurrence of apoptosis needs further investigation. Inhibition of type I IFN using ruxolitinib enhanced viral replication in infected macrophages, resulting in an increase in viral load in macrophages of 8/10 participants. Higher viral loads were also observed when macrophages were pre-treated with ruxolitinib. Results suggest ruxolitinib may prevent the induction of type I IFN in SINV infected macrophages. Such effects indirectly confirm that IFN-α/β may play a major role in protection against SINV infection.

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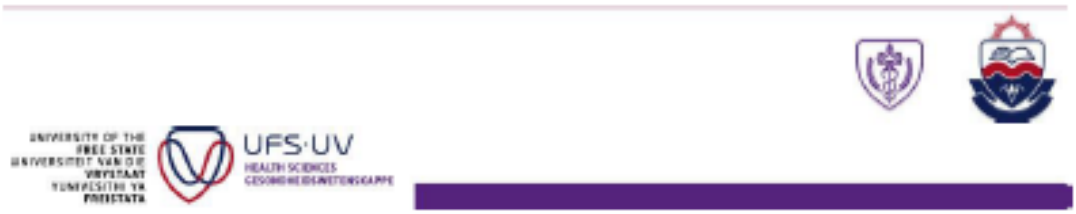
Appendix

Appendix A: Letter of approval from the Health Sciences Research Ethics Committee



06 April 2018

Health Sciences Research Ethics Committee
Faculty of Heath Sciences
University of the Free State.

Dear Chair

I grant permission to the following scientist:

Ms Matefo M Litabe

to conduct the in vitro MMed Sc research study entitled:

IN VITRO IMMUNE RESPONSES TO SINDBIS VIRUS

within the School of Pathology (**Division of Medical Virology**, Universitas Academic Laboratory Complex, Faculty of Health Sciences, Bloemfontein):

Supervisor:
Prof Felicity Burt

I wish Ms Litabe much success in her study.

Yours Sincerely

Jocelyn Naicker
Head: School of Pathology
Faculty of Health Sciences
University of the Free State
Tel: 051 405 2914 | Cell: 0829071925
jocelyn.naicker@nhls.ac.za | www.nhls.ac.za

Appendix B: Biosafety and Environmental Research Ethics Committee Approval Letter

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

Biosafety & Environmental Research Ethics Committee

05-Jul-2018

Dear Miss **Matefo Litabe**

Project Title: **In vitro immune responses to Sindbis virus**

Department: **Medical Microbiology (Bloemfontein Campus)**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Biosafety & Environmental Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2018/0002**

Please note the following:

1. **This ethical clearance is valid for one year from the issuance of this letter.**
2. **If the research takes longer than one year to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Biosafety & Environmental Research Ethics Committee
University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smitham@ufs.ac.za
Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa
P.O. Box 339 | Bloemfontein 9300 | South Africa | www.ufs.ac.za

Appendix C: Section 20 from the Department of Agriculture and Forestry

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4

Prof Felicity Burt
Department of Medical Microbiology and Virology
University of the Free State
Tel: 051 405 3348
E-mail: burtfj@ufs.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Dear Prof Burt

Your application dated 21 February 2017 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form dated 21 February 2017 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
4. A Veterinary Import Permit must be obtained prior to the importation of Vero cells CCL-81 from the American Tissue Culture Collection (ATCC);

5. All other cell lines and foetal bovine serum used in this study must have been legally imported in compliance with all relevant legislation;
6. Only the local Sindbis virus strain SAAR 86 may be used in this study;
7. No live animals may be used in this study;
8. All potentially infectious material utilised or generated during or by the study is to be destroyed at completion of the study by incineration;
9. Only a registered waste disposal company may be used for the removal of waste generated by or during the study;
10. Records must be kept for five years for auditing purposes;
11. A dispensation for the storage of Vero cells and cell culture derived SAAR 86 Sindbis virus is attached.

Title of research/study: Arboviruses and Zoonotic Diseases: Innate Responses
Researcher: Prof Felicity Burt
Institution: Department of Medical Microbiology and Virology; University of the Free State
Permit Expiry date: 30 March 2023
Our ref Number: 12/11/1/4
Your ref: UFS 95/2016

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -08- 23

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4

Prof Felicity Burt
Department of Medical Microbiology and Virology
University of the Free State
Tel: 051 405 3348
E-mail: burtfj@ufs.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ARBOVIRUSES AND ZOONOTIC DISEASES: INNATE RESPONSES"

A dispensation is hereby granted on Point 8 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Vero cells and cell culture derived SAAR 86 Sindbis virus must be stored under access control at the Department of Medical Microbiology and Virology; University of the Free State

ii) Vero cells and cell culture derived SAAR 86 Sindbis virus may not be outsourced or used for further research without prior written approval from the Director: Animal Health.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2017 -08- 23

Appendix D: Viral loads obtained post-infection with Sindbis virus

Table A1: qRT-PCR estimated viral load results following infection of macrophages with SINV S.A.AR86

Participant	Time post-infection	C_p value	Estimated viral load (copy number/µl)	Average viral load (copy number/µl) ± standard deviation
Participant 1	0 hours	31.44 29.54 29.15	$1.82x10^5$ $6.14x10^6$ $8.78\ x10^6$	$5.03x10^6$ $+/-4.40x10^6$
	6 hours	27.44 28.54 29.25	$7.28x10^6$ $1.28x10^7$ $8.06x10^6$	$9.38x10^6$ $+/-2.99x10^6$ ↑
	12 hours	25.93 30.76 30.10	$2.67x10^7$ $4.97x10^5$ $3.65x10^6$	$1.03x10^7$ $+/-1.43x10^7$ ↑
	24 hours	29.49 29.31 28.91	$6.01x10^5$ $7.65x10^6$ $1.1x10^7$	$6.42x10^6$ $+/-5.31x10^6$ ↑
Participant 2	0 hours	30.08 29.24 27.97	$5.99x10^5$ $8.55x10^5$ $1.81x10^6$	$1.09x10^6$ $+/-6.38x10^5$
	6 hours	30.04 28.50 29.28	$6.22x10^5$ $1.32x10^6$ $8.38x10^5$	$9.27x10^5$ $+/-3.57x10^5$ ↓
	12 hours	28.88 26.60 28.98	$1.82x10^6$ $4.04x10^6$ $9.96x10^5$	$5.27x10^6$ $+/-4.64x10^5$ ↑
	24 hours	29.60 29.73 28.09	$9.44x10^5$ $6.24x10^5$ $1.68x10^6$	$1.08x10^6$ $+/-5.41x10^5$ ↑
Participant 3	0 hours	28.93 30.26 30.52	$1.00x10^6$ $1.77x10^5$ $1.37x10^5$	$4.38x10^5$ $+/-4.87x10^5$

	6 hours	29.20 30.51 32.77	$5.60x10^5$ $1.39x10^5$ $1.55x10^4$	$2.37x10^5$ $+/-2.85x10^5$
	12 hours	29.87 32.17 30.45	$3.01x10^5$ $2.82x10^4$ $1.47x10^4$	$1.14x10^5$ $+/-1.62x10^5$
	24 hours	30.43 30.14 30.80	$1.77x10^5$ $1.97x10^5$ $1.05x10^5$	$1.60x10^5$ $+/-4.84x10^4$
Participant 4	0 hours	28.71 26.88 28.83	$1.02x10^5$ $7.91x10^6$ $1.40x10^6$	$3.14x10^6$ $+/-4.18x10^6$
	6 hours	29.89 29.87 29.15	$3.45x10^4$ $5.38x10^5$ $1.05x10^6$	$5.41x10^5$ $+/-2.73x10^5$
	12 hours	30.14 29.70 31.03	$2.74x10^4$ $6.31x10^5$ $3.84x10^5$	$3.47x10^5$ $+/-3.03x10^5$
	24 hours	28.71 29.15 28.27	$1.02x10^5$ $1.05x10^6$ $2.32x10^6$	$1.16x10^6$ $+/-1.11x10^6$
Participant 5	0 hours	30.13 31.10 28.17	$1.59x10^5$ $8.26x10^4$ $1.25x10^6$	$4.97x10^5$ $+/-6.53x10^5$
	6 hours	30.14 30.74 30.89	$3.26x10^5$ $1.16x10^4$ $1.05x10^4$	$1.16x10^5$ $+/-1.82x10^5$
	12 hours	29.20 31.06 30.91	$3.77x10^5$ $8.57x10^4$ $9.93x10^4$	$1.87x10^5$ $+/-1.64x10^5$
	24 hours	30.31 30.93 29.22	$1.34x10^5$ $9.75x10^4$ $4.82x10^5$	$2.38x10^5$ $+/-2.12x10^5$
Participant 6	0 hours	30.97 28.97 29.29	$8.99x10^4$ $8.63x10^4$ $6.43x10^4$	$8.02x10^4$ $+/-1.39x10^4$

	6 hours	30.09 29.14 29.03	$2.08x10^5$ $7.36x10^4$ $8.14x10^4$	$1.21x10^5$ $+/-7.54x10^4$
	12 hours	30.17 28.63 28.87	$1.92x10^5$ $1.17x10^5$ $9.46x10^4$	$1.35x10^5$ $+/-5.10x10^4$
	24 hours	30.75 28.63 29.75	$1.11x10^5$ $1.17x10^5$ $1.29x10^6$	$5.06x10^5$ $+/-6.79x10^5$
Participant 7	0 hours	28.33 24.24 27.66	$6.74x10^5$ $2.87x10^6$ $1.67x10^5$	$1.24x10^6$ $+/-1.44x10^6$
	6 hours	29.46 28.05 27.60	$2.41x10^5$ $1.13x10^5$ $1.54x10^5$	$1.69x10^5$ $+/-6.54x10^4$
	12 hours	30.48 27.90 28.00	$2.38x10^5$ $1.28x10^5$ $1.17x10^5$	$1.61x10^5$ $+/-6.69x10^4$
	24 hours	29.70 27.66 27.88	$1.93x10^5$ $1.58x10^5$ $1.30x10^5$	$1.60x10^5$ $+/-3.16x10^4$
Participant 8	0 hours	30.29 30.47 29.88	$2.58x10^4$ $2.46x10^4$ $4.31x10^4$	$3.12x10^4$ $+/-1.04x10^3$
	6 hours	29.60 31.12 30.91	$4.90x10^4$ $1.33x10^4$ $1.63x10^4$	$2.62x10^4$ $+/-1.98x10^4$
	12 hours	30.43 29.80 29.55	$2.26x10^4$ $4.64x10^4$ $5.83x10^4$	$4.24x10^4$ $+/-1.82x10^4$
	24 hours	28.90 25.36 27.50	$9.37x10^4$ $2.32x10^6$ $3.71x10^5$	$9.28x10^5$ $+/-1.21x10^6$
Participant 9	0 hours	29.68 29.54 29.23	$6.46x10^4$ $5.69x10^4$ $7.58x10^4$	$6.58x10^4$ $+/-9.50x10^3$

	6 hours	30.58 27.74 29.56	$1.94x10^4$ $2.89x10^5$ $5.60x10^4$	$1.21x10^5$ $+/-1.46x10^5$ ↑
	12 hours	27.37 29.45 29.70	$3.60x10^5$ $6.17x10^4$ $4.91x10^4$	$1.57x10^5$ $+/-1.76x10^5$ ↑
	24 hours	30.17 26.92 29.70	$2.85x10^4$ $5.93x10^5$ $8.47x10^4$	$2.35x10^5$ $+/-3.11x10^5$ ↑
Participant 10	0 hours	30.29 26.51 29.71	$2.58x10^6$ $7.71x10^5$ $4.43x10^4$	$1.13x10^6$ $+/-1.31x10^6$
	6 hours	28.13 29.85 28.75	$9.68x10^5$ $3.88x10^4$ $1.07x10^5$	$3.71x10^5$ $+/-5.18x10^5$ ↓
	12 hours	30.26 29.13 28.89	$1.37x10^5$ $2.73x10^4$ $9.39x10^4$	$8.61x10^4$ $+/-5.53x10^4$ ↓
	24 hours	30.83 29.13 30.00	$8.04x10^4$ $7.55x10^4$ $3.38x10^4$	$6.32x10^4$ $+/-2.56x10^4$ ↓

The green arrows represent an increase in viral load, whereas the red represents a decrease in viral RNA titer relative to the viral load at time 0. Results are represented as mean ±standard deviation.

Table A2: qRT-PCR viral load results following pre-treatment of macrophages with ruxolitinib immediately (0 hours) prior to infection

Participant	Time after infection	C_p value	Estimated viral load (copy number/µl)	Average viral load (copy number/µl) +/- standard deviation
Participant 1	0 hours	29.90 29.19 28.78	$2.19x10^7$ $2.94x10^7$ $4.24x10^7$	$3.12x10^7$ $+/-1.04x10^7$
	6 hours	28.80 29.23 29.49	$6.07x10^7$ $2.82x10^7$ $2.23x10^7$	$3.71x10^7$ $+/-2.07x10^7$ ↑
	12 hours	26.86 29.22 28.96	$3.39x10^7$ $2.86x10^7$ $3.62x10^7$	$3.29x10^7$ $+/-3.90x10^6$ ↑
	24 hours	26.83 29.76 29.13	$3.48x10^8$ $1.73x10^7$ $3.08x10^7$	$1.32x10^8$ $+/-1.87x10^6$ ↑
Participant 2	0 hours	30.96 27.23 30.57	$1.23x10^8$ $1.54x10^8$ $7.37x10^6$	$9.48x10^7$ $+/-7.73x10^7$
	6 hours	31.00 26.59 29.52	$1.18x10^8$ $6.27x10^7$ $1.97x10^7$	$6.68x10^7$ $+/-4.93x10^7$ ↓
	12 hours	30.71 29.76 28.57	$1.56x10^8$ $1.57x10^7$ $4.69x10^7$	$7.29x10^7$ $+/-7.37x10^7$ ↓
	24 hours	30.66 30.57 26.31	$1.64x10^8$ $7.37x10^6$ $3.42x10^8$	$1.71x10^8$ $+/-1.67x10^8$ ↑
Participant 3	0 hours	29.92 24.20 24.19	$4.68x10^7$ $3.63x10^8$ $3.66x10^8$	$2.59x10^8$ $+/-1.83x10^8$
	6 hours	28.19 28.92 27.24	$3.55x10^7$ $7.96x10^6$ $3.58x10^7$	$2.64x10^7$ $+/-1.60x10^7$ ↓
	12 hours	30.33 29.27 29.25	$3.20x10^7$ $4.60x10^6$ $4.65x10^6$	$1.38x10^7$ $+/-1.58x10^7$ ↓
	24 hours	27.54 27.87 28.32	$6.07x10^7$ $1.62x10^7$ $1.08x10^7$	$2.29x10^7$ $+/-2.74x10^7$ ↓
Participant 4	0 hours	29.63 29.70 29.67	$6.75x10^6$ $3.93x10^6$ $4.04x10^6$	$4.91x10^6$ $+/-1.60x10^6$
	6 hours	29.76 29.15	$8.53x10^7$ $6.52x10^7$	$3.29x10^7$ $+/-4.54x10^7$ ↑

		29.08	$6.96x10^6$	
	12 hours	27.71 28.42 29.47	$5.50x10^8$ $1.26x10^7$ $4.83x10^6$	$1.89x10^8$ $+/-3.13x10^8$
	24 hours	29.13 27.98 28.53	$1.53x10^8$ $1.88x10^7$ $1.15x10^7$	$6.11x10^7$ $+/-7.97x10^7$
Participant 5	0 hours	29.45 26.93 30.27	$2.57x10^8$ $3.62x10^7$ $1.76x10^6$	$9.83x10^7$ $+/-1.38x10^8$
	6 hours	28.68 28.33 28.82	$5.23x10^8$ $1.05x10^7$ $6.78x10^6$	$1.80x10^8$ $+/-2.97x10^8$
	12 hours	29.00 29.14 26.86	$3.89x10^8$ $5.04x10^6$ $3.83x10^7$	$1.44x10^8$ $+/-2.13x10^8$
	24 hours	28.91 29.32 28.75	$4.21x10^8$ $4.28x10^6$ $7.17x10^6$	$1.44x10^8$ $+/-2.40x10^8$
Participant 6	0 hours	26.59 28.87 28.49	$9.43x10^7$ $1.61x10^7$ $5.58x10^6$	$3.87x10^7$ $+/-4.85x10^7$
	6 hours	29.68 28.87 28.84	$6.46x10^6$ $3.95x10^6$ $4.06x10^6$	$4.82x10^6$ $+/-1.42x10^6$
	12 hours	29.67 26.25 26.36	$3.63x10^6$ $3.98x10^7$ $3.62x10^7$	$2.65x10^7$ $+/-1.99x10^7$
	24 hours	28.73 28.00 28.10	$1.55x10^7$ $8.66x10^6$ $1.67x10^7$	$1.36x10^7$ $+/-4.34x10^7$
Participant 7	0 hours	28.68 29.01 28.70	$1.25x10^8$ $2.73x10^7$ $3.61x10^7$	$6.28x10^7$ $+/-5.40x10^7$
	6 hours	29.83 28.62 28.77	$4.37x10^7$ $3.88x10^7$ $3.39x10^7$	$3.88x10^7$ $+/-4.90x10^6$
	12 hours	29.53 28.65 28.75	$5.80x10^7$ $3.79x10^7$ $3.46x10^7$	$4.35x10^7$ $+/-1.27x10^7$
	24 hours	26.63 28.47 20.08	$7.73x10^8$ $4.46x10^7$ $6.29x10^7$	$2.94x10^8$ $+/-4.15x10^8$
Participant 8	0 hours	29.81 29.19 29.42	$5.60x10^7$ $8.92x10^7$ $7.26x10^7$	$7.25x10^7$ $+/-1.65x10^7$
	6 hours	29.68	$1.02x10^8$	$8.59x10^7$

		29.61 29.12	$6.06x10^7$ $9.51x10^7$	$+/-2.22x10^7$ ↑
	12 hours	29.00 26.12 28.45	$1.19x10^8$ $1.35x10^8$ $1.76x10^8$	$1.43x10^8$ $+/-2.94x10^7$ ↑
	24 hours	26.16 28.19 28.86	$1.45x10^9$ $2.22x10^8$ $1.21x10^8$	$1.63x10^8$ $+/-5.28x10^7$ ↑
Participant 9	0 hours	29.90 30.08 29.70	$1.96x10^7$ $2.81x10^6$ $3.99x10^6$	$8.80x10^6$ $+/-9.37x10^6$
	6 hours	28.88 29.74 29.70	$5.03x10^7$ $3.86x10^6$ $3.89x10^6$	$1.94x10^7$ $+/-2.68x10^7$ ↑
	12 hours	28.42 29.84 29.59	$7.57x10^7$ $3.15x10^6$ $4.42x10^6$	$2.78x10^7$ $+/-4.15x10^7$ ↑
	24 hours	29.61 28.73 29.27	$2.58x10^7$ $9.69x10^6$ $5.93x10^6$	$1.38x10^7$ $+/-1.06x10^7$ ↑
Participant 10	0 hours	30.54 28.54 28.62	$1.04x10^7$ $1.22x10^7$ $1.14x10^7$	$1.13x10^7$ $+/-9.02x10^5$
	6 hours	28.65 28.61 28.47	$3.79x10^8$ $1.14x10^7$ $1.30x10^7$	$1.34x10^8$ $+/-2.12x10^8$ ↑
	12 hours	30.32 28.81 28.74	$8.13x10^7$ $1.04x10^7$ $1.02x10^7$	$3.40x10^7$ $+/-4.10x10^7$ ↑
	24 hours	30.93 28.81 28.43	$7.15x10^6$ $9.63x10^6$ $1.35x10^7$	$1.01x10^7$ $+/-3.20x10^6$ ↓

The green arrows represent an increase in viral load, whereas the red represents a decrease in viral load relative to viral loads at baseline (time=0). Final results are represented as mean ±standard deviation.

Table A3: qRT-PCR viral load results following pre-treatment macrophages with ruxolitinib 2 hours prior to infection

Participant	Time after infection	C_p value	Estimated viral loads (copy number/µl)	Average viral load (copy number/µl) +/- standard deviation
Participant 1	0 hours	29.59 27.72 29.21	$2.92x10^7$ $1.89x10^7$ $4.49x10^6$	$1.77x10^7$ $+/-1.22x10^7$
	6 hours	28.90 29.55 28.53	$5.54x10^7$ $3.57x10^6$ $9.08x10^6$	$2.27x10^7$ $+/-2.85x10^7$ ↑
	12 hours	28.79 28.75 28.32	$9.47x10^7$ $7.44x10^6$ $1.10x10^7$	$3.77x10^7$ $+/-4.94x10^7$ ↑
	24 hours	28.26 28.85 28.49	$9.84x10^7$ $6.79x10^6$ $9.47x10^6$	$3.82x10^7$ $+/-5.12x10^7$ ↑
Participant 2	0 hours	30.08 29.55 29.54	$2.05x10^7$ $5.69x10^6$ $5.75x10^6$	$1.06x10^7$ $+/-8.53x10^6$
	6 hours	30.51 28.69 29.54	$2.69x10^7$ $1.26x10^7$ $5.78x10^6$	$1.51x10^7$ $+/-1.08x10^7$ ↑
	12 hours	27.70 29.55 27.93	$1.46x10^7$ $5.72x10^6$ $2.48x10^7$	$1.50x10^7$ $+/-9.55x10^6$ ↑
	24 hours	26.01 28.46 28.63	$6.35x10^8$ $1.54x10^7$ $1.32x10^7$	$2.21x10^8$ $+/-3.58x10^8$ ↑
Participant 3	0 hours	29.69 28.27 27.48	$4.08x10^8$ $1.31x10^7$ $2.64x10^7$	$1.49x10^8$ $+/-2.24x10^8$
	6 hours	30.43 27.78 26.18	$2.02x10^8$ $2.03x10^7$ $8.16x10^7$	$1.01x10^8$ $+/-9.24x10^7$ ↓
	12 hours	31.25 28.92 25.09	$9.27x10^7$ $7.30x10^7$ $2.03x10^7$	$4.01x10^7$ $+/-4.60x10^7$ ↓
	24 hours	30.29 29.05 28.06	$2.32x10^8$ $6.47x10^6$ $1.59x10^7$	$8.48x10^7$ $+/-1.28x10^8$ ↓
Participant 4	0 hours	29.95 29.07 30.00	$7.19x10^7$ $6.73x10^6$ $2.83x10^6$	$2.72x10^7$ $+/-3.88x10^7$
	6 hours	28.25	$3.14x10^8$	$1.07x10^8$

		29.72 30.42	$3.67x10^{6}$ $1.92x10^{6}$	$+/-1.80x10^{8}$
	12 hours	25.71 26.59 29.75	$3.09x10^{8}$ $6.27x10^{7}$ $3.58x10^{6}$	$1.25x10^{8}$ $+/-1.62x10^{8}$
	24 hours	25.58 24.59 29.92	$3.44x10^{9}$ $3.35x10^{8}$ $1.11x10^{8}$	$1.30x10^{9}$ $+/-1.86x10^{9}$
Participant 5	0 hours	31.01 28.73 28.62	$5.93x10^{7}$ $7.53x10^{6}$ $8.35x10^{6}$	$2.15x10^{7}$ $+/-2.97x10^{7}$
	6 hours	29.94 30.14 28.77	$1.64x10^{8}$ $2.05x10^{6}$ $7.27x10^{6}$	$5.78x10^{7}$ $+/-9.20x10^{7}$
	12 hours	26.54 28.51 27.43	$3.44x10^{9}$ $9.25x10^{6}$ $2.41x10^{7}$	$1.16x10^{9}$ $+/-1.98x10^{9}$
	24 hours	30.02 27.68 28.62	$1.52x10^{8}$ $1.93x10^{6}$ $8.37x10^{6}$	$5.41x10^{7}$ $+/-8.48x10^{7}$
Participant 6	0 hours	29.63 29.79 29.77	$6.75x10^{6}$ $1.52x10^{7}$ $1.55x10^{7}$	$1.25x10^{7}$ $+/-4.97x10^{6}$
	6 hours	29.85 26.24 27.94	$2.09x10^{7}$ $3.58x10^{8}$ $8.17x10^{7}$	$1.54x10^{8}$ $+/-1.80x10^{8}$
	12 hours	25.86 29.27 27.67	$1.90x10^{8}$ $2.46x10^{7}$ $1.04x10^{8}$	$1.06x10^{8}$ $+/-8.27x10^{7}$
	24 hours	29.50 27.78 27.47	$7.62x10^{6}$ $9.39x10^{7}$ $1.24x10^{8}$	$7.51x10^{7}$ $+/-6.04x10^{7}$
Participant 7	0 hours	29.50 28.60 29.06	$7.62x10^{6}$ $9.34x10^{6}$ $6.14x10^{6}$	$7.70x10^{6}$ $+/-1.60x10^{6}$
	6 hours	29.65 26.60 27.01	$5.15x10^{7}$ $5.44x10^{7}$ $3.80x10^{7}$	$4.80x10^{7}$ $+/-8.75x10^{6}$
	12 hours	29.34 26.16 28.76	$6.86x10^{7}$ $7.92x10^{7}$ $8.05x10^{6}$	$5.20x10^{7}$ $+/-3.84x10^{7}$
	24 hours	29.19 27.71 28.25	$7.86x10^{7}$ $2.06x10^{7}$ $1.28x10^{7}$	$3.73x10^{7}$ $+/-3.60x10^{7}$
Participant 8	0 hours	28.60 28.16	$1.71x10^{8}$ $1.36x10^{7}$	$7.70x10^{7}$ $+/-8.61x10^{7}$

		27.21	$3.18x10^7$	
	6 hours	28.83 26.12 27.53	$1.39x10^8$ $8.15x10^7$ $2.40x10^7$	$8.15x10^7$ $+/-5.75x10^7$ ↑
	12 hours	27.78 28.83 27.58	$3.54x10^8$ $7.54x10^6$ $2.30x10^7$	$1.28x10^8$ $+/-1.96x10^8$ ↑
	24 hours	26.73 24.65 27.68	$8.88x10^8$ $2.78x10^8$ $2.11x10^7$	$1.29x10^8$ $+/-1.33x10^8$ ↑
Participant 9	0 hours	30.05 27.77 30.33	$1.70x10^7$ $2.24x10^7$ $2.35x10^6$	$1.39x10^7$ $+/-1.04x10^7$
	6 hours	29.90 29.21 29.43	$1.97x10^7$ $6.11x10^6$ $5.44x10^6$	$1.04x10^7$ $+/-8.05x10^6$ ↓
	12 hours	26.81 29.21 28.59	$3.16x10^8$ $6.65x10^6$ $1.17x10^8$	$1.11x10^8$ $+/-1.77x10^8$ ↑
	24 hours	26.69 30.21 27.53	$3.48x10^8$ $2.62x10^6$ $2.76x10^7$	$1.26x10^8$ $+/-1.93x10^8$ ↑
Participant 10	0 hours	27.56 28.09 28.10	$1.57x10^8$ $1.76x10^7$ $1.74x10^7$	$6.40x10^7$ $+/-8.05x10^7$
	6 hours	30.48 28.50 28.31	$1.09x10^7$ $1.22x10^7$ $1.45x10^7$	$1.25x10^7$ $+/-1.82x10^6$ ↓
	12 hours	29.91 28.51 27.97	$1.87x10^7$ $1.20x10^7$ $1.96x10^7$	$1.68x10^7$ $+/-4.15x10^6$ ↓
	24 hours	29.76 28.66 27.97	$2.15x10^7$ $1.05x10^7$ $1.15x10^7$	$1.45x10^7$ $+/-6.08x10^6$ ↓

The green arrows represent an increase in viral RNA load, whereas the red represents a decrease in viral RNA load relative to viral load at baseline (time=0). Final results are represented as mean ±standard deviation.

Appendix E: ELISA Plates Raw Data

IFN-α

<>	1	2	3	4	5	6	7	8	9	10	11	12
a	2,162	2,067	0,335	0,449	0,529	0,365	1,281	0,524	0,52	0,389	0,631	0,559
b	1,151	1,151	0,29	0,302	0,363	0,402	0,677	0,597	1,587	1,986	1,519	1,452
c	1,067	1,058	0,28	0,803	1,988	0,288	1,222	0,352	0,481	0,484	0,452	0,394
d	0,694	0,773	0,252	0,261	0,277	0,286	0,567	0,341	0,352	0,285	0,364	0,22
e	0,429	0,472	0,836	1,267	1,484	1,153	1,23	0,29	1,003	1,16	0,414	0,759
f	0,417	0,497	0,329	0,798	0,727	1,12	0,882	0,345	0,683	0,708	0,416	0,507
g	0,291	0,296	0,684	1,284	2,221	2,684	2,592	0,294	0,349	0,497	0,599	1,062
h	0,296	0,235	0,327	0,434	0,631	0,546	0,493					

TNF-α

<>	1	2	3	4	5	6	7	8	9	10	11	12
a	2,674	2,1	0,34	0,535	0,475	0,564	0,462	0,653	0,458	0,459	0,833	0,701
b	1,611	1,793	0,442	0,502	0,414	0,493	0,488	0,552	0,513	0,499	0,538	0,625
c	0,882	0,952	0,447	0,539	0,47	0,519	0,492	0,624	0,632	0,5659	0,827	0,508
d	0,687	0,608	0,385	0,722	0,5	0,646	0,603	0,533	0,46	0,579	0,577	0,636
e	0,573	0,613	0,444	0,476	0,371	0,473	0,526	0,386	0,366	0,457	0,554	0,578
f	0,417	0,585	0,44	0,573	0,426	0,502	0,46	0,499	0,624	0,599	0,563	1,001
g	0,634	0,585	0,64	0,521	0,533	0,566	0,711	0,54	0,585	0,619	0,62	0,66
h	0,209	0,399	0,517	0,586	0,586	0,506	0,709					

IL-1β

<>	1	2	3	4	5	6	7	8	9	10	11	12
a	2,36	2,277	0,156	0,206	0,265	0,296	0,271	0,248	0,359	0,312	0,309	0,258
b	2,29	2,077	0,219	0,164	0,161	0,148	0,208	0,259	0,188	0,271	0,363	0,205
c	1,56	1,227	0,177	0,22	0,117	0,119	0,158	0,219	0,206	0,169	0,227	0,29
d	0,844	0,747	0,094	0,1	0,128	0,188	0,15	0,181	0,298	0,204	0,195	0,366
e	0,558	0,426	0,273	0,215	0,193	0,167	0,16	0,228	0,247	0,21	0,157	0,443
f	0,337	0,346	0,272	0,177	0,263	0,136	0,162	0,223	0,286	0,199	0,086	0,353
g	0,312	0,269	0,273	0,35	0,266	0,205	0,202	0,296	0,283	0,251	0,254	0,406
h	0,199	0,111	0,122	0,376	0,324	0,197	0,199					

IL-6

<>	1	2	3	4	5	6	7	8	9	10	11	12
a	2,546	2,317	0,279	0,379	0,593	0,261	1,143	0,164	0,197	0,134	0,291	0,219
b	1,854	2,102	0,186	0,451	0,237	0,635	0,727	0,979	1,899	2,185	1,837	2,397
c	1,326	1,35	0,455	1,083	2,68	1,255	1,771	0,481	0,453	0,46	0,521	0,693
d	1,097	0,863	0,213	0,229	0,256	0,19	0,738	0,222	0,332	0,538	0,545	0,548
e	0,655	0,682	1,801	2,32	2,453	2,039	1,678	0,923	2,557	2,767	1,569	1,185
f	0,677	0,809	0,509	2,193	2,279	1,935	2,772	0,437	1,298	1,309	1,01	1,033
g	0,391	0,441	2,137	2,892	2,894	2,899	2,929	0,731	0,645	1,06	1,165	1,83
h	0,048	0,046	0,669	1,224	1,364	1,722	1,366					

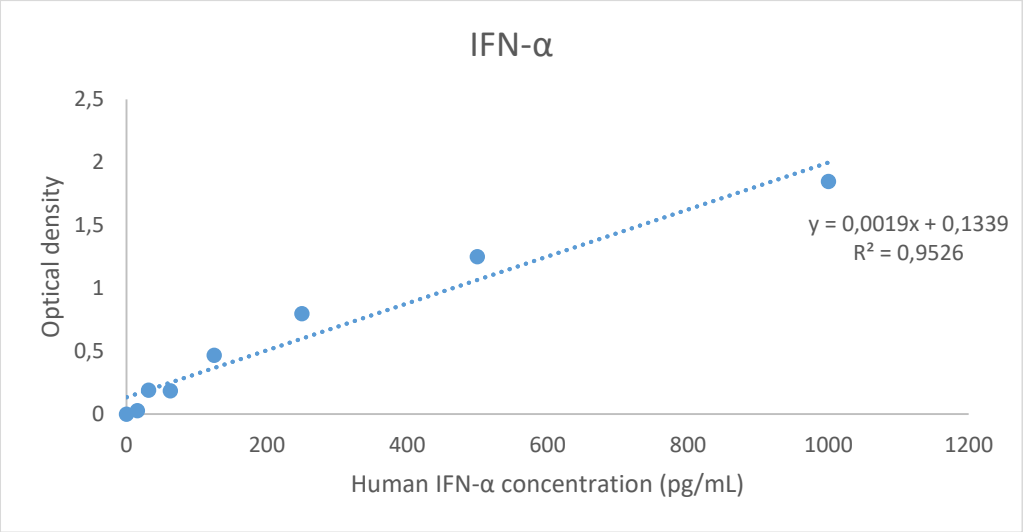
IL-8

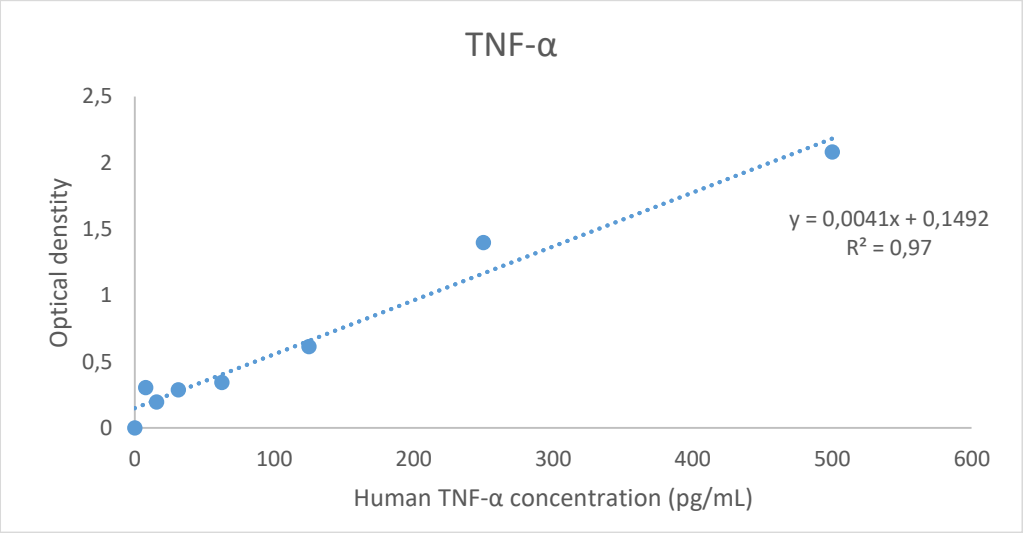
<>	1	2	3	4	5	6	7	8	9	10	11	12
a	2,304	2,263	0,145	0,2	0,264	0,175	1,063	0,173	0,175	0,244	0,329	0,412
b	2,147	1,831	0,143	0,16	0,286	0,53	1,019	0,956	1,886	2,339	2,293	2,842
c	1,5	1,347	0,25	1,096	0,375	1,646	2,286	0,32	0,434	0,52	0,372	0,553
d	0,736	0,797	0,248	0,212	0,243	0,177	0,458	0,215	0,232	0,269	0,331	0,27
e	0,463	0,461	0,892	1,256	1,428	1,137	1,203	0,347	1,436	1,558	0,572	0,72
f	0,318	0,33	0,223	1,038	1,199	0,869	1,327	0,242	0,494	0,583	0,52	0,772
g	0,211	0,231	0,755	1,383	2,671	2,785	2,813	0,325	0,473	0,521	0,865	1,67
h	0,079	0,081	0,205	0,451	0,586	0,646	0,579					

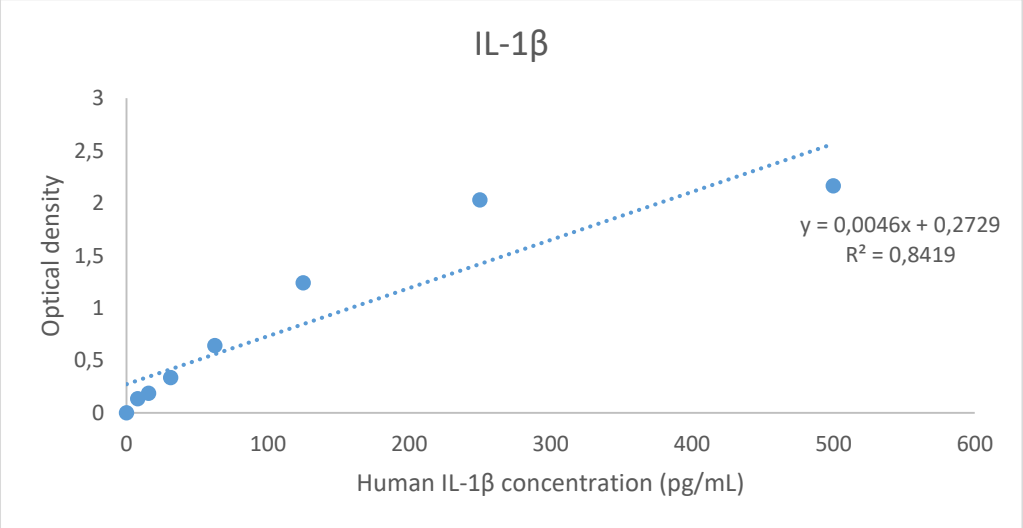
IL-12

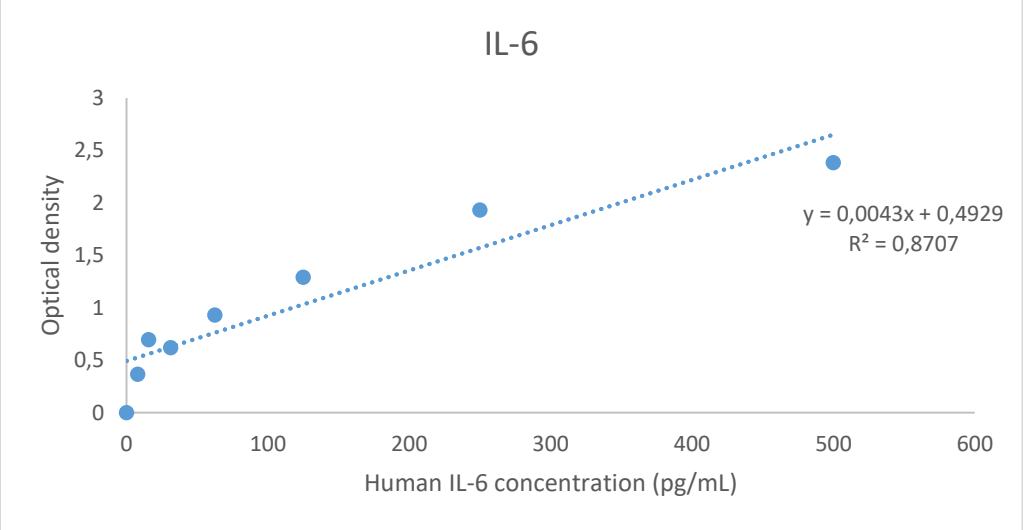
<>	1	2	3	4	5	6	7	8	9	10	11	12
a	1,99	1,838	0,083	0,358	0,231	0,279	0,263	0,248	0,234	0,313	0,221	0,574
b	1,562	1,527	0,222	0,204	0,29	0,202	0,153	0,074	0,216	0,193	0,386	0,592
c	0,996	1,025	0,056	0,193	0,053	0,161	0,137	0,058	0,334	0,103	0,314	0,669
d	0,659	0,555	0,056	0,056	0,071	0,328	0,056	0,084	0,136	0,174	0,354	0,63
e	0,418	0,268	0,261	0,109	0,062	0,088	0,088	0,17	0,267	0,131	0,138	0,508
f	0,411	0,329	0,37	0,528	0,112	0,374	0,403	0,389	0,22	0,286	0,102	0,63
g	0,57	0,368	0,477	0,235	0,3	0,498	0,393	0,497	0,355	0,182	0,078	0,776
h	0,357	0,082	0,404	0,042	0,49	0,417	0,597					

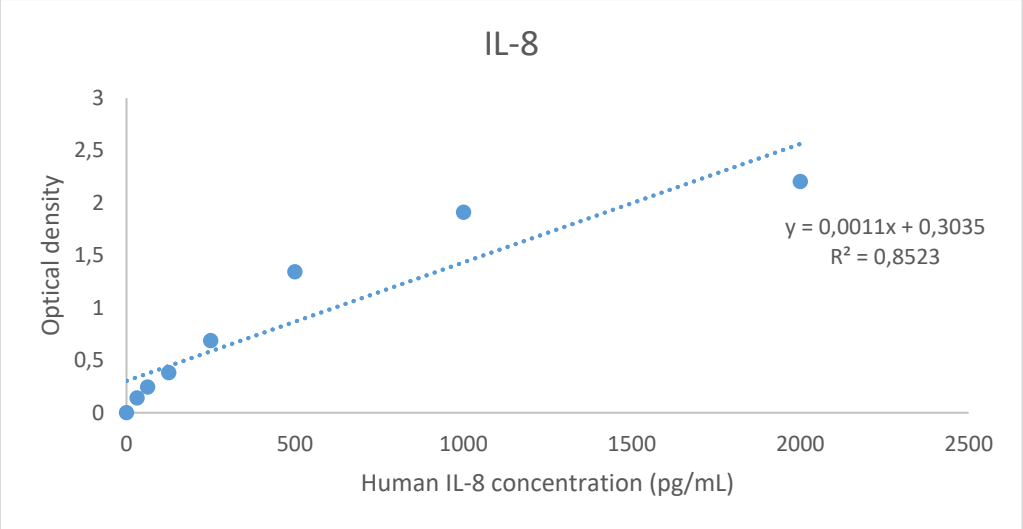
Appendix F: Pro-inflammatory cytokine ELISA standard curve

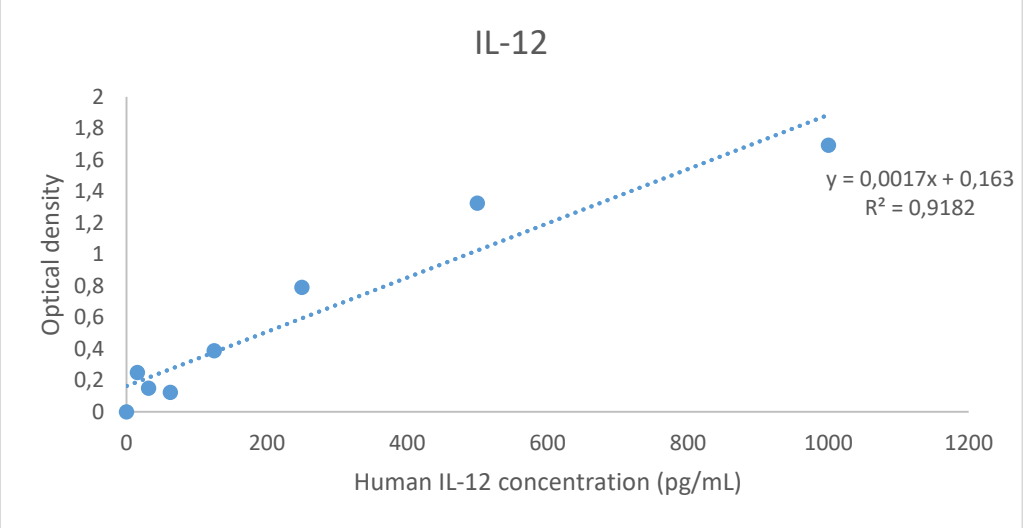







IL-6
Optical density
3
2,5
2
1,5
1
0,5
0
0
100
200
300
400
500
600
Human IL-6 concentration (pg/mL)
y = 0,0043x + 0,4929
R² = 0,8707


IL-8
Optical density
3
2,5
2
1,5
1
0,5
0
0
500
1000
1500
2000
2500
Human IL-8 concentration (pg/mL)
y = 0,0011x + 0,3035
R² = 0,8523


IL-12
Optical density
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
200
400
600
800
1000
1200
Human IL-12 concentration (pg/mL)
y = 0,0017x + 0,163
R² = 0,9182

Appendix G: Concentration levels of secreted cytokines in SINV, mock-infected and uninfected macrophages

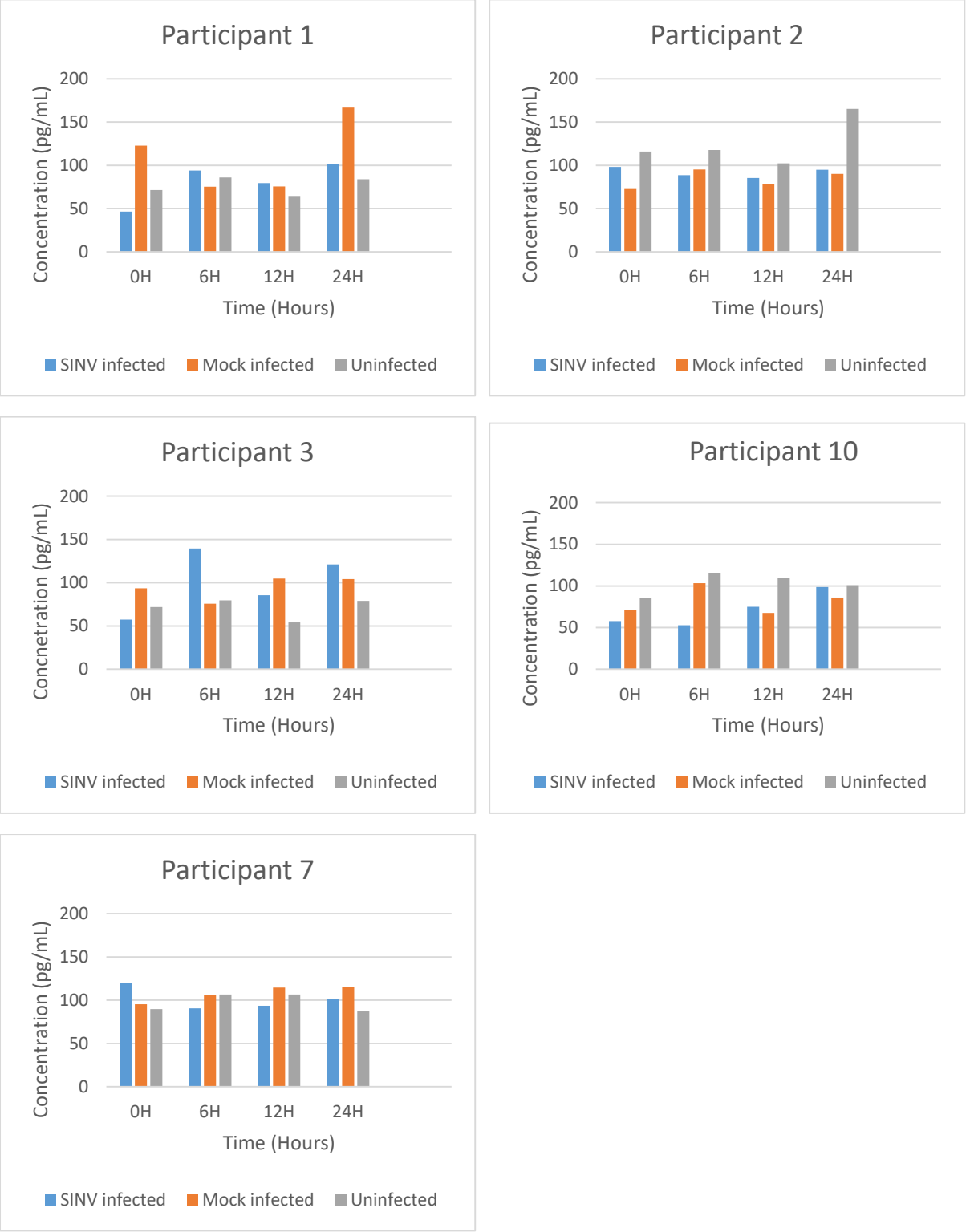


Figure A.1: Concentration of TNF-α in macrophages of five participants measured during the 24 hours post-infection. Cell-free supernatant was collected at baseline, 6, 12, and 24 hours post-infection from SINV infected, HI SINV infected and uninfected macrophages and concentration was determined by an ELISA

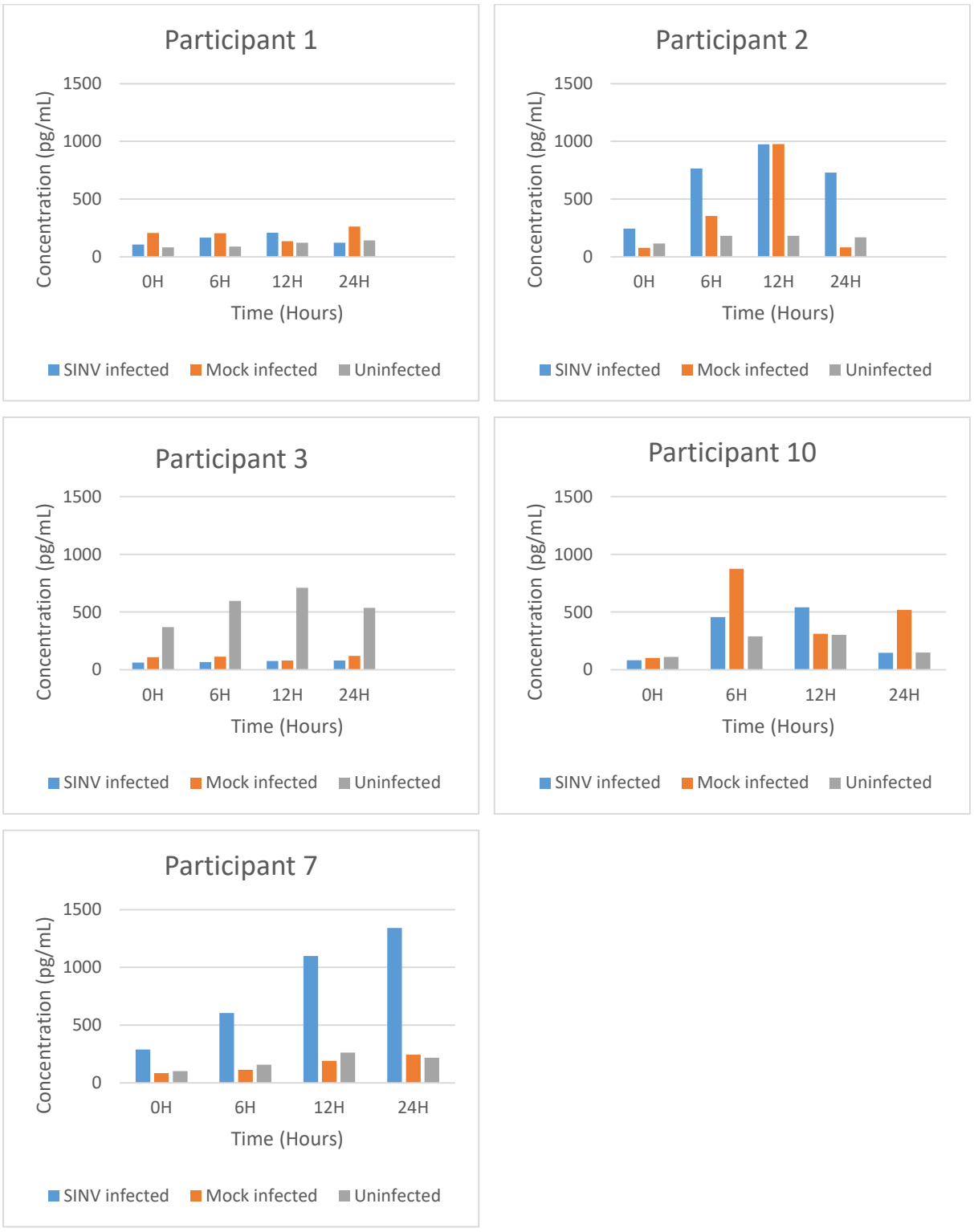


Figure A.2: Concentration of IFN-α in macrophages of five participants measured during infection with SINV. Cell-free supernatant was collected at baseline, 6, 12, and 24 hours post-infection from SINV infected, HI SINV infected and uninfected macrophages and concentrations were determined by an ELISA.

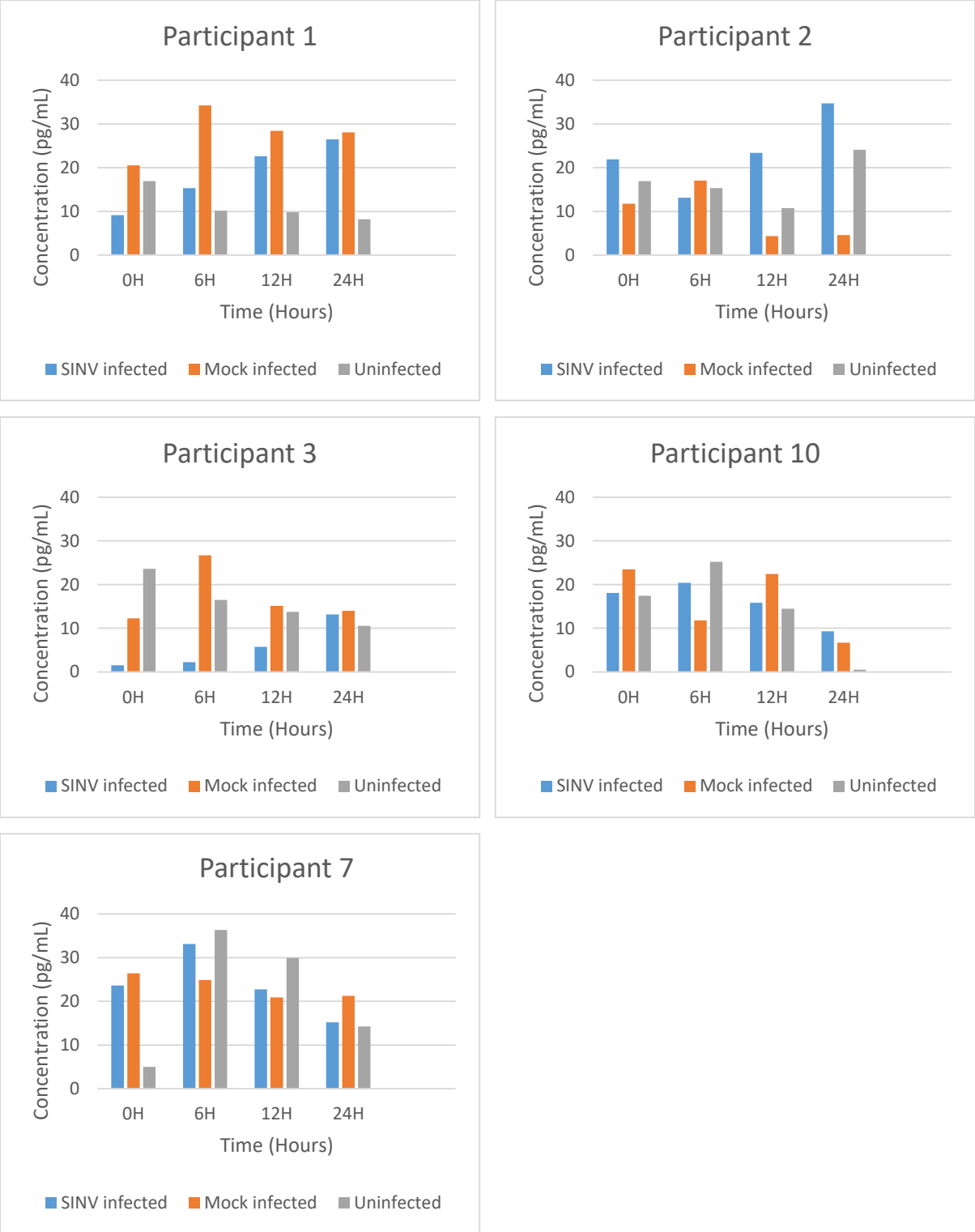


Figure A.3: Concentration of IL-1β in macrophages of five participants measured during infection with SINV. Cell-free supernatant was collected at baseline, 6, 12, and 24 hours post-infection from SINV infected, HI SINV infected and uninfected macrophages and concentration were determined by an ELISA.

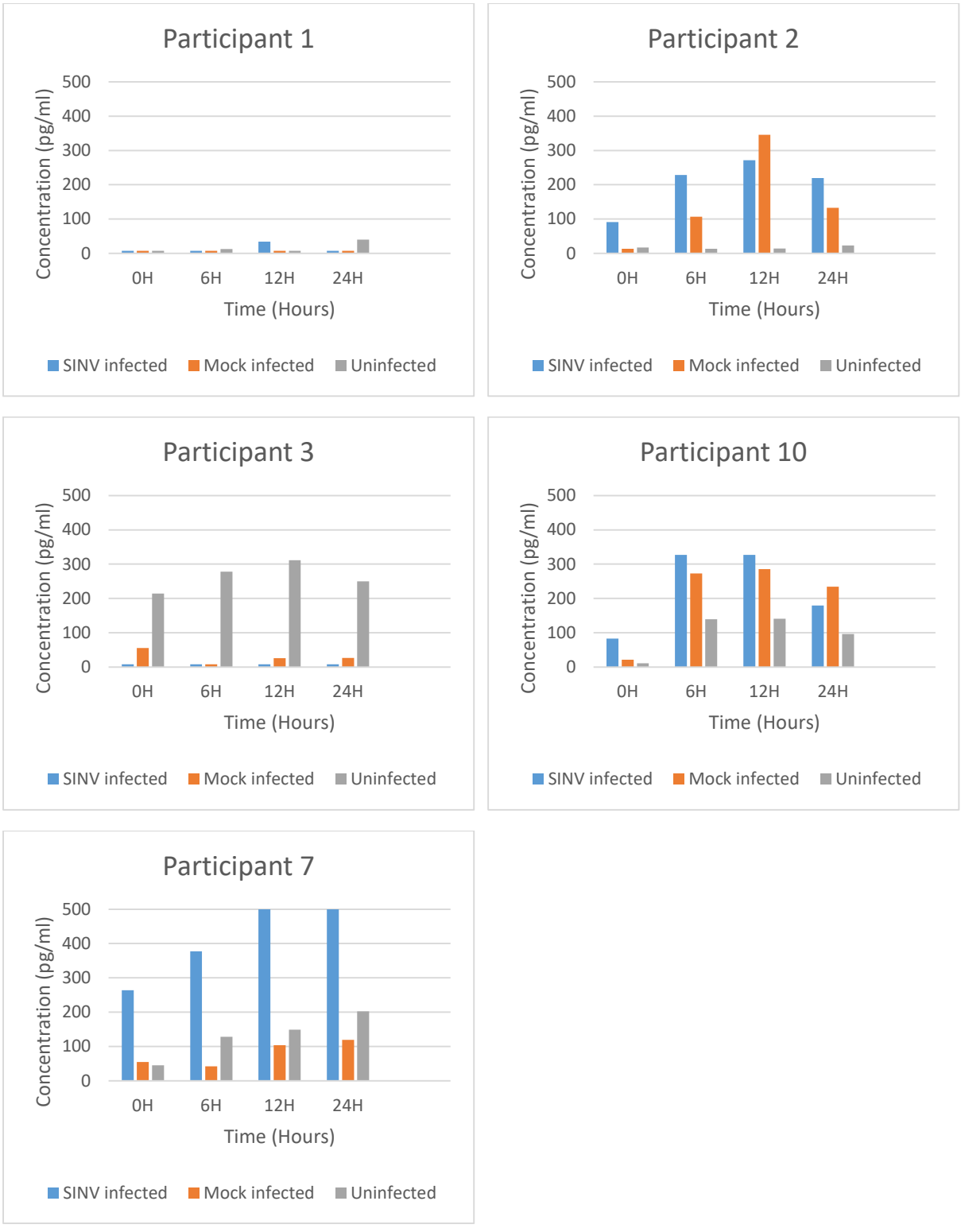


Figure A.4: Concentration of IL-6 in macrophages of five participants measured during infection with SINV. Cell-free supernatant was collected at baseline, 6, 12, and 24 hours post-infection from SINV infected, HI SINV infected and uninfected macrophages and concentration were determined by an ELISA.

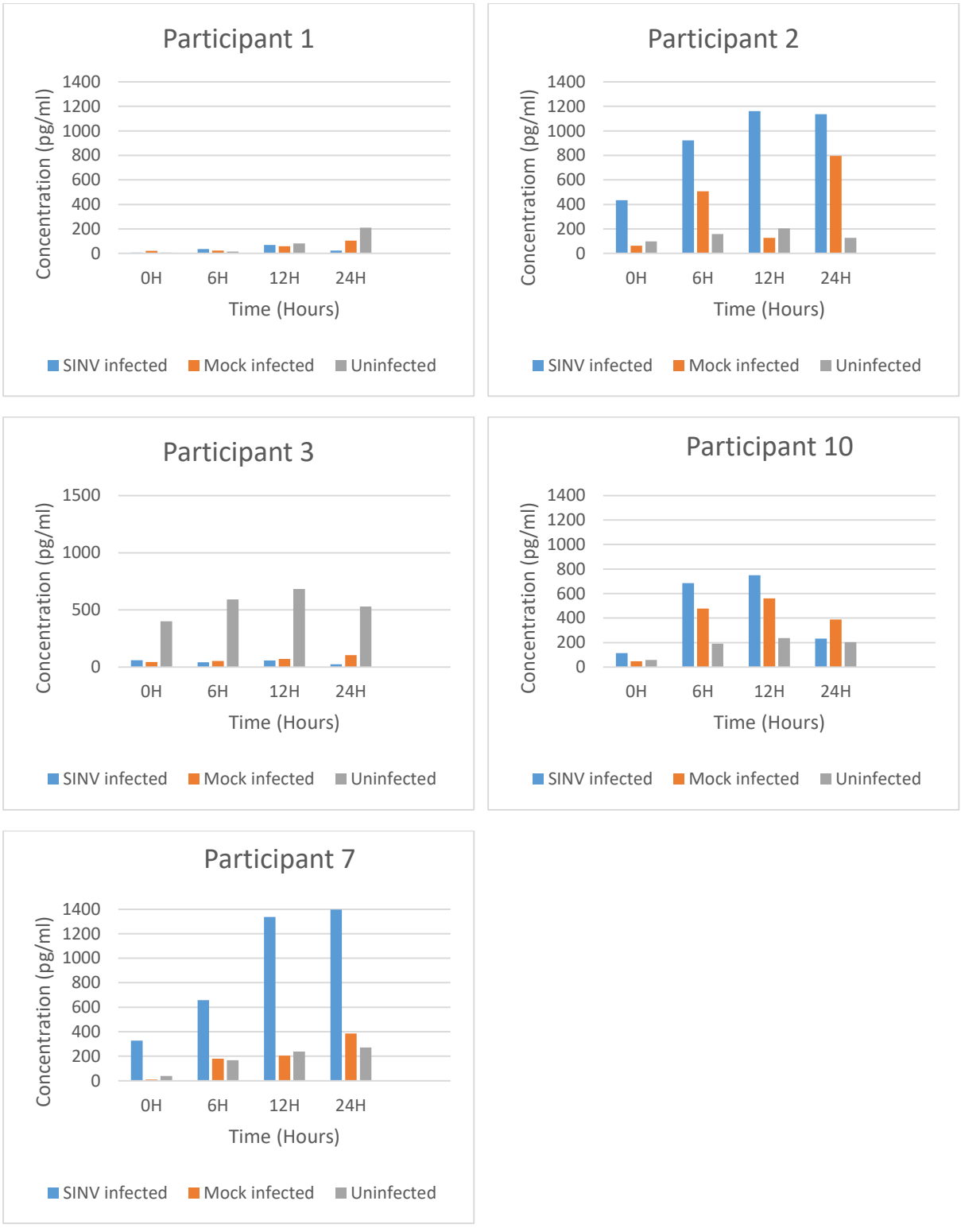


Figure A.5: Concentration of IL-8 in macrophages of five participants measured during infection with SINV. Cell-free supernatant was collected at baseline, 6, 12, and 24 hours post-infection from SINV infected, HI SINV infected and uninfected macrophages and concentration were determined by an ELISA.

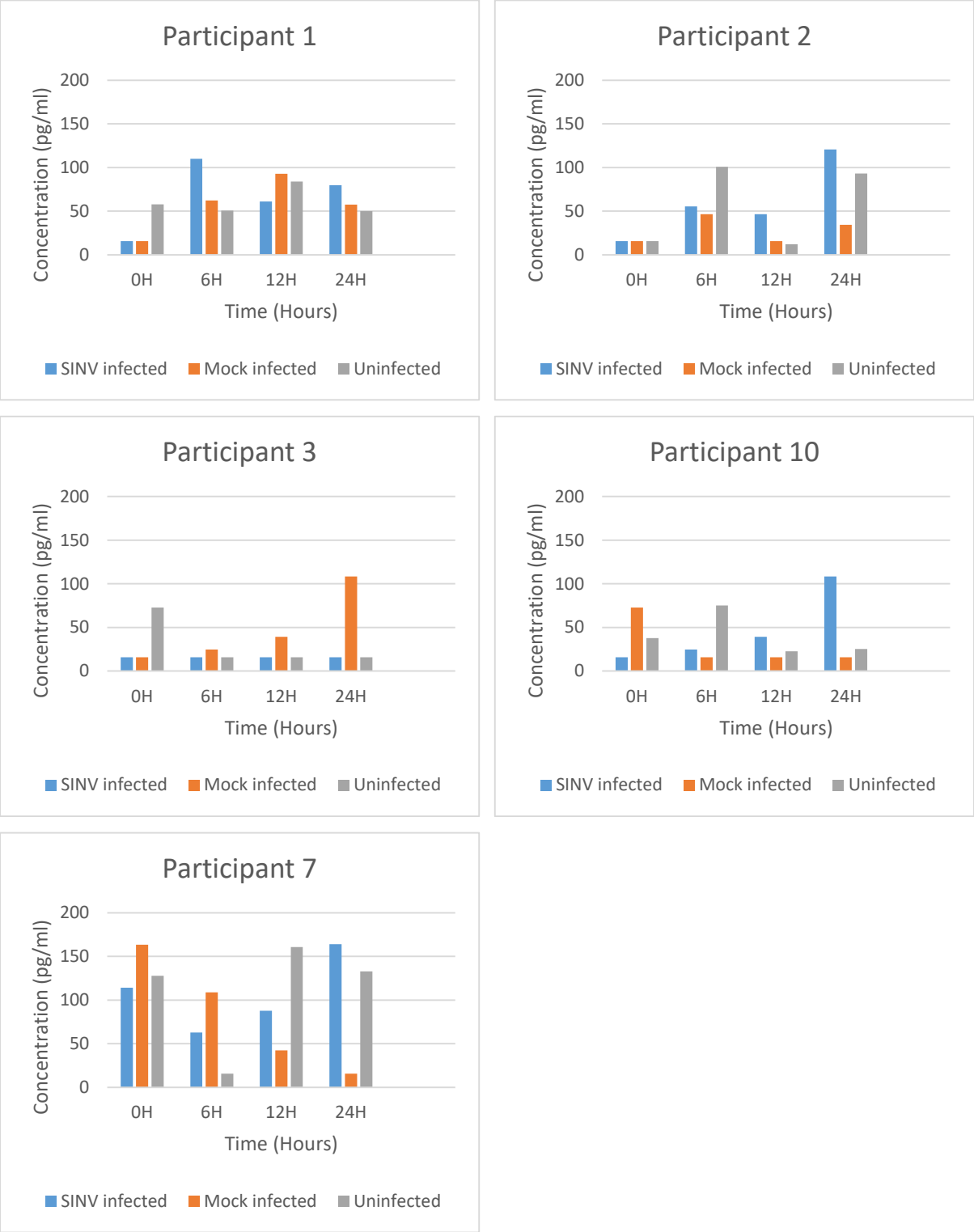


Figure A.6: Concentration levels of secreted IL-12 from cell-free supernatant of SINV-infected, mock-infected and uninfected cells were measured at different times post-infection by ELISA. Graphical representation shows the amount of secretion at times 0, 6, 12, and 24 hours post-infection.