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Characterization of T cell responses to the non-structural proteins of the M segment in survivors of Crimean-Congo haemorrhagic fever

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Declaration

I, Makgotso Golda Maotoana, certify that the dissertation hereby submitted for the M.Med.Sc. Medical Virology qualification at the University of the Free State is my independent effort and has not previously been submitted for a qualification at another university/faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

Makgotso Golda Maotoana

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“Don’t be afraid, for I am with you. Don’t be discouraged, for I am your God. I will strengthen you and help you. I will hold you up with my victorious right hand.”

–Isaiah 41:10 (New Living Translation)

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
%	Percent
mers	Monomers
mg	Milligram
ml	Millilitres
µg	Microgram
µl	Microliter
ACD-A	Anticoagulant citrate dextrose solution A
AP	Alkaline phosphatase
APC	Antigen presenting cell
APC	Allophycocyanin
BD	Becton Dickinson
BD FACS	Becton Dickinson fluorescence-activated cell sorting
BFA	Brefeldin A
BSA	Bovine serum albumin
^{51}Cr	Cromium isotope
C1	Initial concentration
C2	Desired concentration
CCHF	Crimean-Congo haemorrhagic fever
CCHFV	Crimean-Congo haemorrhagic fever orthonairovirus
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CO_2	Carbon dioxide
Cy7	Cyanine7
DMSO	Dimethyl sulfoxide

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assays
ELISpot	Enzyme linked immunospot assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothicyanate
g	Gravitational force
HLA	Human leucocyte antigen
HSREC	Health Science Research Ethics Committee
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFN-γ ELISpot	Inteferon gamma enzyme linked immunospot assay
IF	Indirect immunofluorescence
$IFNAR^{-/-}$	Type 1 interferon receptor knockout
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
L	Large segment
LAMPs	Lysosomal associated membrane glycoproteins
M	Medium segment
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MVA	Modified vaccinia virus Ankara
NaN3	Sodium azide
Neg	Negative
NK	Natural killer cell

NP	Nucleocapsid protein
NS_M	Non-structural protein M segment
NS_S	Non-structural protein S segment
OAS	Oligoadenylate synthetase
OTU	Ovarian tumour
PacB	Pacific blue
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohaemagglutinin
PKR	RNA-activated protein kinase
Pos	Positive
PPE	Personal protective equipment
RdRp	RNA dependent RNA polymerase
RPMI	Rosewell Park Memorial Institute
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Small segment
SA	South Africa/ African
SEB	Staphylococcal enterotoxin B from Styphlococcus aureus
$SFC/10^6$	Spot forming cells per million
ssRNA	Single stranded RNA
$STAT\text{-}1^{-/-}$	Signal transducer and activator of transcription 1 knockout
T_{CM}	Central memory T cell
TCR	T cell receptor
tc-VLPs	Transcriptionally competent virus-like particle
T_{EM}	Effector memory T cell

T_{EMRA}	Terminally differentiated T cell
T_N	Naïve T cell
TNF	Tumour necrosis factor
USA	United States of America
V1	Initial volume
V2	Volume in which cells are to be suspended in
WWII	World War II

ABSTRACT

Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) is one of the most widely distributed arboviruses globally. The disease caused by the virus, Crimean-Congo haemorrhagic fever (CCHF), continues to emerge and re-emerge across the globe. There are currently various vaccines under development for CCHF prevention. The non-structural M protein (NS_M), GP38, highly variable mucin-like domain and N-terminus of G_C regions in CCHFV have proved to be immunogenic in vaccine studies. Furthermore, both arms of the immune system have been found to be fundamental for protection in mice. However, there is limited information about immunity in patients following natural infection.

The aim of the study was to characterize T cell immune responses against the NS_M, GP38 and the highly variable mucin-like domain of CCHFV in survivors of CCHF. This was achieved by first identifying immunogenic peptides in the regions of interest and determining the amino acid conservation of the identified peptides. An overlapping peptide library spanning the NS_M, GP38 and highly variable mucin-like domain was designed using the South African CCHFV isolate SPU 103/87. The secretion of interferon-gamma (IFN-γ) by peripheral blood mononuclear cells isolated from 12 participants was screened using 24 peptide pools in an IFN-γ enzyme linked immunospot (ELISpot) assay.

IFN-γ secretion was detected in eight of the twelve participants. Two participants showed no detectable IFN-γ responses to any of the peptide pools, and another two were excluded from the analysis due to a high background in the negative controls indicating non-specific reactivity. Nine peptides were identified with the IFN-γ ELISpot, including five peptides in the GP38 region and four in the NS_M region, thus confirming the immunogenic potential of these regions during natural infection. No immunogenic peptides were identified in the highly variable mucin-like domain, which is possibly because of the high genetic diversity in the region.

The identified immunogenic peptides were used to stimulate T cells of participants and a flow cytometry assay was performed to characterize the immune responses, with the focus on detecting the presence of the T cell memory subsets, the expression of CD107a, which is a cytotoxic marker, and the secretion of IFN-γ and tumour necrosis factor-alpha (TNF-α), which are antiviral cytokines.

Cytotoxic $CD8^+$ T cells were detected in six participants in response to nine peptides. IFN-γ and TNF-α secretion within the CD4 and CD8 populations were comparable; thus, highlighting the ability of the $CD8^+$ T cell population to secrete antiviral cytokines, even though the population is known to be predominantly cytotoxic. The secretion of IFN-γ was more frequent than TNF-α secretion in both the CD4 and CD8 populations. Polyfunctional T cells were detected with the phenotypes IFN-γ^+CD107a$^+$ and IFN-γ^+ TNF-α^+, in both the CD4 and CD8 populations. Therefore, the results indicate the possibility of efficient antiviral responses upon stimulation with viral epitopes in survivors of infection. Heterogeneous functionality of the T cell memory subsets was observed, however the terminally differentiated (T_{EMRA}) subset was the most dominant and abundant, followed by the naïve (T_N), effector memory (T_{EM}), with the least abundant being the central memory (T_{CM}) T cell memory subset.

The IFN-γ secretion detected with the IFN-γ ELISpot and the flow cytometry assay was used as basis for comparing the sensitivity of the two techniques. The IFN-γ ELISpot proved to be comparable to the flow cytometry assay. The ELISpot is suited for screening purposes, while the flow cytometry allowed further characterization of the T cell responses. Therefore, it is recommended that these complementary assays be used in combination.

In conclusion, T cell epitopes were identified in the NS_M and GP38 regions of CCHFV. Polyfunctional T cells were found in both the CD4 and CD8 populations, thus suggesting the presence of effective long-term memory T cells responses in survivors of CCHF.

CHAPTER 1
Literature review

1.1 INTRODUCTION

Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) is classified under the order *Bunyavirales*, family *Nairoviridae* and genus *Orthonairovirus* (Adams *et al.*, 2017). It is the pathogenic agent responsible for Crimean-Congo haemorrhagic fever (CCHF), which was proven using Koch's postulate on newborn mice, newborn rats and humans with serum from infected patients. It was also discovered that CCHFV is non-pathogenic for adult mammals (Grasiichenkov, 1945; Hoogstraal, 1978).

CCHF was first documented towards the end of World War II (WWII) in the years 1944-1945, when Soviet military personnel in Crimea showed symptoms of disease (Grasiichenkov, 1945; Hoogstraal, 1978). In 1956, a child in the Belgian Congo (now referred to as the Democratic Republic of Congo) had a clinically similar disease. The isolation of the virus in 1967 showed that the virus that caused disease in both the Soviet personnel in Crimea and the child in Congo were antigenically identical, hence the naming of the virus as CCHFV (Casals, 1969; Chumakov *et al*., 1969; Hoogstraal, 1978). In the 1960s, CCHF outbreaks were associated with environmental changes caused by war, new agricultural practices, floods, and the distribution and density of dairy herds (Hoogstraal, 1978).

CCHF has a mortality rate of 10%-40%. This wide range of mortality collectively takes into account the differences between geographic regions were cases have been reported. However, the higher mortality rates of 30%-40% are seen in large CCHF outbreaks (WHO, 2013). Research on the disease and the virus has been limited because of the listing of CCHFV as a hazard group 4 pathogen, thus requiring biosafety level 4 laboratories for studies and experimentation and due to the absence of an animal model. The lack of resources for building biosafety level 4 laboratories with all the requirements for safe operation when working with dangerous pathogens has been a limitation for research into CCHFV and many other

group 4 pathogens. Hence the need to understand the virus, disease pathogenesis and immune responses, as this knowledge would allow for better management and prevention of the disease.

1.1.1 VIRUS CHARACTERISTICS

CCHFV is a spherical virion with a glycoprotein studded lipid envelope (Chumakov *et al.*, 1968). The envelope encapsidates the negative-sense, single-stranded RNA (ssRNA) genome, consisting of three segments which are the large (L), medium (M) and small (S) segments (Clerx *et al.*, 1981), as shown in Figure 1.1. Upon viral characterization in the 1970s, it was observed that CCHFV is highly sensitive to sodium desoxycholate, ether and chloroform. While it was found to be resistant to prolonged freezing on dry ice and lyophilized well, it was inactivated when exposed to 60°C for 15 minutes or 37°C for 7 hours (Hoogstraal, 1978).

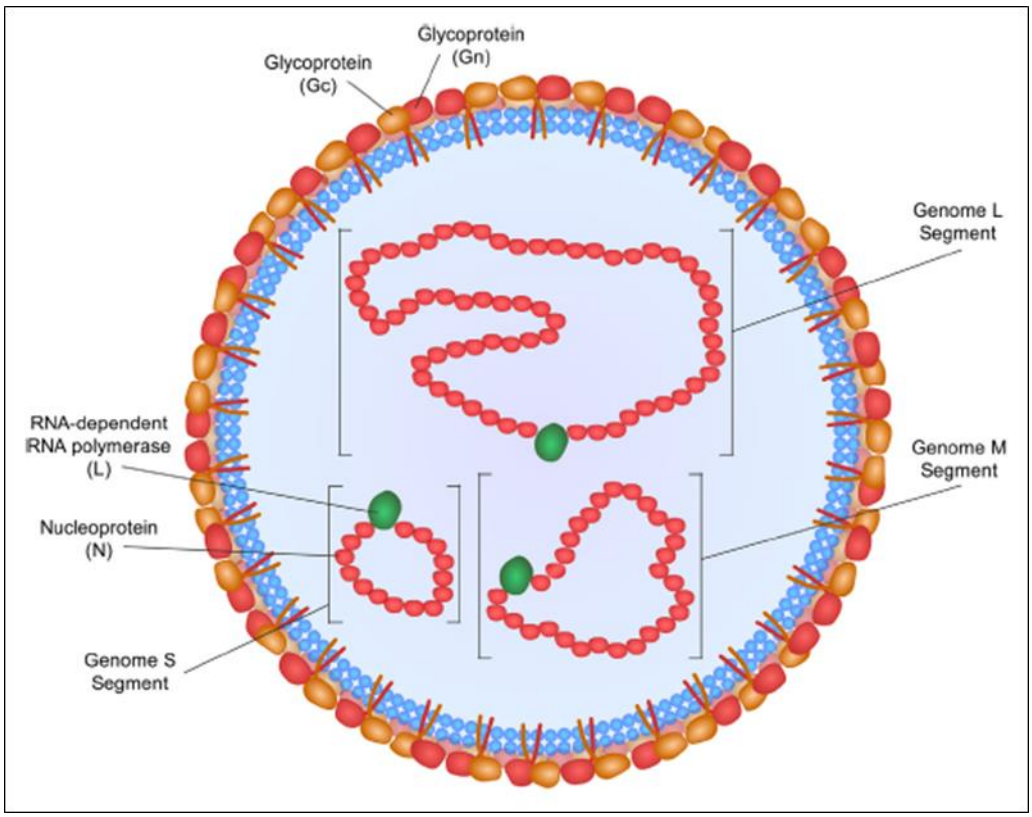


Figure 1.1. The virion structure of CCHFV (Bente *et al.*, 2013; permission from Elsevier attached in appendix G)

The S segment encodes the nucleocapsid protein (NP) and the non-structural protein (NS_S) in an ambisense orientation (Barnwal *et al.*, 2016; Hewson *et al.*, 2004). The main function of the NP is to encapsidate the RNA of the virus resulting

in the formation of the ribonucleocapsid, a number of putative RNA binding regions have been identified recently (Carter *et al.*, 2012). The NP may have other functions during virus assembly, morphogenesis, and suppression of apoptosis in the early phase of infection (Karlberg *et al.*, 2011; Karlberg *et al.*, 2015; Zhou *et al.*, 2011).

The function of the NS_S has not yet been confirmed, but it is hypothesized to be involved in the disturbance of interferon alpha and beta (IFN α/β) production, host cell protein synthesis, and degradation of double stranded RNA dependent protein kinase, as seen with other viral NS_S in the order *Bunyavirales* (Barnwal *et al.*,2016). However, it has been confirmed that the NS_S of CCHFV induces apoptosis through both the intrinsic and extrinsic pathways in a caspase dependent activity, although the mechanism is unknown (Barnwal *et al.*, 2016).

The M segment is translated to a polyprotein referred to as the M-polyprotein, and encodes the glycoproteins G_N and G_C, variable mucin-like domain, the secreted non-structural glycoproteins GP38, GP85, and GP160, and the non-structural protein NS_M (Altamura *et al.*, 2007; Sanchez *et al.*, 2006).

The L segment of orthonairoviruses is twice the size of other viruses in the *Bunyavirales* order (Honig *et al.*, 2004), and it encodes the RNA dependent RNA polymerase (RdRp). However, due to the large size and the presence of the ovarian tumour (OTU) like protease motif, which is linked to autoproteolytic activity, and a helicase domain, it is suspected that the L segment of CCHFV could encode additional proteins such as helicase (Honig *et al.*, 2004).

The three genomic segments of CCHFV have complementary sequences at the termini (Clerx-Van Haaster *et al.*, 1982). This sequence complementarity results in closed circular RNA strands that form a stable panhandle structure, as illustrated in Figure 1.1. This structure serves as the functional promoter region to which the RdRp will bind to initiate transcription and replication in the host cell (Clerx-Van Haaster *et al.*, 1982).

1.1.2 REPLICATION

The replication cycle of CCHFV begins when the glycoproteins G_N and G_C recognise the receptors on CCHFV susceptible cells as demonstrated in Figure 1.2. Further research on the pathogenesis of CCHFV could give an indication of the specific host

cellular receptor that the glycoproteins interacts with to initiate virion entry (Connolly-Andersen *et al.*, 2009; Connolly-Andersen *et al.*, 2011; Kaya *et al.*, 2014; Peyrefitte *et al.*, 2010).

The presence of nucleolin, a host protein, has been observed to play an essential role in CCHFV cell entry (Xiao *et al.*, 2011). Although it is a nucleolar protein, it has been observed to shuttle to cell surfaces and has various functions, one of which is possibly to act as a receptor for viruses (Xiao *et al.*, 2011). In their study, Xiao *et al.*, also observed the expression of nucleolin on the surface of all CCHFV susceptible cells, but this expression was absent in CCHFV resistant cells, thus the role of nucleolin as a possible receptor was proposed (Xiao *et al.*, 2011).

The glycoproteins G_N and G_C are responsible for the binding of the virion to target cell receptors. The G_C has been observed to be more important for viral entry than G_N, as is evidenced by the observation that monoclonal antibodies against G_C neutralized viral infection in SW-13 cells (Bertolotti-Ciarlet *et al.*, 2005). Other evidence includes the ability of G_C fragments to bind to susceptible cell lines while G_N had a limited binding ability (Xiao *et al.*, 2011). G_N is believed to be more important for localization of the M polyprotein (pre-G_N and pre-G_C precursors) to the Golgi apparatus, since it was transported to the Golgi apparatus at a faster rate than the G_C ectodomain (Bertolotti-Ciarlet *et al.*, 2005).

CCHFV enters the host cell through clathrin dependent endocytosis (Simon *et al.*, 2009), as in Figure 1.2. This was observed by the decrease in CCHFV NP when the clathrin dependent pathway was inhibited, and the reduction in detected CCHFV RNA in clathrin knocked-out cells (Simon *et al.*, 2009).

Once in the cytoplasm, the viral RdRp interacts with the genome to initiate replication and transcription by binding to the panhandle regions of the segments (Clerx-Van Haaster *et al.*, 1982). The RdRp produces complementary positive sense strands which act as templates for both replication and translation. Replication occurs in the cytoplasm while the synthesis of viral glycoprotein precursors occurs in the endoplasmic reticulum (ER) (Brooks *et al.*, 2013). This is proposed, as it is generally accepted for RNA viruses, but is yet to be confirmed for CCHFV.

The synthesized nucleocapsid interacts with the newly synthesized viral RNA forming the ribonucleocapsid. At this point, the M polyprotein is also synthesized and the cytoplasmic region of G_N and G_C interacts with the ribonucleocapsids, which triggers the transition of the complex from the ER to the Golgi apparatus where the M-polyprotein is modified to G_N and G_C. The maturation of the structural glycoproteins results in the budding of the virus and the secretion of the NS_M, variable mucin-like domain and the non-structural glycoproteins. The NS_M has been observed to accumulate in the Golgi apparatus, and is suspected to play a role in virion assembly (Altamura *et al.*, 2007). The function of the variable mucin-like domain, GP38, GP85 and GP160 has not yet been determined.

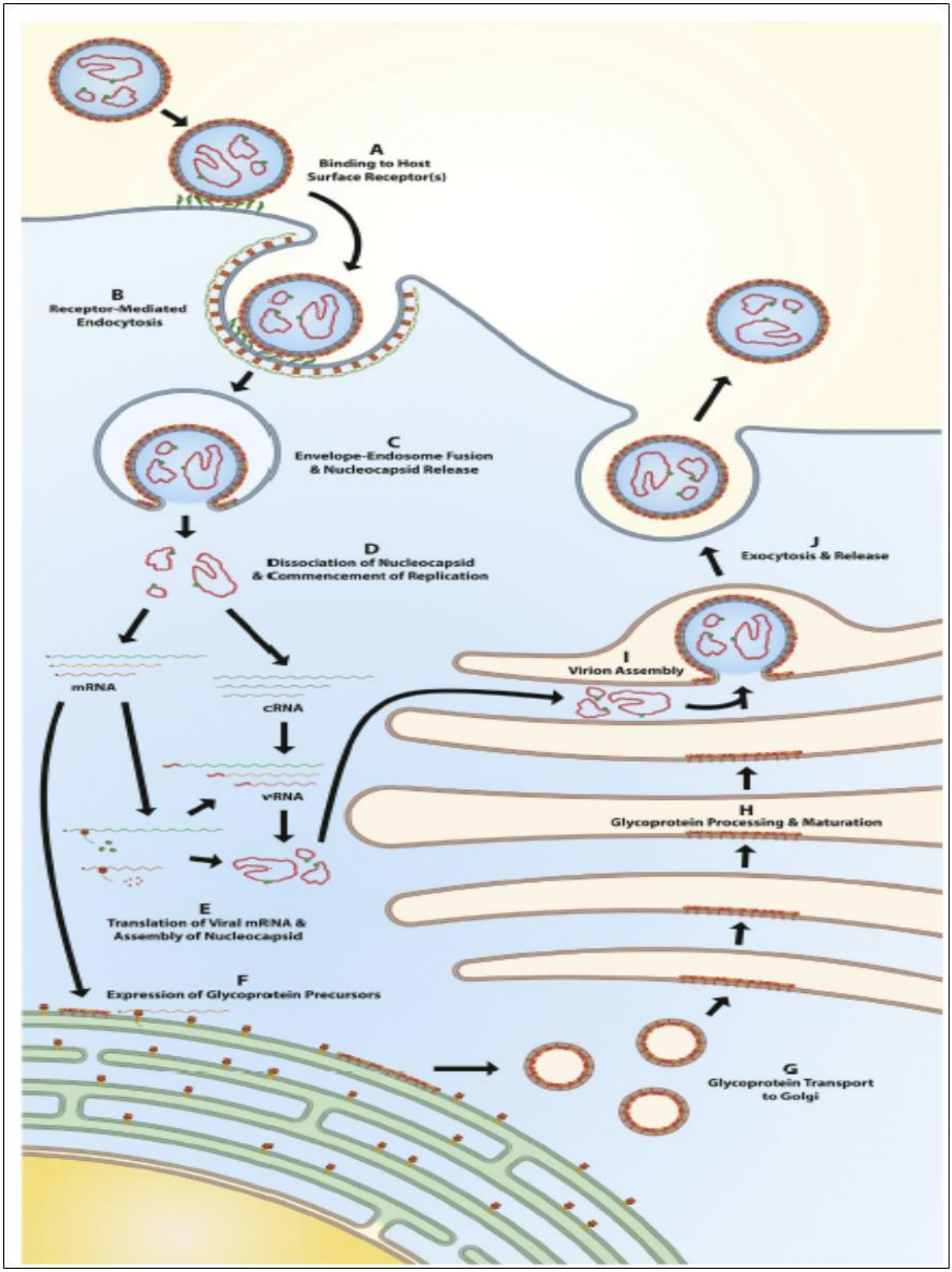


Figure 1.2. Replication of CCHFV (Bente *et al.*, 2013; permission obtained from Elsevier attached in appendix G).

1.1.2.1 The M segment

The M segment is translated as a polyprotein and is cleaved by host proteases to produce the structural glycoproteins G_N and G_C, variable mucin-like domain, the secreted non-structural glycoproteins (GP38, GP85, and GP160) and NS_M (Altamura *et al.*, 2007; Sanchez *et al.*, 2006).

According to a model described by Sanchez *et al.* (Figure 1.3), the M polyprotein is cleaved by signalase on the N-terminus of the $preG_N$ and $preG_C$ precursors, resulting in the $preG_N$ precursor and $preG_C$ precursor. Once in the Golgi apparatus, the $preG_C$ precursor is cleaved in the N-terminus at the motif RKPL by a SKI-1-like protease, resulting in the matured G_C glycoprotein (Sanchez *et al.*, 2006).

The $preG_N$ precursor is cleaved at the motif RKLL by an unknown protease resulting in the release of the NS_M, and at the RRLL by the SKI-1 protease resulting in the release of the matured G_N glycoprotein and an N-terminus domain. The N-terminus domain is further cleaved by a furin protease at an RSKR motif resulting in the release of the variable mucin-like domain, the GP38 and possibly other secreted glycoproteins. It is possible that the other secreted glycoproteins are a result of differential cleavage by other unidentified host proteases (Sanchez *et al.*, 2006).

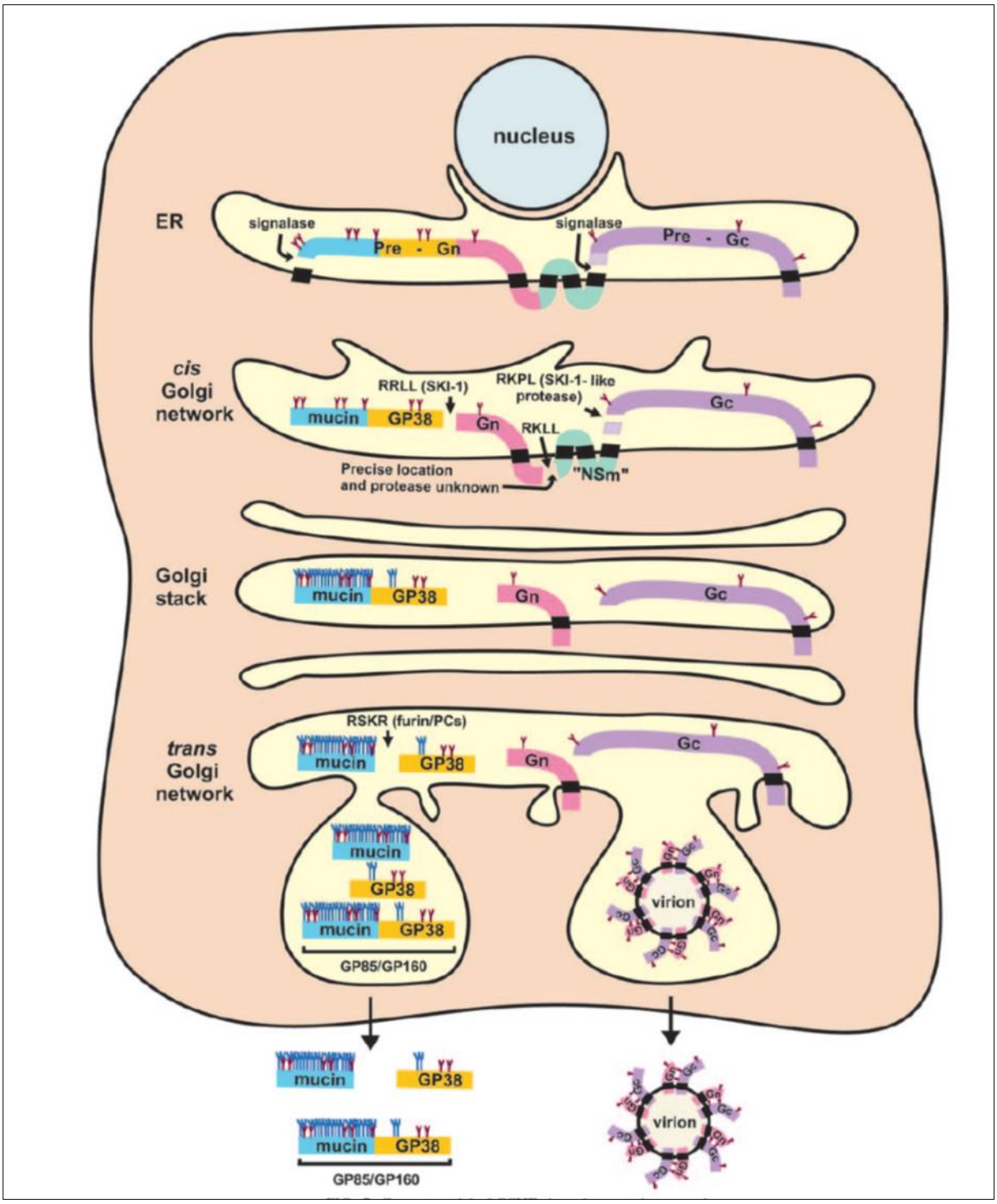


Figure 1.3. The cleavage of the M-polyprotein by host proteases according to a model by Sanchez *et al*. (Sanchez *et al.,* 2006; permission from American Society for Microbiology attached in appendix G).

1.1.3 EPIDEMIOLOGY

Historically, the geographical distribution of CCHFV was thought to have been limited to the war devastated areas in Crimea; however, post WWII outbreaks occurred in Uzbekistan, Khazakhstan, Astrakhan and Bulgaria amongst others. After the development of serological tests, the range was confirmed to include Palestine, Democratic Republic of Congo (previously Zaire), Uganda and Nigeria (Hoogstraal, 1978), proving that the disease was more widely distributed than previously known.

More recently, cases of CCHF have been reported in more than 30 countries in Africa, South Eastern Europe, the Middle East and Asia (Gou *et al.*, 2017; WHO, 2013). CCHFV is one of the most widely distributed arboviruses globally. CCHF is categorised as an emerging disease due to reports of naturally occurring cases and outbreaks in areas where it was not previously known to circulate, such as Turkey in 2002, Greece in 2008, Georgia in 2009, and Spain in 2016 (Messina *et al.*, 2015, Negredo *et al.*, 2017; Papa *et al.*, 2010). CCHF is also considered a re-emerging disease due to reports of infection after long periods of absence in areas such as in South-Western Russia and Central Africa (Messina *et al.*, 2015).

The global distribution of CCHF is due to the primary vector, namely ixodid ticks from the genus *Hyalomma* (Shepherd *et al.*, 1989a). This is evident by the parallel presence of CCHF cases and the distribution of *Hyalomma* ticks (WHO, 2013). CCHFV RNA has been isolated from at least 28 species of thermophilic *Ixodidae* ticks, which includes ticks in the genera *Hyalomma, Boophilus, Ixodes, Rhipicephalus, Amblyomma, Haemaphysalis* and *Dermacentor* (Akuffo *et al.*, 2016; Orkun *et al.*, 2017; Shepherd *et al.*, 1989b). Although viral RNA has been recovered from ticks in these genera, the vector competency, role in natural transmission and maintenance of CCHFV by ticks in these genera (excluding *Hyalomma*) has not been confirmed using vector competency studies, hence ticks belonging to the genus *Hyalomma* are considered the principal vectors of the virus (Jameson *et al.*, 2012; Orkun *et al.*, 2017).

Surveillance studies have shown that CCHFV distribution is affected by a number of factors such as presence of the vector, environmental conditions and the ecological niche (Messina *et al.*, 2015). Messina *et al.*, developed a global map using an ecological niche modelling approach, in which specific information from CCHFV high

risk areas (such as Turkey, Iran and Pakistan amongst others) was collected to predict the risk of CCHFV cases on a global scale, especially in areas where less is known about CCHFV circulation (Messina *et al.*, 2015). Ecologically, CCHFV was found to circulate in lowlands, foothills and low mountain belts with arid/ semiarid climates, long dry seasons, warm temperatures during summer and relatively mild winters, basically deserts, semi-deserts, steppes and savannas (Hoogstraal, 1978). The model developed by Messina *et al.*, observed similar patterns of high risk associated with a high proportion of grass and shrubs, increased land surface area and a less frequent mean annual precipitation (Messina *et al.*, 2015). Consequently, the model successfully highlighted areas where there is a high potential of CCHFV circulation based on the ecological data, thus suggesting the need for surveillance in these regions, which are highlighted in Figure 1.4. This map may help with evaluating control and prevention strategies and also guide targeted distribution should an effective vaccine or treatment become available (Messina *et al.*, 2015).

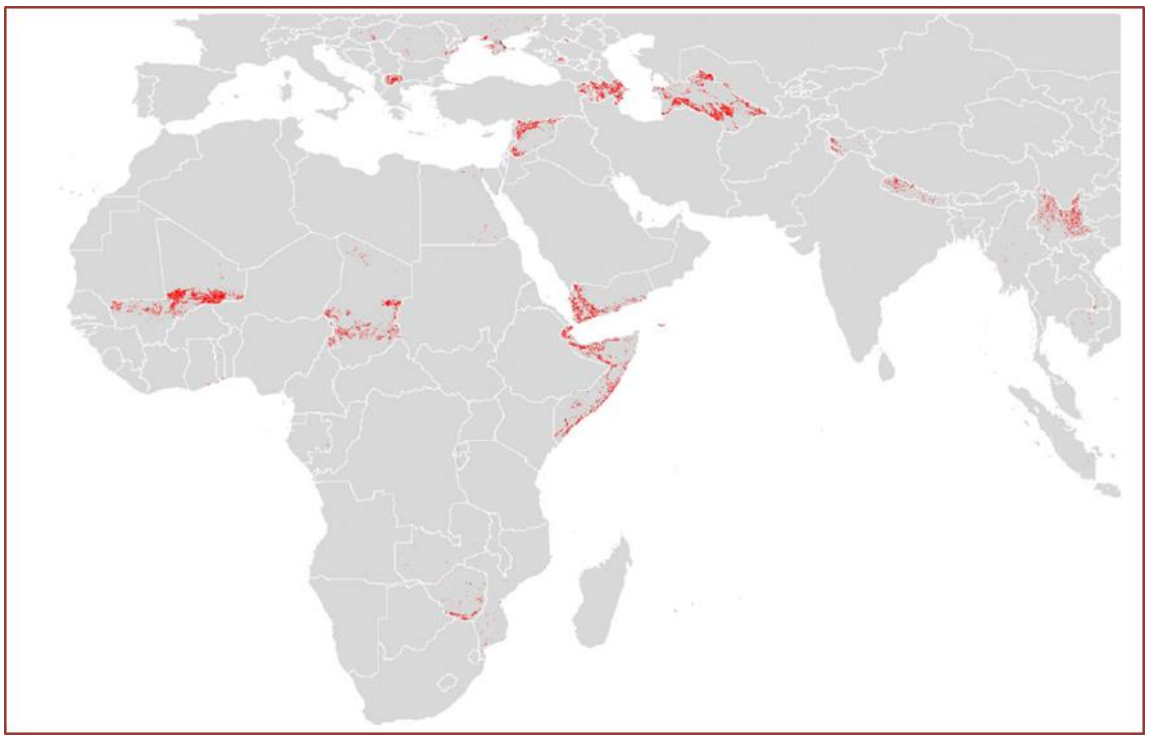


Figure 1.4. The surveillance map developed to predict occurrence of CCHF in areas where the virus is not known to circulate. In red are the areas where surveillance is needed as humans are predicted to be at potential risk, yet evidence is lacking on the presence of CCHF in these areas. Places highlighted are Zimbabwe, Djibouti, Somalia, Chad, Mali in Africa, Syria, Macedonia, Azerbaijian, Armenia, Turkmenistan and Yemen in Eastern Europe and Central Asia, and Kashmir,

Nepal and Yunnan in Southern Asia (Messina *et al.*, 2015; permission from Royal Society of Tropical Medicine and Hygiene attached in appendix G).

Hyalomma ticks maintain the existence of CCHFV through a tick-vertebrate-tick enzoonotic cycle including transovarial, transstadial and venereal transmission between ticks (Shepherd, *et al.*, 1989b) as shown in Figure 1.5. Transstadial transmission is when the pathogen remains in the vector through the different stages of metamorphosis. Transovarial transmission is when the pathogen is transferred from female to egg.

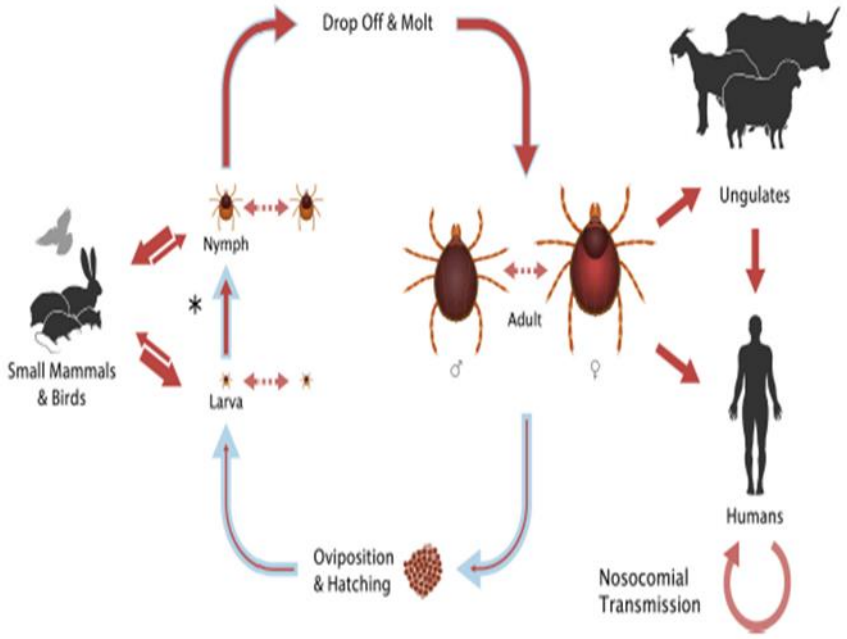


Figure 1.5. Demonstration of CCHFV transmission by ticks (Bente *et al.*, 2013; permission from Elsevier attached in appendix G). Transovarial and transstadial transmissions are indicated by the blue arrows. The tick-vertebrate-tick enzoonotic cycle is indicated by the arrows pointing towards the small mammals and birds; this also highlights the phase when the nymph and larva of the ticks feed for nutrition to grow. The arrows pointing towards the human and the livestock highlight the phase when the adult female feeds for nutrition to lay eggs. The dotted arrows represent the tick to tick transmission of CCHFV by feeding on viraemic animals.

The virus is transmitted to susceptible wild and domestic animals in the period when ticks feed for nutrients for maturation and egg production (Figure 1.5). During the feeding season, the reservoir animals experience a lot of tick bites and are thus repeatedly inoculated with the virus. These animals then become highly viraemic. This high viraemia is necessary for transmission to uninfected ticks (Hoogstraal,

1978; Shepherd, *et al.*, 1989b). Low viraemia levels in reservoirs may limit transmission to feeding uninfected ticks (Shepherd, *et al.*, 1989a).

In African studies, low level viraemia was found in small African mammals including rabbits, hares, guinea pigs and hamsters (Shepherd, *et al.*, 1989a), while high level viraemia was found in ostriches indicating the potential of ostriches as CCHFV reservoirs (Swanepoel *et al.*, 1998). However, experiments have found unconvincing evidence of other bird species as hosts of CCHFV, and no evidence of viral RNA in ticks isolated from birds (Hoogstraal, 1978, Jameson *et al.*, 2012).

Although the role of birds as reservoirs for CCHFV is in question, they may play a role as hosts for ticks and distribution of ticks during local and global migration (Hoogstraal, 1978). Orkun *et al.*, found that the majority of ticks that tested positive for CCHFV RNA were collected from cattle, wild boar, goats and hares, thus the role of these animals as reservoirs is convincing (Orkun *et al.*, 2017). However, some studies suspect that the abundance of reservoirs may just be an indication of the presence of the virus in nature, and not necessarily an indication of viral transmission (Messina *et al.*, 2015).

1.1.3.1 CCHFV in South Africa

The first recorded case in South Africa (SA) was in 1981 when a boy died of haemorrhagic fever after he returned from camping and was found to have a *Hyalomma* tick on his scalp (Gear, 1982). Between 1981 and 2013, approximately 192 cases of CCHF have been recorded in SA (Msimang *et al.*, 2013).

It has been observed that on average, five cases per annum are recorded in the country (Msimang *et al.*, 2013). Cases usually occur sporadically and have been reported from all of the nine provinces in SA. However, the majority of cases in SA are recorded in the Free State and the Northern Cape provinces (Msimang *et al.*, 2013).

Although CCHF is known to occur sporadically, a number of larger outbreaks have been recorded in SA. In 1996 an outbreak was recorded among ostrich abattoir workers in Oudtshoorn, and this was caused by exposure to ticks and highly viraemic body fluids of infected birds while slaughtering and skinning the birds (Swanepoel *et al.*, 1998). SA is the only country to have documented an outbreak

due to exposure to infected ostriches (Swanepoel *et al.*, 1998). A nosocomial outbreak was reported at Tygerberg Hospital in the Western Cape province; beginning with admission of the index case and followed by six secondary cases of CCHF including medical personnel (van Eeden *et al*., 1985).

1.1.4 GENETIC DIVERSITY OF CCHFV

CCHFV is a genetically diverse virus. The open reading frame lengths and important motifs (including the protease cleaving sites) have been found to be conserved through all isolates globally (Deyde *et al.*, 2006). However, the nucleotide variations among isolates have been found to be at 20% for the S segment, 22% for the L segment and 31% for the M segment (Deyde *et al.*, 2006). The high genetic diversity of the M segment is proposed to be influenced by the presence of the highly variable mucin-like domain and the coding of the glycoproteins (Carroll *et al.*, 2010; Deyde *et al.*, 2006).

The highly variable mucin-like domain is rich in serine, threonine and proline and is heavily O-glycosylated. It is therefore proposed that the high level of amino acid diversity in this region is tolerated as the heavy glycosylation is the main constraint, instead of any related functionality, although this is yet to be confirmed (Deyde *et al.*, 2006). The diversity associated with the structural glycoproteins is linked to immune selection and effective attachment of the virus in both the vector and the host cells (Deyde *et al.*, 2006; Lukashev, 2005).

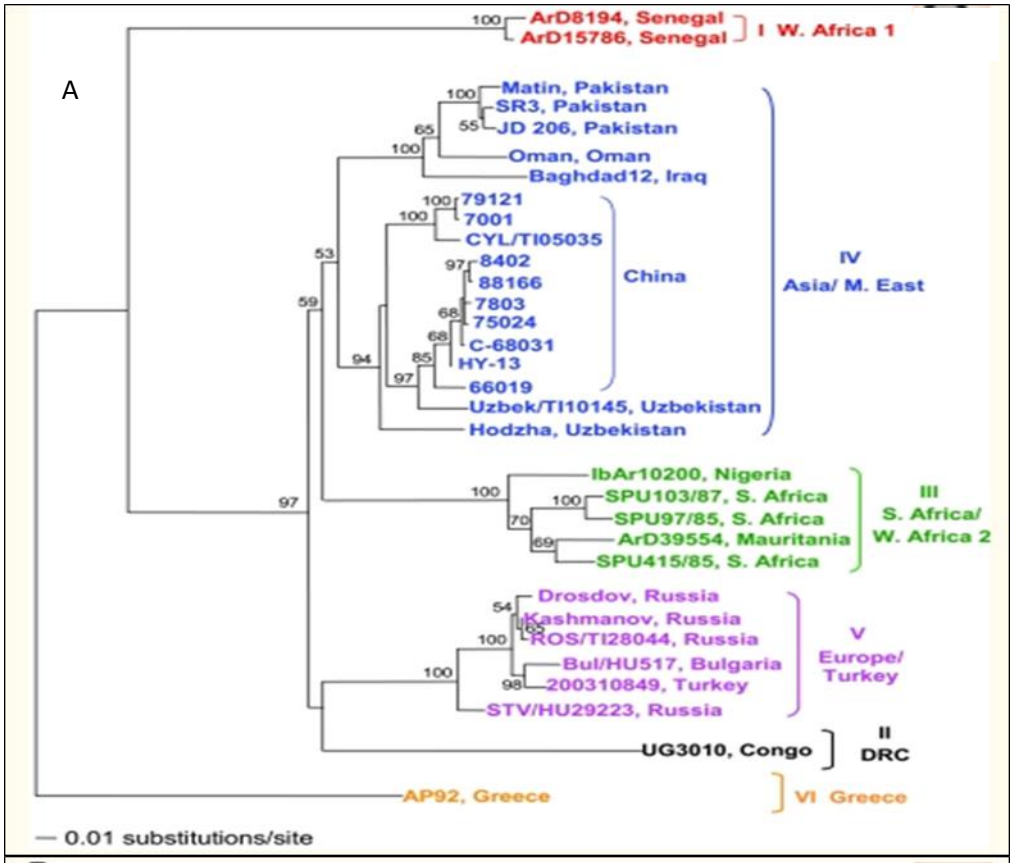
The high genetic variation of the overall viral genome is believed to have been influenced by events including mutation accumulation, segment reassortment and RNA recombination amongst the isolates of the virus (Burt *et al.*, 2009; Carroll *et al.*, 2010; Deyde *et al.*, 2006; Hewson *et al.*, 2004b). Mutation accumulation was proposed to occur due to the lack of proof reading ability of the CCHFV RdRp (Holland *et al.*, 1998).

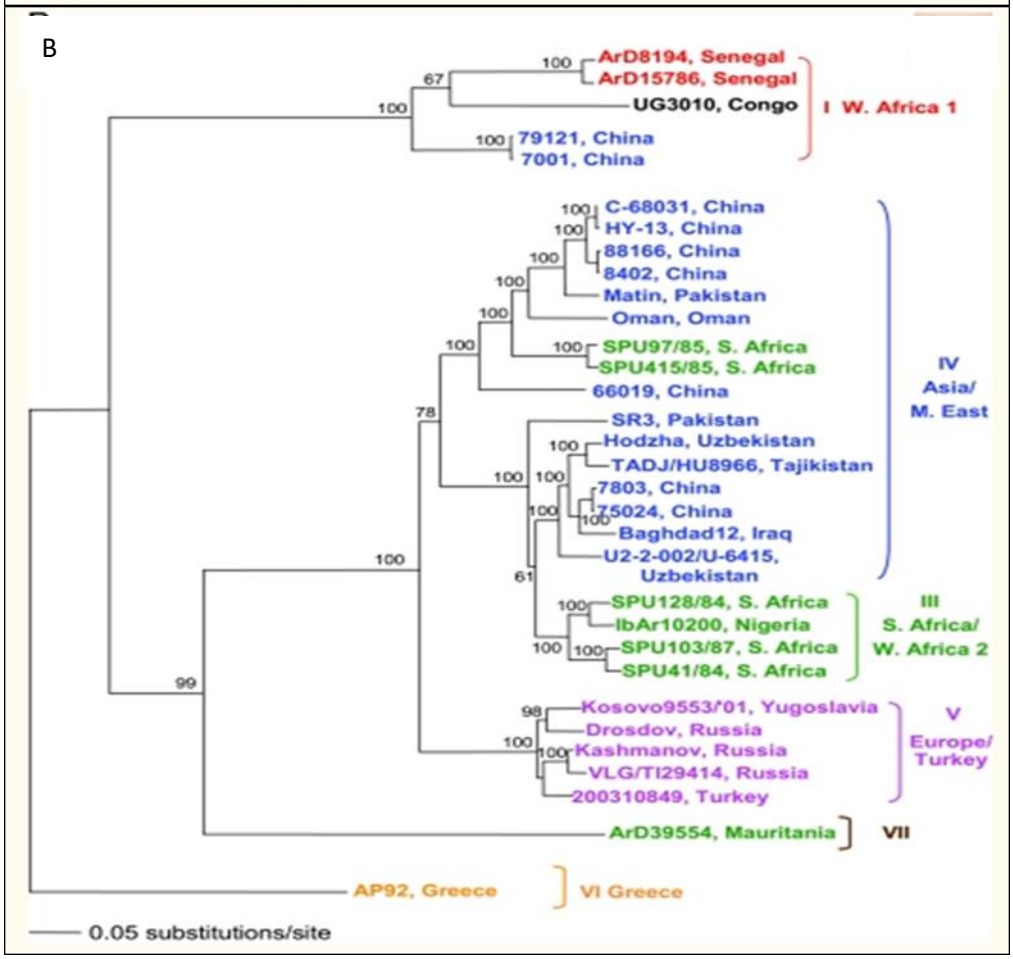
Phylogenetic analysis of global CCHFV sequences has observed evidence of recombination in the S segment, but unconvincing observations in the M and L segments (Chare *et al.*, 2003; Deyde *et al.*, 2006; Lukashev, 2005). On the other hand, genetic reassortment has been observed in all three segments, but occurred more frequently in the M segment than in the S and L segments (Burt *et al.*, 2009; Carroll *et al.*, 2010; Deyde *et al.*, 2006; Hewson *et al.*, 2004b).

Recombination and reassortment are proposed to occur in the tick vectors during co-infection, as the virus persists and undergoes transovarial transmission through the ticks (Burt *et al.*, 2009; Deyde *et al.*, 2006; Goedhals *et al.*, 2014; Lukashev, 2005). The observation that reassortment and recombination varies amongst the isolates over a wide geographical distribution, points to the likelihood of a global reservoir, which are proposed to be migratory birds, or animals that are frequently exchanged between and within continents, such as livestock (Burt *et al.*, 2009; Carroll *et al.*, 2010; Goedhals *et al.*, 2014; Lukashev, 2005).

The phylogenetics of CCHFV can be studied by the alignment of the complete or partial sequences of the L, M or S segments (Deyde *et al.*, 2006). Studies have found that CCHFV has seven distinct lineages reflecting their approximate geographical distribution (Carroll *et al.*, 2010; Deyde *et al.*, 2006). These seven lineages are designated with roman numerals as follows: I West Africa 1, II Demographic Republic of Congo, III Southern Africa and West Africa 2, IV Asia and Middle East, V Europe and Turkey, VI Greece, VII Mauritiana.

Research based on southern African isolates observed that the L and S segments cluster in clade III with the western African strains while the M segments of some southern African isolates cluster in clade IV with the Asian and Middle East strains (Carroll *et al.*, 2010; Deyde *et al.*, 2006; Goedhals *et al.*, 2014), in agreement with the model in Figure 1.6 below.


A
ArD8194, Senegal
ArD15786, Senegal
I W. Africa 1
Matin, Pakistan
SR3, Pakistan
JD 206, Pakistan
Oman, Oman
Baghdad12, Iraq
79121
7001
CYL/TI05035
8402
88166
7803
75024
C-68031
HY-13
66019
China
Uzbek/TI10145, Uzbekistan
Hodzha, Uzbekistan
IV
Asia/ M. East
IbAr10200, Nigeria
SPU103/87, S. Africa
SPU97/85, S. Africa
ArD39554, Mauritania
SPU415/85, S. Africa
III
S. Africa/
W. Africa 2
Drosdov, Russia
Kashmanov, Russia
ROS/TI28044, Russia
Bul/HU517, Bulgaria
200310849, Turkey
STV/HU29223, Russia
V
Europe/
Turkey
UG3010, Congo
II
DRC
AP92, Greece
VI Greece
0.01 substitutions/site


B
ArD8194, Senegal
ArD15786, Senegal
UG3010, Congo
I W. Africa 1
79121, China
7001, China
C-68031, China
HY-13, China
88166, China
8402, China
Matin, Pakistan
Oman, Oman
SPU97/85, S. Africa
SPU415/85, S. Africa
66019, China
SR3, Pakistan
Hodzha, Uzbekistan
TADJ/HU8966, Tajikistan
7803, China
75024, China
Baghdad12, Iraq
U2-2-002/U-6415,
Uzbekistan
IV
Asia/
M. East
SPU128/84, S. Africa
IbAr10200, Nigeria
SPU103/87, S. Africa
SPU41/84, S. Africa
III
S. Africa/
W. Africa 2
Kosovo9553/'01, Yugoslavia
Drosdov, Russia
Kashmanov, Russia
VLG/TI29414, Russia
200310849, Turkey
V
Europe/
Turkey
ArD39554, Mauritania
VII
AP92, Greece
VI Greece
0.05 substitutions/site

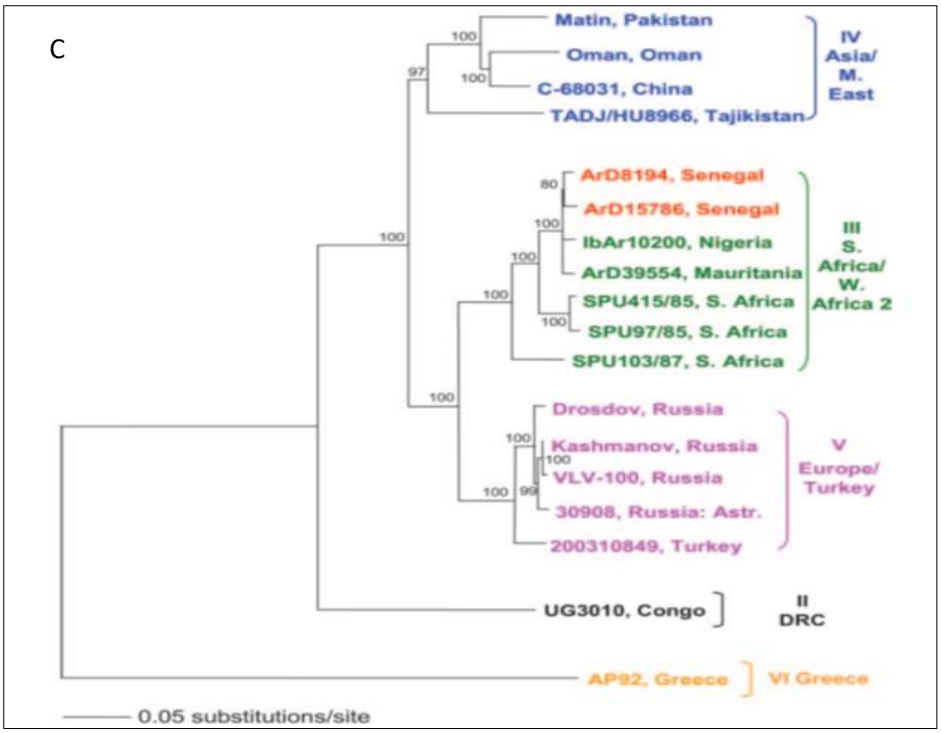


Figure 1.6. Phylogenetic trees of the CCHFV complete S, M and L segments of isolated strains using the Bayesian algorithm. A. Depicts the phylogenetic relationship based on 32 sequences targeting the S segment. B. Depicts the phylogenetic relationship based on the M segment of 32 sequences. C. Depicts the phylogenetic relationships based on the L segment of 18 sequences (Deyde *et al.*, 2006; permission obtained from American Society for Microbiology attached in appendix G).

An example of the M segment reassortment is evidenced by the southern African isolates SPU415/85 and SPU97/85, which cluster with group III (Southern Africa and West Africa 2) on the S and L phylogenetic trees, but cluster with group IV (Asia and Middle East) on the M phylogenetic tree. The clustering of the M segment of some southern African isolates with the Asian clades is proposed to have been influenced by the pattern of migratory birds, thus introducing the spill over of virus circulation between the two continents (Burt *et al.*, 2009).

1.1.5 HUMAN INFECTIONS AND CLINICAL PRESENTATION

Humans are dead end hosts for CCHFV as they do not play a role in maintaining CCHFV in nature. Only humans develop disease, while other mammals clear infection without any signs of illness (Bente *et al.*, 2010; Canakoglu *et al.*, 2015; Saksida *et al.*, 2010).

Humans acquire CCHFV through tick bites, squashing of ticks with bare hands and contact with body fluids of viraemic animals or infected patients (Shepherd, *et al.*, 1989b; Swanepoel *et al.*, 1998). Individuals with a higher risk of CCHFV infection are people who spend long periods outdoors such as campers and hikers, individuals who work in close proximity to animals such as farmers, butchers, veterinarians and abattoir workers, and lastly healthcare personnel (Akuffo *et al.*, 2016; Hoogstraal, 1978; Swanepoel *et al.*, 1998).

The knowledge of the risk groups has, in the past, allowed for better control of the disease and prevention of further spread through targeted population prevention strategies. These include the use of personal protective equipment (PPE) for health care professionals, education for the community and teaching the removal of ticks correctly for targeted population groups (Hoogstraal, 1978). The use of insecticide aerial spray has seldom been used, but was recommended in specific habitats, for example, the spraying of cattle in 1970s in Astrakhan Oblast (Hoogstraal, 1978).

In a study by Gozel *et al.*, it was found that compliance to using PPE such as masks and gloves plays a role in the prevention of human to human transmission in health care professionals (Gozel *et al.*, 2013). In another surveillance study by Akuffo *et al.*, a seroprevalence of 5.7% was observed in abattoir workers in Ghana performing duties involving handling animal parts and clean-up of the abattoir without the use of PPE (Akuffo *et al.*, 2016).

CCHF occurs in four clinical phases: incubation, pre-haemorrhagic, haemorrhagic and convalescence. The incubation period usually lasts one to three days after inoculation following a tick bite, but it has been found that in cases when a patient is inoculated through exposure to infected body fluids, the incubation period is longer at approximately five to six days.

The pre-haemorrhagic phase usually lasts less than a week (three to four days) and is characterized by non-specific influenza like symptoms and may include vomiting (van Eeden *et al.*, 1985). Patients may have neurological symptoms or show confusion (van Eeden *et al.*, 1985). The haemorrhagic phase occurs three to six days after onset of illness and is characterized by skin rash, conjunctival hyperaemia, cutaneous ecchymosis and bleeding from the gastrointestinal tract,

urinary tract and mucous membranes (Leshchinskaya, 1967; Smirnov *et al.*, 1948, van Eeden *et al.*, 1985).

Convalescent patients show signs of improvement from the ninth to tenth day of illness. The rash fades but patients may remain weak along with suffused eyes, slight confusion and amnesia at the time when they are discharged from hospital (Swanepoel *et al.*, 1987). Patients infected during pregnancy may have premature labour and may die due to profuse haemorrhage (Hoogstraal, 1978).

1.1.6 LABORATORY DIAGNOSIS

During the 1960s, CCHFV experimentation included techniques such as intracerebral inoculation of newborn white mice, tissue culture and serological assays (Hoogstraal, 1978). CCHF is diagnosed through a combination of laboratory techniques such as virus isolation, serological techniques including enzyme linked immunosorbent assays (ELISA), indirect immunofluorescence assay (IFA) and molecular techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) (Burt *et al.*, 1994; Burt *et al.*, 1998; Drosten *et al.*, 2002; Osman *et al.*, 2013; Shepherd *et al.*, 1986; Shepherd *et al.*, 1988; Shepherd *et al.*, 1989c; Swanepoel *et al.*, 1983).

The most commonly used sample for CCHF laboratory diagnosis is blood (serum or plasma) (Burt *et al.*, 1994; Drosten *et al.*, 2002; Osman *et al.*, 2013; Shepherd *et al.*, 1986; Shepherd *et al.*, 1988; Shepherd *et al.*, 1989c). Liver tissue can be collected by needle biopsy *post mortem*, as this is a much safer option by reducing exposure to viraemic tissue (Burt *et al.*, 1997). Liver tissue collected *post mortem* can be used for virus isolation, RT-PCR and immunohistochemical assays. The immunohistochemical assays use a cocktail of polyclonal antibodies and monoclonal antibodies directed against GP and NP (Burt *et al.*, 1997).

Virus isolation is usually performed on days one to six after onset of illness, as these are the days when patients are highly viraemic. Virus is isolated in susceptible mammalian cell cultures or in suckling mice after inoculation with infected patient sera (Shepherd *et al.*, 1986). However, in fatal cases patients continue to be highly viraemic for two to eleven days after onset of illness (Shepherd *et al.*, 1989c).

Viral nucleic acid has been detected for up to 16 to 18 days after onset of symptoms (Burt *et al.*, 1998; Wölfel *et al.*, 2007). RT- PCR is performed for the detection of viral nucleic acid with primers mainly targeting the S segment as this segment shows the highest genetic conservation (Burt *et al.*, 1998; Wölfel *et al.*, 2007;). Real time PCR is an alternative method for viral nucleic acid detection (Wölfel *et al.*, 2007). Molecular techniques are being further improved for resource poor settings, such as the development of a loop-mediated isothermal amplification assay (Osman *et al.*, 2013).

From day 5 after onset of illness, antibodies are detected by ELISA or IFA (Burt *et al.*, 1994; Shepherd *et al.*, 1988; Shepherd *et al.*, 1989; Swanepoel *et al.*, 1983). Antigens for IF are prepared from CCHFV infected cell cultures (Saijo *et al.*, 2002). Inactivated antigens (usually NP) for ELISA are extracted from infected mouse brain suspension (Shepherd *et al.*, 1988). Other serological techniques including reverse passive haemagglutination, complement fixation and agar gel immunodiffusion have been replaced due to the lack of sensitivity of these assays (Casals *et al.*, 1980; Shepherd *et al.*, 1989c; Swanepoel *et al.*, 1983).

In convalescent patients, IgM continues to be detected at decreasing concentrations for three to five months, and IgG remains detectable for many years post infection (Shepherd *et al.*, 1989c). Antigen detection by capture ELISA has been described during the acute phase but is not widely used for the diagnosis of CCHF due to the lack of sensitivity relative to virus isolation and RT-PCR techniques (Saijo *et al.*, 2005; Shepherd *et al.*, 1988).

1.1.7 IMMUNOLOGY

The immune system is divided into innate and adaptive immunity. With regards to innate immunity, the interferon system is the most efficient against viruses. The type 1 interferons (IFN α/β) are mediators secreted by virus infected cells as a chemical messenger to neighbouring cells (Gargani, 2012). This results in the production of antiviral proteins such as 2',5'-oligoadenylate synthetase (OAS), the double-stranded RNA-activated protein kinase (PKR), and the Mx proteins (Andersson *et al.*, 2006) by the neighbouring cells which halts early viral replication and consequently the spread of infection, thus allowing the host to gain enough time for the development of the adaptive immune response (Andersson *et al.*,2004; Kaya *et al.*, 2014).

CCHFV was found to be IFN-α and consequently MxA protein sensitive, as the presence of these proteins was found to inhibit CCHFV infection in endothelial cells (Andersson *et al.*, 2006). MxA proteins which are exclusively activated by IFN α/β, interfere with replication possibly through mechanisms of interacting with NP of CCHFV or cellular factors associated with viral NP, as was evidenced by the sequestration of the NP in the peri-nuclear location where it could have co-localized with MxA proteins (Andersson *et al.*, 2004).

Although the host has mechanisms of inhibiting the progression of CCHFV infections, disease progression is believed to be due to three possible mechanisms. These mechanisms are insufficient secretion of antiviral proteins, delayed production of antiviral proteins and viral mechanisms of suppressing interferon production, as has been found in related viruses (Andersson *et al.*, 2004; Andersson *et al.*, 2006).

For protective immunity against viral pathogens, adaptive immunity must be induced. This involves the stimulation of the cell mediated immune system, responsible for defense against intracellular pathogens, including viruses. The cell mediated immune system includes T-lymphocytes, B-lymphocytes, macrophages and natural killer cells (NKs). T cells are mostly comprised of $CD4^+$ (helper) and $CD8^+$ (cytotoxic) cells (Gargani, 2012).

$CD8^+$ T cells recognize peptides bound to class I major histocompatibility complex (MHC) molecules for antigen recognition and act on target cells by two pathways; either through a granule secretory pathway or through ligand-ligand induced target cell death (Betts *et al.*, 2003; Epstein, 2003; Gargani, 2012). The function of the $CD4^+$ T cells is to recognise antigens in association with class ll MHC molecules, and to activate the cells of the innate immunity, B-cells and cytotoxic T cells (Epstein, 2003; Gargani, 2012).

The ligand-ligand induced cell death is triggered when a specific intracellular or extracellular epitope interacts with the T cell receptor (TCR) resulting in the release of cytokines thus attracting cells of the innate immunity (Epstein, 2003; Gargani, 2012). The granule secretion pathway involves the secretion of lytic granules, stimulated by the attachment of an epitope to TCR by a process known as degranulation (Betts *et al.*, 2003).

Granules are vesicles consisting of a lipid bilayer containing lysosomal associated membrane glycoproteins (LAMPs) and cytolytic proteins such as granzymes and perforins which induce death in target cells (Gargani, 2012). This group of proteins includes CD107a, CD107b and CD63. The LAMP proteins are normally found on activated $CD8^+$ T cells undergoing degranulation or cells producing interferon gamma (IFN-γ). However, this does not occur in all antigen specific $CD8^+$ T cells (Betts *et al.*, 2003). The presence of CD107a is proposed to protect against the leakage of lytic proteins from the granules, and it is used as a marker of T cells with cytotoxic activity (Betts *et al.*, 2003).

One of the main factors that influence the susceptibility to or survival of infection of the human host is the genetic composition of the MHC, known as the human leucocyte antigen (HLA) (Epstein, 2003). Therefore, identifying the MHC molecules associated with disease susceptibility allows for identification of the population at risk (Akinci *et al.*, 2013). This has been studied and observed for susceptibility towards HIV, dengue, hepatitis B and C and Epstein Barr infections (Akinci *et al.*, 2013). The association of CCHF susceptibility with MHC alleles has been studied and it was observed that only three alleles may influence susceptibility to CCHFV infection (Akinci *et al.*, 2013).

Akinci *et al*. compared a group of CCHF patients to a control group of healthy solid organ donors from the same geographic region. They observed that the HLA-A*02 allele was more frequent in the patient group, while the HLA-B*27 was lower in the patient group compared to the control group. HLA-A*23 was associated with severe CCHF cases and might be a genetic risk factor for progression to severe CCHF (Akinci *et al.*, 2013). Although in the study a wide variety of HLA alleles were evaluated, the three mentioned above were the only statistically significant observations (Akinci *et al.*, 2013).

The HLA-A, -B and –C are expressed in tissues, and are associated with class I MHC proteins, whereas HLA-DQ and –DR are expressed on antigen presenting cells (including macrophages, T and B cells) and are associated with class II MHC proteins (Epstein, 2003). Thus, according to the study by Akinci *et al.*, CCHF and the severity of disease may be influenced by the expression of certain molecules on the tissues of the host (Akinci *et al.*, 2013).

There is a high degree of polymorphism in the MHC alleles, indicating that the host will express co-dominant heterozygosity (Gargani, 2012). The diversity is beneficial, as it indicates that the MHC class I and II proteins will bind to a wide variety of oligopeptides with high affinity, because the MHC proteins interact with the backbones of the antigenic peptides (Epstein, 2003), thus indicating a high chance of mounting an immune response to a wide variety of diverse pathogenic antigens (Gargani, 2012).

It has been observed that in CCHF survivors, viral loads decrease with time, blood platelet concentration increases with time and there is a production of IgG which results in the subsidisation of symptoms (Kaya *et al.*, 2014). High levels of pro-inflammatory cytokines such as interleukin (IL)-6, IL-10, IL-12, IFN-γ and tumour necrosis factor (TNF)-α are suspected to play a role in disease progression (Connolly-Andersen *et al.*, 2009). The high levels of these cytokines are believed to be associated with endothelial damage, which results in vascular dysfunction (Connolly-Andersen *et al.*, 2009; Kaya *et al.*, 2014; Saksida *et al.*, 2010). Alternatively, endothelial damage could be caused by direct viral invasion in infected cells (Connolly-Andersen *et al.*, 2009; Saksida *et al.*, 2010).

Higher levels of IL-6 and TNF-α were found in fatal CCHF cases compared to non-fatal cases (Kaya *et al.*, 2014; Saksida *et al.*, 2010). IFN-γ levels were high in fatal cases of some studies, while the levels were comparable in both fatal and non-fatal cases of other studies (Kaya *et al.*, 2014; Saksida *et al.*, 2010). A high and increasing viral load between the ranges $\geq 10^8$ or $\geq 10^9$ copies/ml has also been associated with fatality (Kaya *et al.*, 2014; Saksida *et al.*, 2010). However, it seems that these ranges differ between regions and studies (Kaya *et al.*, 2014). Saksida *et al.* found higher levels of IL-10 in fatal cases, which contradict the findings of Kaya *et al.*

It has also been observed that high levels of NK cells, $CD3^+$ T cells and $CD8^+$ T cells are associated with death (Akinci *et al.*, 2009). However, these studies were focused on the association of high levels of immune cells and mediators with death, and thus not a lot is known about immune responses associated with protection against CCHFV infection.

1.1.8 PATHOGENESIS

The pathogenesis of CCHFV has not been fully studied due to the requirement of a BSL-4 facility for the handling of virus and due to the lack of animal models (Bereczky *et al.*, 2010). In 2010, adult type 1 interferon receptor knockout ($IFNAR^{-/-}$) and the signal transducer and activator of transcription 1 knockout ($STAT\text{-}1^{-/-}$) mouse models were developed for CCHF (Bente *et al.*, 2010; Bereczky *et al.*, 2010). Both mouse models have homozygous defective interferon responses and are susceptible to CCHFV infection (Dowall *et al.*, 2016a; Karlberg *et al.*, 2015). Thus, the design was based on "knocking-out" the expression of the normal gene, causing a dominant negative mutation (Epstein, 2003). The $STAT\text{-}1^{-/-}$ mice exhibit selective signalling defects in their response to all three IFNs, while the $IFNAR^{-/-}$ mice are a good model for investigating the adaptive immune response and vaccine efficacy (Dowall *et al.*, 2016a; Hinkula *et al.*, 2017).

The development of these mouse models was a breakthrough, because mammals and adult mice are asymptomatic to the infection. Thus, if the disease does not develop, it would not be possible to study the progression of the disease and the efficacy of treatment and vaccines. Newborn mice succumb to CCHFV infection, but due to the lack of a mature immune system, newborn mice are not suitable for investigating vaccine efficacy (Hinkula *et al.*, 2017).

Histopathological examination of CCHFV infected $IFNAR^{-/-}$ mice showed swollen and congested livers with well-defined haemorrhagic areas, whereas in mice that survived infection, the livers were enlarged with no signs of disease (Bereczky *et al.*, 2010). This gave an indication that the liver and spleen are the main targets of CCHFV (Bereczky *et al.*, 2010).

Histopathological examination of infected $STAT1^{-/-}$ mice showed discoloured livers and spleens, serosal petechiae and intestinal hyperaemia, whereas organs of infected wild type mice (WT129) appeared normal (Bente *et al.*, 2010). High plasma levels of IFN α/β were noted which indicated that IFN was produced, but unable to induce an IFN-mediated antiviral state due to the knocked out STAT1 gene. It was therefore hypothesized that the ability of CCHFV to disable or evade components of the IFN responses may be human specific (Bente *et al.*, 2010).

In summary, it was found that in STAT$^{-/-}$ mice, lymphocyte and APC populations were activated within three days post infection, possibly influenced by early cytokine production or other factors resulting from early viral replication in the spleen. Although there was an increase in the cytokine levels, it was not sustained, suggesting that the innate immunity was unable to control infection long enough for the adaptive immunity to ramp up (Bente *et al.*, 2010). NK cell loss was observed and additionally, there was insufficient upregulation of MHC-II in macrophages and DC that would be necessary for correct activation of the adaptive immune response (Bente *et al.*, 2010). The study was also able to prove that ribavirin is an effective treatment but is highly influenced by the viral load (Bente *et al.*, 2010), with the highest efficacy in a host with a low viral load.

From a variety of CCHF challenge studies in mice, it was concluded that CCHFV targets the spleen and liver, as these were the most affected organs in the body of challenged mice (Bente *et al.*, 2010; Bereczky *et al.*, 2010; Karlberg *et al.*, 2015), although, CCHFV has also been detected in other organs such as brain. The targeting of spleen and liver was also observed in a study based on human biopsy samples, in South Africa (Burt *et al.*, 1997). CCHFV has been found to have successful replication cycles in human dendritic cells, macrophages, hepatocytes and endothelial cells. CCHF has been characterized by loss of endothelial cell function, which leads to changes in vascular permeability, imbalanced fluid distribution between the intra and extra-vascular tissue space, resulting in coagulation disorders, haemorrhage and multi-organ failure (Karlberg *et al.*, 2015).

CCHFV is known to regulate apoptosis, which plays an important role in viral dissemination, survival and killing of uninfected immune cells (Barnwal *et al.*, 2016). Theoretically, viruses may suppress apoptosis, because the activation of apoptosis may negatively affect the replication and thus production of virions.

Two main apoptotic pathways will be discussed; the intrinsic and extrinsic pathways. The intrinsic pathway is characterized by the permeabilization of the mitochondrial membrane. The overexpression of Bax proteins (pro-apoptotic proteins) results in the formation of pores in the mitochondrial membrane, through which cytochrome c leaks into the cytosol and activates a pro-apoptotic pathway (Epstein, 2003). Therefore, this pathway is intrinsic because it is triggered within the cell. The

extrinsic pathway is stimulated by external signals from neighbouring cells, which bind to the TNF receptor family through the TNF and Fas-Fas-ligand mediated model (Epstein, 2003). The binding of the ligands to cellular receptors triggers a cascade of pro-apoptotic proteins within the cell.

The process of apoptosis (both the intrinsic and extrinsic pathways) is mediated by death enzymes referred to as caspases, which are cysteine dependent aspartate-specific proteases and are grouped into initiator and effector caspases. Initiator caspases include caspases 2, 8, 9, 10, 11 and 12, while the effector (also referred to as executor) caspases include 3, 6 and 7 (Epstein, 2003). All caspases are proteolytically activated. The initiator caspases induce downstream caspases, whereas effector caspases activate target proteins by proteolytic activity (Epstein, 2003). The target proteins may be cytosol proteins to be cleaved and trigger cellular apoptosis.

It was observed in a study by Barnwal *et al.*, that the expression of the CCHFV NS_S protein in cells significantly increased the activities of caspases 3/7. It was determined that both the extrinsic and intrinsic pathways were activated in the NS_S induced apoptosis, although the actual mechanism has not been identified (Barnwal *et al.*, 2016).

To study the role NP plays in apoptosis regulation, Karlberg *et al.* demonstrated that CCHFV NP has a conserved cleavage site at the motif DEVD for caspase 3 (Karlberg *et al.*, 2011). It was discovered that NP underwent cleavage at the late stages of infection in SW13 cells (Karlberg *et al.*, 2011). Numerous hallmarks of apoptosis and the 30 kDa NP product were clearly observed in CCHFV infected cells, especially at 72 hours post infection (Karlberg *et al.*, 2011). It was also demonstrated that the inhibition of apoptosis results in the inhibition of the cleavage of CCHFV NP (Karlberg *et al.*, 2011).

It is possible that the activation of the caspase 3 may be induced by host cells as a protective anti-viral response (Karlberg *et al.*, 2015). The activation of caspase 3 leads to the cleavage of the viral NP, and this cleavage has a negative effect on viral replication (Karlberg *et al.*, 2015). It was also observed that apoptotic signalling pathways are suppressed early in the CCHFV replication cycle and the overexpression of the viral NP in a recombinant expression system could inhibit Bax-

induced apoptosis, therefore the NP may play a role in the control of cell death in the early phase of infection (Karlberg *et al.*, 2015).

In summary, the information gathered so far indicates that CCHFV may bypass apoptosis in the early phase of infection, to allow for the mass production of viral progeny. During the late phase, host responses activate apoptosis while simultaneously causing the cleavage of viral NP as a strategy to halt viral replication.

1.1.9 VACCINES

The CCHFV Bulgarian vaccine is the only available vaccine and is administered in Bulgaria and surrounding areas (Hoogstraal, 1978; Mousavi-Jazi *et al.*, 2012). The Bulgarian vaccine has not been internationally approved and only one immunogenicity study has been performed on the vaccine, concluding that the administration of the Bulgarian vaccine stimulates the production of IFN-γ secreting T cells and neutralizing antibodies (Mousavi-Jazi *et al.*, 2012). The approval of the Bulgarian vaccine has been held back by the lack of efficacy studies and lack of convincing evidence that the vaccine stimulates potent long lasting protective immune response against the virus.

Dowall *et al.*, developed a vaccine in which the S segment of CCHFV was incorporated into a modified vaccinia virus Ankara (MVA) vector (Dowall *et al.*, 2016b). The NP was believed to be a desirable target because it is a predominant antigen and has been found to induce a high immune response in hantavirus and Rift Valley fever virus (Dowall *et al.*, 2016b). Antibody and specific T cell responses against CCHFV NP were observed in vaccinated mice. However when faced with a virus challenge, there was no observable difference in survival between mice injected with empty vector and those immunized with the MVA-NP vaccine (Dowall *et al.*, 2016b).

A CCHFV cell culture based vaccine composed of the PreG_N, NP, G_N, and G_C viral proteins was tested in IFNAR$^{-/-}$ mice (Canakoglu *et al.*, 2015). Neutralizing antibodies were detected at titres linked to the dose of the vaccine, therefore immunized mice showed varying degrees of protection (Canakoglu *et al.*, 2015). The highest percentage of survival in challenged mice was 80% after three injections with the vaccine (Canakoglu *et al.*, 2015). At least two immunizations were required for the generation of neutralizing antibodies and it was observed that a strong neutralizing

antibody response is essential for effective protection with the cell culture based vaccine (Canakoglu *et al.*, 2015).

Although the cell culture based vaccine was designed based on the Turkey-Kelkit06 CCHF strain, the CCHF IbAr10200 strain was used to test the antigenicity of the vaccine (Canakoglu *et al.*, 2015). Cross-reactivity towards the G_C and NP proteins of the CCHF IbAr10200 strain was observed, indicating the potential of cross-reactivity of antibodies between these strains of CCHFV (Canakoglu *et al.*, 2015) and further suggests that in the future it will be necessary to evaluate cross reactivity with other CCHFV strains.

Hinkula *et al.*, investigated the immunogenicity and protective efficacy of two vaccine candidates. One was a DNA vaccine coding for CCHFV G_C, G_N and NP, and the other was a transcriptionally competent virus-like particle (tc-VLPs) which was composed of the two structural glycoproteins (G_N and G_C), and a reporter minigenome encapsidated by the NP and bound by a viral polymerase (Hinkula *et al.*, 2017). The $IFNAR^{-/-}$ mice were divided into three groups and the results showed 100% survival in mice vaccinated with the DNA plasmid vaccine at three doses, 80% survival in mice vaccinated with a combination of the DNA plasmid and the tc-VLP vaccine and a 40% survival rate in mice vaccinated with just the tc-VLPs (Hinkula *et al.*, 2017).

Furthermore, the levels of immune stimulation were measured before and after challenge with virus. It was found that although neutralizing antibodies were detected in all groups, the group vaccinated with just the tc-VLPs had higher neutralizing antibody titres compared to the other groups (Hinkula *et al.*, 2017). After challenge, the group vaccinated with the DNA plasmid vaccine and the group vaccinated with a combination of both vaccines had cleared infection and had no detectable viral genome in the sera, but had detectable viral genome at very low levels in the spleen and liver of the mice (Hinkula *et al.*, 2017).

Amongst other tests, a B-cell epitope test was performed after challenge using a strain from Turkey (the study was based on a Nigerian strain), and it was found that the highest responses were towards the N-terminal G_N protein, followed by the G_C epitope and the least positive responses were found towards the mucin-like antigen epitope (Hinkula *et al.*, 2017). This suggests that these may be regions of interest for

diagnostic tools or vaccine development (Hinkula *et al.*, 2017). It was concluded that adaptive immunity combined with neutralizing antibodies provides the most efficient protective immunity against CCHF challenge in IFNAR$^{-/-}$ mice (Hinkula *et al.*, 2017).

Another research group designed a transgenic plant vaccine expressing the structural glycoproteins G_N and G_C of CCHFV in transgenic tobacco leaves and in hairy roots (Ghiasi *et al.*, 2011). The vaccine was aimed to be orally administered to domestic animals followed by an injection with a purified glycoprotein solution as a final boost (Ghiasi *et al.*, 2011). The rationale was that the reduction of CCHFV carriage in domesticated animals would consequently reduce transmission to humans. Significant concentrations of IgG and IgA in faecal pellets were detected in all vaccinated mice (Ghiasi *et al.*, 2011), but unfortunately there have not been any challenge studies performed for this vaccine.

The recombinant DNA vaccine constructs expressing the complete M segment of CCHFV (Spik *et al.*, 2006), is another example. Of the mice vaccinated with this DNA vaccine, only 50% developed neutralizing antibodies, but as there was no mouse model available at the time of this study, no challenge studies were performed to evaluate vaccine efficacy (Spik *et al.*, 2006).

The recombinant MVA-GP is another potential vaccine. It consists of MVA expressing the full M segment open reading frame (Buttigieg *et al.*, 2014). An IFN-γ ELISpot was performed on A129 mouse splenocytes to test the cellular immune response to MVA-GP, and the results showed strong responses towards the NS_M, variable mucin-like domain, GP38 and the N-terminus of the G_C, thus making these proteins epitopes that can potentially stimulate a strong T cell immune response (Buttigieg *et al.*, 2014). Humoral and cell mediated immunity was observed in all vaccinated mice, and all mice were protected when faced with a challenge (Buttigieg *et al.*, 2014).

In a follow up study by a different research group, the $CD3^+$ T cells (crude mixture of $CD4^+$ T and $CD8^+$ T cells) and sera of the MVA-GP vaccinated mice, were transferred into control mice, and the control group were then challenged with CCHFV. It was found that the MVA-GP vaccinated mice once again demonstrated 100% efficacy when challenged (Dowall *et al.*, 2016a). Mice which received both the sera and $CD3^+$ T cells exhibited a significant delayed time to death, when compared

to the group of mice that received only the sera or the $CD3^+$ T cells (Dowall *et al.*, 2016a). It is suspected that the failure to protect the mice was due to insufficient continuous stimulus by the antigen or due to the breakdown of cells or antibodies (Dowall *et al.*, 2016a). It was also observed that the transferred antibodies and $CD3^+$ T cells alone failed to protect mice during virus challenge, and therefore both arms of the immunity are needed for protection against CCHFV infection (Dowall *et al.*, 2016a).

1.1.10 PROBLEM IDENTIFICATION

The understanding of a disease, how to manage and prevent it, requires knowledge of the protective immunity the host must develop in order to prevent disease progression. In the field of CCHFV, immunology research in infected patients has only investigated the association between the levels of inflammatory mediators and death.

Thus far there is limited knowledge about protective immunity in patients following infection with CCHFV; however, challenge studies in mouse models have brought to light, that for protection against infection, both arms of the immune system are required. The need to investigate protective immunity is related to vaccine development and other control measures for the disease. The identification of T cell responses in survivors of CCHF will give an indication of the immunity a vaccine should be able to stimulate in order for the host to mount an effective response for protection. T cell responses are the main targets because of the feature of memory cells, which suggests that stimulation of T cell responses would be favourable for long term immune protection against CCHFV.

One approach to solving the problem is identifying viral epitopes that stimulate T cell responses. The identified epitopes will thus be the main component in tools that will be designed for disease intervention and control. Several studies have investigated the B cell epitopes for CCHFV, but there is still a lack of data regarding T cell responses (Ahmed *et al.*, 2005; Fritzen *et al*, 2018; Goedhals *et al.*, 2015). Thus far research has proved the potential of the NS_M, mucin like variable domain, GP38 and the N-terminus of the G_C protein as potential epitopes for T cell stimulation. This was suggested by a 100% survival rate of challenged mice that were vaccinated with the MVA-GP vaccine (Buttigie g *et al.*, 2014). Hence, it was considered appropriate to

further investigate the role of the non-structural proteins NS_M, GP38 and variable mucin like domain in survivors of CCHFV infections.

1.1.11 AIM

The aim of the study was to identify and characterize T cell responses specific to the non-structural proteins (mucin-like variable domain, GP38 and NS_M) of the M segment of CCHFV in survivors of infection.

1.1.12 OBJECTIVES

The objectives of the study were:

- To identify the presence of T cell responses to the non-structural proteins of the M segment of CCHFV;
- To determine if the reactive epitopes are conserved within all CCHFV strains;
- To confirm cytolytic activity against identified T cell epitopes.

CHAPTER 2
The identification of immunogenic peptides using an IFN-γ ELISpot

2.1 INTRODUCTION

Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) shows a high level of genetic diversity as compared to other arboviruses (Deyde *et al.*, 2006). This is largely due to the accumulation of mutations in the M segment (Carroll *et al.*, 2010; Deyde *et al.*, 2006). The M segment encodes for a glycoprotein precursor which is post translationally cleaved to generate seven proteins as illustrated in Figure 2.1. Genetic studies observed nucleotide variation of 31%, 20% and 22% in the M, S and L segments respectively, and variation in predicted amino acids of 27%, 8% and 10% within the M, S and L segments based on the alignment of 32 full length M segment sequences (Deyde *et al.*, 2006). The majority of the amino acid variations in the M segment are located in the highly variable mucin-like domain (Deyde *et al.*, 2006).

The high genetic diversity of CCHFV may be the result of genetic drift, natural reassortment or recombination. Genetic drift can be attributed to an error prone RNA dependent RNA polymerase (RdRp), which is common in RNA viruses and is essential for accumulating mutations which allow for adaptation of the virus to various ticks and hosts through natural selection (Holland *et al.*, 1998). Reassortment was observed most commonly for the M segment, while L segment reassortment has been demonstrated in isolated from West Africa (Burt *et al.*, 2009; Deyde *et al.*, 2006; Goedhals *et al.*, 2014; Lukashev, 2005). Recombination is observed to occur less frequently in the M and L segments of CCHFV, as compared to the S segment (Burt *et al.*, 2009; Carroll *et al.*, 2010; Deyde *et al.*, 2006; Hewson *et al.*, 2004b).

It is expected that the structural glycoproteins have nucleotide variations because of the binding function to cell surface receptors in various ticks and hosts, and these proteins also undergo immune selection as these are the most exposed parts of the virus (Ahmed *et al.*, 2005; Brooks *et al.*, 2013; Lukashev, 2005). It was observed that

although a high nucleotide variation exists between the structural glycoproteins, subtle variations occurred at the amino acid level (Ahmed *et al.*, 2005).

Despite the high diversity of the M segment, the functional domains essential for the processing and trafficking of the glycoprotein precursor, such as the protease cleavage sites and portions of the structural proteins, are highly conserved in all strains (Deyde *et al.*, 2006).

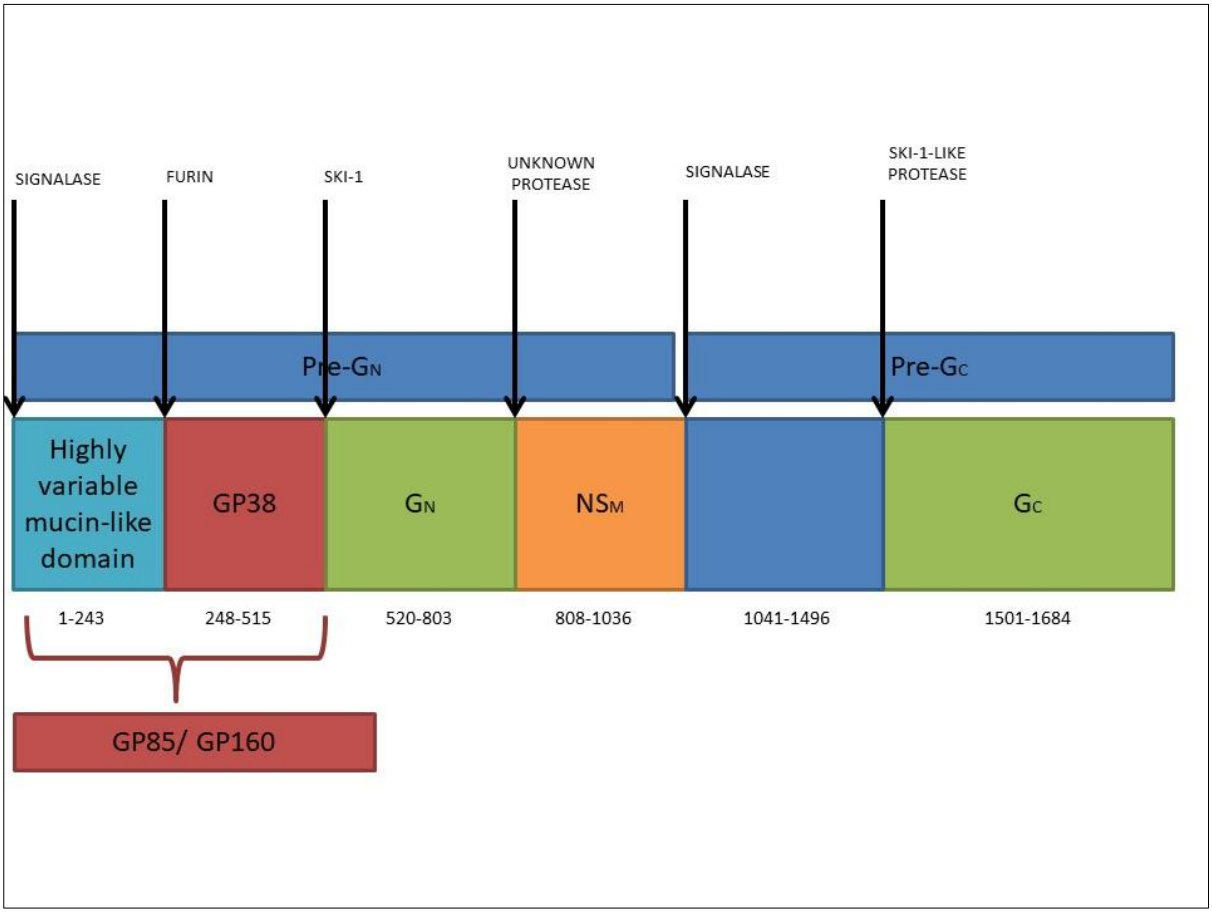


Figure 2.1. Illustration of the glycoprotein precursor. The glycoprotein precursor is post translationally cleaved to generate the non-structural proteins GP38, GP85, GP160, NS_M and highly variable mucin-like domain, and the structural glycoproteins G_N and G_C. The arrows represent each protease cleavage site and the proteases. The proteins are illustrated as coloured blocks. The positions of the proteins are shown under the coloured blocks. GP85 and GP160 may be produced through differential cleavage of the highly variable mucin-like domain and GP38 (Sanchez *et al*., 2006).

Genetically distinct strains of CCHFV are considered antigenically similar. However, there are differences in epitopes. These viral epitopes can be investigated by screening of a peptide library. Peptide libraries are a standard technique for mapping

T cell epitopes. The overlapping peptide library for this study was designed to include the complete regions of the highly variable mucin-like domain, GP38 protein and NS_M, spanning 220 amino acids, 268 amino acid and 229 amino acids, respectively. Synthesis of overlapping peptide libraries allows for the testing of the ability of each peptide to stimulate T cell responses. This approach has been shown to reduce the number of peptides required for coverage of the targeted proteins; the number of cells required for the assays and consequently reduces costs. Figure 2.2 gives an example of an overlapping peptide library.

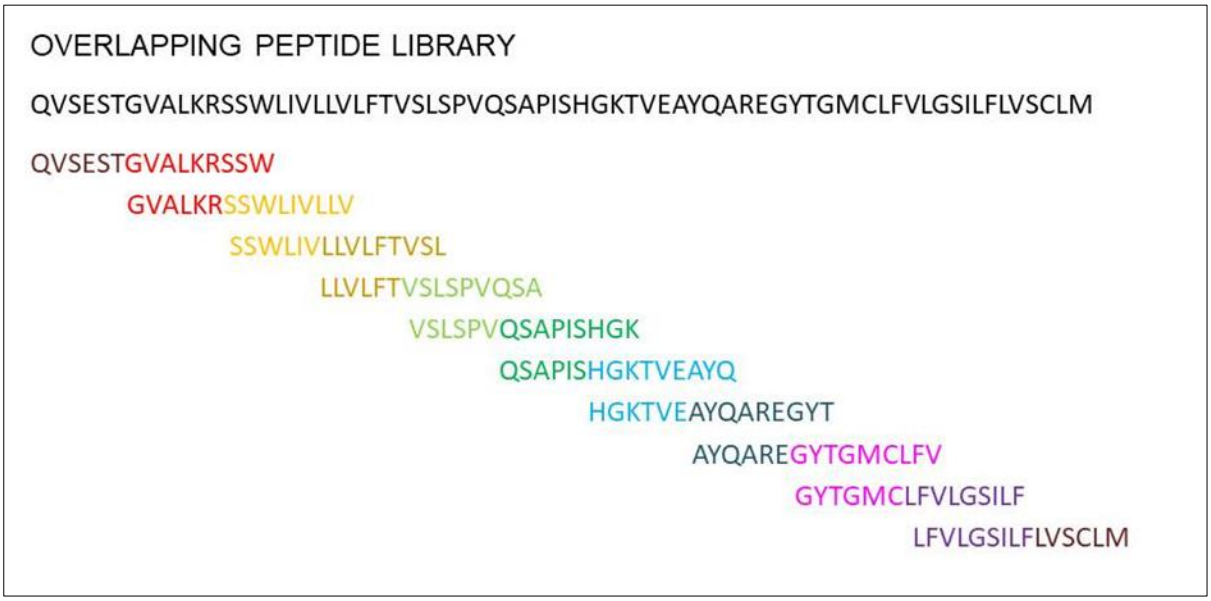


Figure 2.2. An illustration of the overlapping peptide library. The location of the peptides highlights the overlapping nine mer sequences of the peptides by the matching colours. Starting with the red coloured sequence, it is possible to trace how the amino acid sequences overlap. The full reference amino acid sequence is in black at the top.

To investigate epitope cross reactivity, eight monoclonal antibodies directed against G_N and nine monoclonal antibodies against G_C were reacted against five different strains of CCHFV, including two strains from South Africa, one from the Democratic Republic of Congo, one from China and one from Uzbekistan. Of the eight G_N monoclonal antibodies tested, three recognised proteins from all five strains tested, and of the nine G_C monoclonal antibodies tested, only two recognised all strains tested (Ahmed *et al.*, 2005). The rest of the antibodies produced variable recognition patterns. The results indicated that there are differences in epitopes between different genotypes and geographically distinct isolates, also suggesting that the glycoproteins are subject to immune selection (Ahmed *et al.*, 2005).

When exposed to a viral infection, the immune system of the host will be stimulated to fight and clear the infection. The interferon system, which forms part of the innate immunity, is the first line of defence against viruses (Gargani, 2012). Interferons are secreted by virus infected cells and act as cytokines that cause the recruitment of antibodies and send signals to neighbouring cells (Gargani, 2012). Upon arrival of antibodies to the infection site, the virus will be neutralized and simultaneously this activates the recruitment of the cells of the adaptive immunity (Andersson *et al.*, 2004; Gargani, 2012). The adaptive immunity consists chiefly of T cells including $CD4^+$ and $CD8^+$ T cells, amongst others. T cells interact with major histocompatibility complexes (MHC) to clear the virus, by initiating apoptosis or degranulation (Gargani, 2012).

The host immune response stimulated during viral infection must be strong enough to clear the virus. In most cases, an arms race occurs, where the immune system gets stronger and the virus mutates to adapt to the immune pressure within the host. Thus, not all stimulated immunity is efficient enough to clear infection; this is why protective immunity is of importance for disease control measures. As observed in CCHFV vaccine studies, transferred antibodies and $CD3^+$ T cells alone failed to protect mice during challenge, suggesting that both arms of the immune system are required for protection against CCHFV infection (Dowall *et al.*, 2016b). Of interest is the observation that a strong humoral response does not guarantee survival of CCHFV infection. However, in cases where a strong adaptive immunity was induced by a vaccine, all mice survived (Hinkula *et al.*, 2017). This indicates the crucial role adaptive immunity plays in CCHFV protection, with supplementary activity of the innate immunity (Hinkula *et al.*, 2017).

For diagnostic and research purposes, T cells are detected by using markers to identify the cells, whereas T cell activity/responses can be detected by measuring the release of cytokines. Cytokines used as markers for T cell activity mainly include interferons (IFN-γ), interleukins (including IL-2, -10, and -6) and tumour necrosis factors (TNF-α). A number of techniques have been developed for the detection of cytokines as they give an indication of a patient's response to infection (Connolly-Andersen *et al.*, 2009; Kaya *et al.*, 2014). The detection of T cell responses is also useful for monitoring the responses towards vaccines and treatments (Buttigieg *et al.*, 2014; Dowall *et al.*, 2016b).

The enzyme linked immunospot (ELISpot) assay is one such technique, which was designed to detect secreted proteins at single cell level. ELISpots have been developed to detect IFN-γ, TNF-α, IL-2, -1, -4, -6, -8, -10, -12, -13 and cytolytic proteins such as perforins and granzyme B (Czerkinsky *et al.*, 1983; Kuerten *et al.*, 2008). Positive responses to IFN, TNF and IL, give an indication of T cell stimulation and the detection of cytolytic proteins gives an indication of cytotoxic T cell stimulation. The interferon gamma enzyme linked immunospot (IFN-γ ELISpot) assay detects secreted IFN-γ using an anti-IFN-γ capture antibody and a detection antibody linked to an enzyme. A substrate is added, and this results in the visualization of spots in the well which correlates to the positive reaction of secreted IFN-γ, with each spot representing a single cell secreting IFN-γ.

T cells from previously infected individuals can be utilized to screen for epitopes with a potential to stimulate the adaptive immunity. This is because T cells have long term memory against epitopes of viruses that previously infected a host. Therefore, in a case where epitopes that can stimulate T cells need to be identified, T cells from previously infected individuals can be of use.

In this study, survivors of CCHF were recruited, because it was expected that activated memory T cells would recognize CCHFV epitopes to which they were previously exposed. Isolated T cells from recruits can therefore be used to identify epitopes with potential to stimulate T cells. The aim was to identify T cell responses to the non-structural proteins, NS_M, the highly variable mucin-like domain, and GP38, of CCHFV, using peptide libraries representing these proteins to stimulate isolated T cells and an IFN-γ ELISpot to detect any response. Bioinformatic tools were then used to determine if the potential epitopic regions stimulating responses were conserved between different strains of CCHFV.

2.2 METHODS

2.2.1 PARTICIPANT RECRUITMENT

Twelve participants were recruited for the study, under the criteria of having a history of laboratory confirmed CCHF. At the time of acute infection, CCHF diagnosis was confirmed by laboratory tests such as reverse transcription polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA) performed at

the National Institute for Communicable Diseases, Johannesburg, South Africa (Msimang *et al.*, 2013). Participants were identified during a previous study in the Division of Virology or were identified during hospitalization for the acute CCHF infection where the student supervisor was involved in patient management.

Two participants were recruited as negative controls. The negative controls were selected based on the criteria of no history of CCHF, exposure to CCHFV or risk factors for CCHFV such as contact with CCHFV patients or contact with bodily fluids of infected persons or animals. The negative controls were added to confirm that there were no non-specific responses towards the peptides used in the study. The negative controls were not screened serologically for CCHF due to the low prevalence of CCHF in the region, even in high risk groups (Vawda *et al*, 2018).

During the recruitment, all participants signed an informed consent form which was explained in detail, to ensure full understanding of the blood draws and what the blood samples would be used for; attached as Appendix A. Participants were numbered one to twelve for the study, to anonymise the samples. Ethics approval for the study was obtained from the Health Science Research Ethics Committee (HSREC) of the University of the Free State; with the ethics number ECUFS195/2015B (Appendix B).

Eight of the confirmed CCHF participants were recruited from a study previously performed in the department which investigated epitopic regions in the nucleoprotein and structural glycoproteins of CCHFV, using memory T cells from survivors of CCHF (Goedhals *et al.*, 2017). The other four participants experienced acute infection after the timeline of the previous study.

Participant recruitment was centralized in the Free State and North West provinces. All participants resided within four to six hours drive from the laboratory. This was to ensure that the cells could be isolated within a short time after collection to preserve viability and function of the T cells.

2.2.2 SYNTHETIC PEPTIDE LIBRARY

The amino acid sequence of CCHFV isolate SPU 103/87 (GenBank Accession number: ABB30034.1) a South African (SA) isolate, was used for the synthesis of the overlapping peptide library (GenScript, USA). The sequence is attached in appendix

C. A layout of 15 mer peptides, overlapping by nine amino acids, which span all three target proteins was selected, resulting in a total of 120 peptides. The highly variable mucin-like domain was represented by 39 overlapping peptides, while 36 peptides spanned the NS_M protein and 43 peptides spanned the GP38 protein, as in appendix D. However, due to the highly hydrophobic nature of seven of the peptides, these peptides could not be produced, resulting in a total of 113 peptides (GenScript, USA) which were included in the project. Six of the hydrophobic peptides were in the NS_M region and only one was in the GP38 region, these peptides are highlighted in red in appendix D.

2.2.3 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation. Blood was collected from participants in five 10 ml anticoagulant citrate dextrose solution A (ACD-A) tubes (Becton Dickenson, New Jersey), centrifuged at 750 x*g* for 10 minutes and the plasma removed.

The blood cells were poured into a 50 ml centrifuge tube (Sigma-Aldrich, USA) and topped up to 50 ml with Rosewell Park Memorial Institute (RPMI) media (Lonza, Belgium). The RPMI media used for PBMC isolation and ELISpot was supplemented to a final concentration of 1% penicillin-streptomycin (Lonza, Belgium) and 1% L-glutamine (Lonza, Belgium). The blood was layered on Ficoll-Paque™ PLUS (GE Healthcare Biosciences, Sweden) at a ratio of 5:4, with care to keep the two layers distinctively separated. The tubes were then centrifuged at 910 x*g* for 20 minutes with the brake off, to create a density gradient separation, as can be seen in Figure 2.3.

The layers of blood were separated according to the density. The red blood cells are the most dense and thus form a pellet at the bottom, followed by the Ficoll layer, the buffy coat layer and finally the plasma/RPMI layer, which is the least dense. The buffy coat consisting of PBMCs was collected using a sterile pasteur pipette (Plastpro Scientific, South Africa) and transferred to a 50 ml tube. When removing the buffy coat, it was important to make sure that as much as possible of the layer was removed, even if some of the Ficoll and RPMI/plasma layer were also taken with, as the cells were washed in further steps.

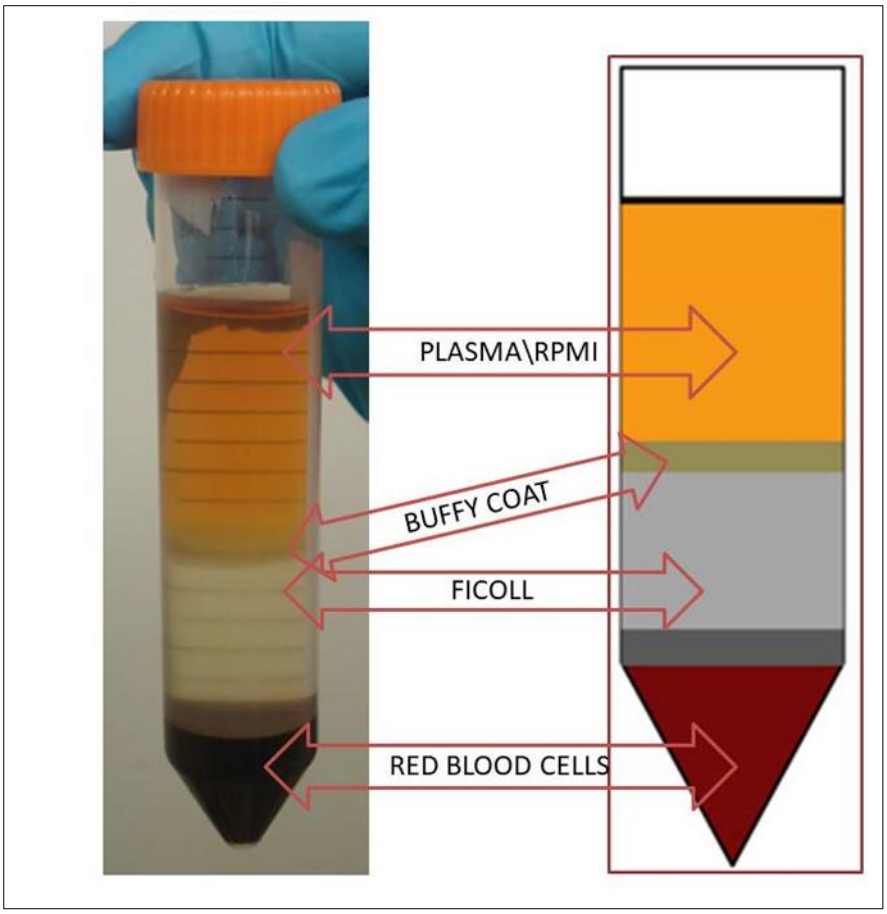


Figure 2.3. Density gradient separation of blood layered on Ficoll, after centrifugation. The picture on the left was taken after the centrifugation step during the PBMC isolation, and the picture on the right is an illustrated demonstration.

The PBMCs were resuspended and washed twice with 50 ml RPMI media and centrifuged at 420 x*g* for eight minutes to remove any Ficoll and RPMI aspirated during buffy coat isolation. The cells were resuspended in 20 ml of RPMI and a cell count was performed to determine the cell concentration and the volume of R10 media required to bring the cells to the desired concentration for subsequent assays using equation 1. The R10 media was made up by combining fetal bovine serum (FBS) (Gibco Life Sciences, South America) and RPMI media at a ratio of 1:9.

EQUATION 1

Total number of cells counted from quadrants/ 400 x 2= ____x10^6 cells/ml

$$V2 = \frac{C1\ V1}{C2}$$

Where
C1 = initial concentration of the cells (cells/ml)

C2= desired concentration (cells/ml)

V1= initial volume (ml)

V2= volume in which cells are to be suspended (ml)

The cells were counted using a haemocytometer (Superior Mareinfeld, Germany) and an inverted microscope (Olympus, Japan). A volume of 20 μl of the cell suspension and 20 μl of trypan blue (Sigma-Aldrich, UK) were mixed in a 0.8 ml microcentrifuge tube (Axygen Incorporated, USA) for the cell count. Trypan blue is a dye used to detect viable cells using an exclusion method. Viable cells have intact cell membranes and thus the dye does not penetrate the cells, and therefore viable cells appear transparent under the microscope. Dead cells have a damaged cell membrane, and so the dye can penetrate the cell, thus causing the non-viable cells to appear blue under the microscope.

The haemocytometer is divided into four quadrants as seen in Figure 2.4. The cells were only counted when situated on the quadrants highlighted in blue squares, and only in a specific direction. In other words, one can count right to down or left to up as illustrated by the arrows in Figure 2.4, this is to make sure the same cells are not counted more than once, especially cells located on the edges. During the count only cells with a perfectly round shape were counted, in order to avoid counting the "potentially" viable cells, thus giving us an estimation that is as close to the accurate number of viable cells as possible. This was necessary because for the IFN-γ ELISpot to be performed, cells had to be viable and functional.

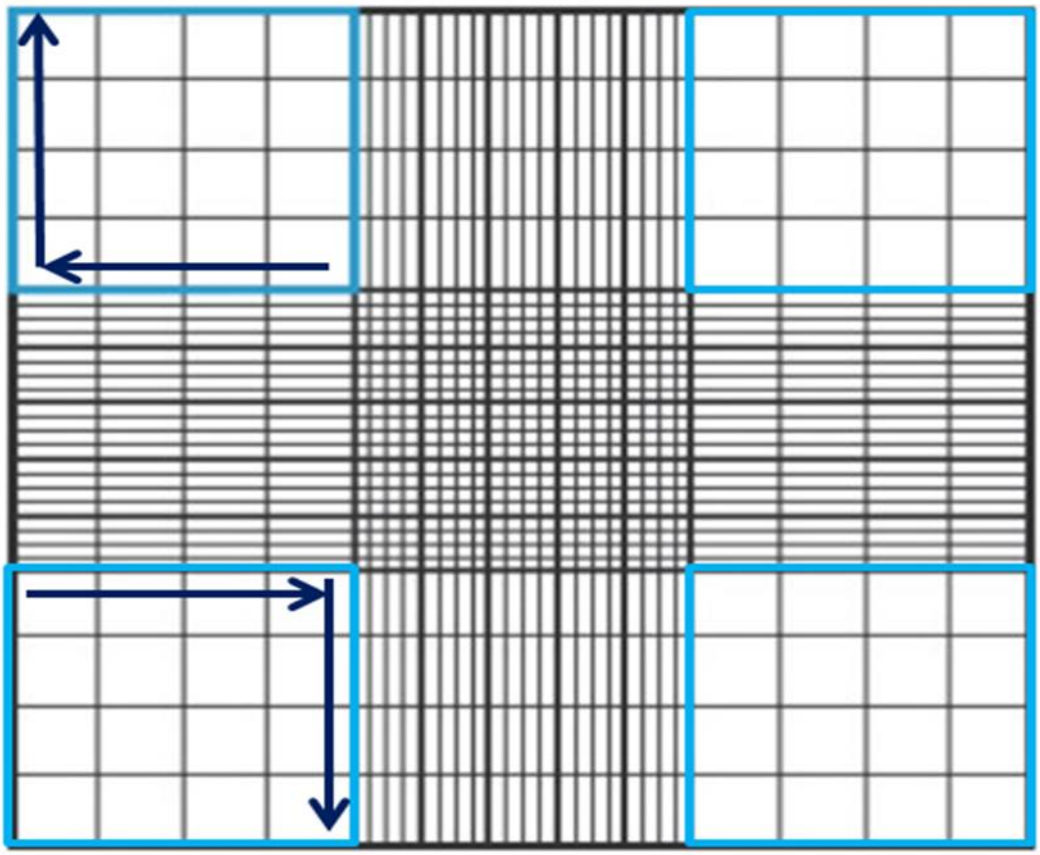


Figure 2.4. Illustration of the haemocytometer used for cell counts. Cells are only counted when located in quadrants highlighted in blue. The arrows illustrate the direction of the cell counts, as either right to up or right to down.

The PBMCs were then resuspended in the calculated volume of R10, to give a concentration of 4 x10^6 cells/ml. A total volume of 2 ml was required for the IFN-γ ELISpot, and the remaining cells were cryopreserved.

For cryopreservation, the remaining cells in R10 were centrifuged at 420 x*g* for 8 minutes. The freezing media was prepared using a ratio of 1:4:5 of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, France), RPMI and FBS. The DMSO prevents formation of crystals during freezing which would cause cell death. After centrifugation the suspension was discarded and the cells were resuspended in freeze media, at the volume V2, calculated according to equation 1.

The suspension was aliquoted into 2 ml cryogenic vials (Corning life sciences, Mexico), stored at -80°C overnight in a Nalgene Mr.Frosty (Sigma-Aldrich, USA) freezing container and then transferred to liquid nitrogen, to be used later. The PBMC thawing protocol is described below.

2.2.3.1 PBMC thawing

The media used for the protocol were R1, R10 and R20, which were prepared by combining FBS and RPMI at ratios 1:100 for R1 media and 1:5 for R20 media. The R10 media was prepared as stated above.

Cryopreserved PBMCs were removed from the liquid nitrogen and immediately thawed in a 37°C water bath (Labotec, South Africa). Once thawed, the PBMCs were transferred into a 50 ml centrifuge tube, and 15 ml of pre-warmed R1 was added dropwise using a pasteur pipette while swirling the cells. The suspension was then topped up to 25 ml with R1 and centrifuged at 750 x*g* for 10 minutes. The suspension was discarded, and cells were resuspended in 25 ml of R1 media and centrifuged at 750 x*g* for 10 minutes. Cells were resuspended in 5 ml of R20 and a cell count was performed to calculate the volume (V2) of R20 needed to bring cells to a concentration of 2 $X10^6$ cells/ml using equation 1 above.

The cells were incubated in 5% CO_2 at 37°C for two hours in order to stimulate growth of the cells. PBMCs were centrifuged at 750 x*g* for 10 minutes and resuspended in 25 ml of R1. The suspension was centrifuged at 750 x*g* for 10 minutes and the cell pellet resuspended in 1 ml of R10. A cell count was performed to determine the volume of R10 (V2) needed to bring cells to a concentration of $4x10^6$ cells/ml, using equation 1. The PBMCs were then resuspended in the appropriate volume of R10 and used for the IFN-γ ELISpot.

2.2.4 INTERFERON GAMMA ENZYME LINKED IMMUNOSPOT (IFN-γ ELISpot)

Cells were initially screened using 113 peptides (15 mers) (GenScript, USA) grouped into 24 overlapping pools, meaning that each peptide was included in two separate pools. The 24 pools each contained between three and ten peptides (appendix E). Confirmation of reactive peptides was then performed using individual 15 mer peptides, which were identified from the pools which were positive for IFN-γ secretion. Individual peptide ELISpots were performed using thawed PBMCs.

The IFN-γ ELISpot was performed using a 96 well Multiscreen filter plate (Millipore™, Ireland). Plates were coated with 2 μg/ml mAb 1-DIK capture antibody (Mabtech, Sweden) and incubated overnight at 4°C. The mAb 1-DIK capture antibody is an anti-human IFN-γ specific monoclonal antibody produced in mice. The plate was washed three times with 200 μl/well of phosphate buffered saline (PBS) (Sigma-

Aldrich, USA) pH 7.4 and blocked with 100 µl/ well of R10 media for two hours to prevent non-specific binding in the wells.

Isolated PBMCs were seeded at 200 000 cells/well and stimulated with peptide antigens for the secretion of IFN-γ. Peptides were tested at a final concentration of 5µg/ml for both the pools and individual peptides. The plate layout is shown in Figure 2.5. Column 1 of the plate was reserved for the controls. Two positive controls were used; namely phytohaemagglutinin (PHA) (Sigma-Aldrich, USA) and anti-CD3 antibodies (clone CD3-2, Mabtech, Sweden). PHA is a mitogen and induces T lymphocyte cell division. Anti-CD3 antibody binds to $CD3^+$ T cells, and as CD3 is an invariant subunit of the T cell receptor it is expected to always produce a positive response. The PHA stock (1 mg/ml) was diluted in R10 media at ratio of 1:50 and anti-CD3 stock at 0.1 µg/ml. Both positive controls were tested in duplicate for each test, in other words, two wells per positive control. PBMCs and R10 media with no peptides or stimulants, and a well with just media and no cells or stimulants were used as negative controls (Figure 2.5). The plate was incubated overnight (~16 hours) at 37°C with 5% CO_2.

	1	2	3	4
A	No Antibody (neg.control)	POOL 1	POOL 9	POOL 17
B	R10 MEDIA (neg control)	POOL 2	POOL 10	POOL 18
C	R10 MEDIA + PBMCS (neg control)	POOL 3	POOL 11	POOL 19
D	R10 MEDIA + PBMCS (neg control)	POOL 4	POOL 12	POOL 20
E	Anti-CD3 + PBMCs (pos control)	POOL 5	POOL 13	POOL 21
F	Anti-CD3 + PBMCs (pos control)	POOL 6	POOL 14	POOL 22
G	PHA + PBMCs (pos control)	POOL 7	POOL 15	POOL 23
H	PHA + PBMCs (pos control)	POOL 8	POOL 16	POOL 24

Pos- positive, neg- negative.

Figure 2.5. An illustration of the plate layout for performing the IFN-γ ELISpot. The first column demonstrates in detail the layout of the controls for every IFN-γ ELISpot performed. The subsequent columns (2-12, not all shown) were used for the pools or individual peptides to be screened.

The plate was washed six times with 200 µl/well of PBS and reacted with 50 µl biotinylated monoclonal antibody 7-B6-1(Mabtech, Sweden) at 2 µg/ml. The plates were incubated at room temperature for three hours and washed as described

previously. Positive reactors were detected using streptavidin alkaline phosphatase (Mabtech, Sweden) diluted 1:2000 for one hour at room temperature and alkaline phosphatase (AP) conjugate substrate kit (BioRad, USA). The plate was incubated at room temperature in the dark for 10 minutes, which resulted in the development of purple coloured spots in the positive wells. The biotinylated antibody 7-B6-1 and the streptavidin alkaline phosphatase form an IFN-γ detection unit, where the utilization of the substrate by the phosphatase resulted in the appearance of the purple coloured spots as illustrated in Figure 2.6.

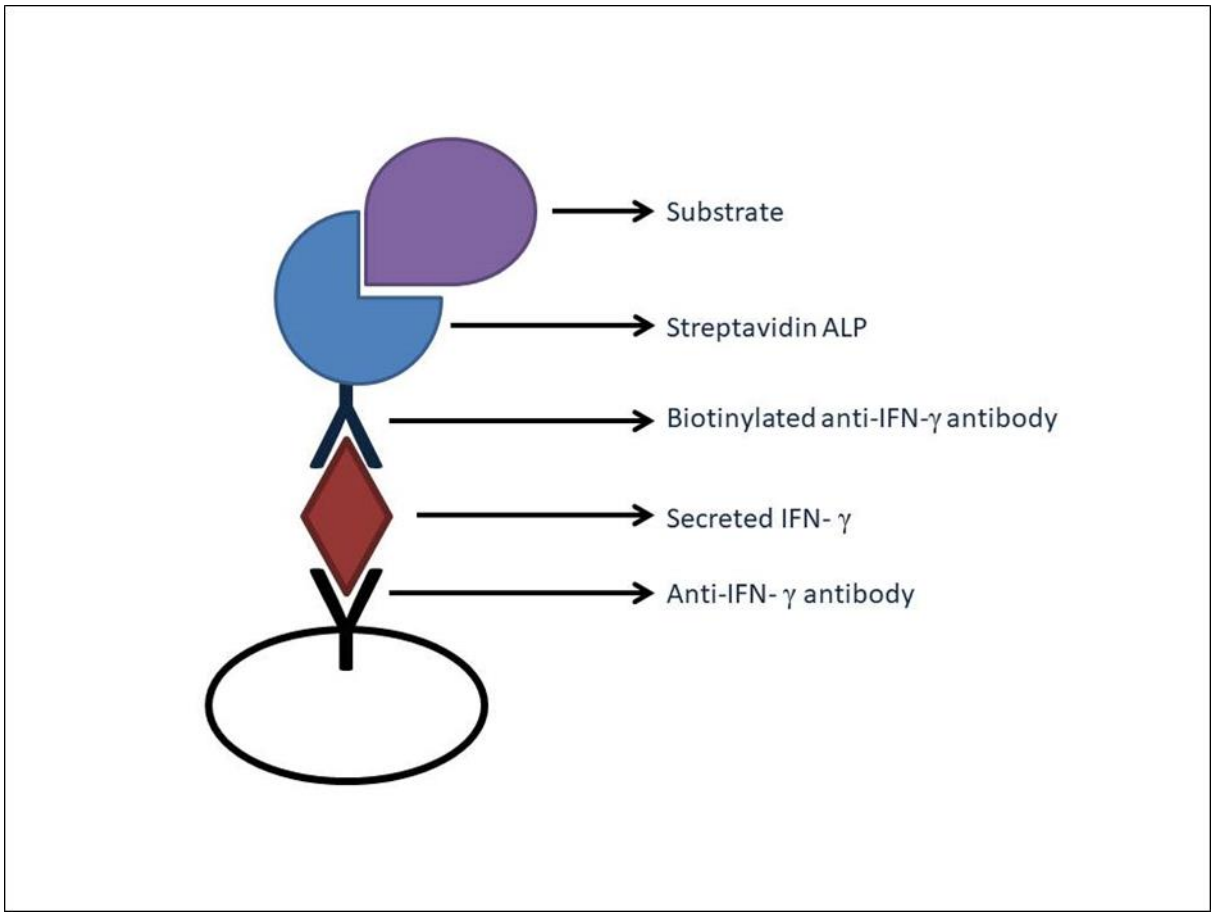


Figure 2.6. An illustration of the principle of the IFN-γ ELISpot.

After the 10 minutes incubation with the substrate, 100 μl/well of distilled water was added to stop the reaction and to prevent development of background colouring. Plates were left to dry at room temperature. Results were read as spot forming cells (SFC) per million PBMCs (SFC/10^6) by manual counting, using the Nikon SMZ800 stereomicroscope (Nikon, USA). The average background SFC/10^6 cells of the negative control wells were subtracted from each reading as per equation 2 below.

EQUATION 2

SFC/10^6 (counts from a tested well) **-** SFC/10^6 (counts from the negative control wells) **×**5 = ___ SFC/10^6

Any count above 50 SFC/10^6 after the subtraction of the background was regarded as significant. The individual peptides were tested in duplicate to confirm the responses.

2.2.5 BIOINFORMATICS ANALYSIS OF AMINO ACID CONSERVATION

The peptides that were identified as immunogenic based on the IFN-γ ELISpot screening were analysed using bioinformatics software. A total of 138 complete CCHFV M segment amino acid sequences were retrieved from GenBank in Fasta format. The sequences were then aligned to form a multiple alignment document using Clustal X 2.1 software (Larkin *et al.*, 2007; Thompson *et al.*, 1997).

In all the bioinformatics analysis, the SA strain used for the development of peptides in this project was used as the reference sequence. The global strains of CCHFV and the SA strains of CCHFV were analysed separately, although the SA strains were included in the analysis of global strains.

The multiple alignment documents were loaded on the ConSurf software website http://consurf.tau.ac.il, and the amino acid conservation score was calculated using the empirical Bayesian algorithm (Ashkenazy *et al.*, 2010; Ashkenazy *et al.*, 2016; Berezin *et al.*, 2004; Celniker *et al.*, 2013). The score is graded on a scale of 1 to 9, where 1 indicates that the amino acid at the specific location is highly variable, 5 indicates moderately variable and 9 indicates conserved. Since the amino acids for each isolate were graded individually, in order to find the grade for the peptide of interest, the location of the amino acids of the peptide of interest was identified and the average grade of the peptide was calculated using equation 3 below. The average grade was used for analysing the conservation of the peptide of interest.

EQUATION 3

Average grade = sum of individual grades / number of amino acids

The multiple alignments were analysed using BioEdit sequence alignment editor software, version 7.2.5 (Hall, 1999). The software was set to analyse the sequences using a standard dot to indicate identical amino acids which would imply that the region is conserved. The location of the amino acids of interest was identified, and the level of amino acid conservation was analysed manually by the presence of dots in the region of interest.

The sequences were manually aligned for accurate analysis. The analysis with this software only allows for visual observation of the amino acid conservation. The BioEdit analysis also included a comparison of the variability amongst the three domains of interest in the project. The two software programmes were used in conjunction to analyse the conservation of the peptides of interest.

2.3 RESULTS

2.3.1 PARTICIPANTS

The twelve participants recruited for the study resided within the Free State and North West provinces. The profile of each participant is summarized in Table 2.1.

The locations mentioned in this analysis are where the participants resided at the time of infection. At the time of infection, three participants were located in Heilbron, two in Jacobsdal and one each from Klerksdorp, Bloemfontein, Senekal, Fauresmith, Wepener, Steynsrus and Bloemhof, as on the map in Figure 2.7. The most recent cases of CCHF (2014-2017), were located in Wepener, Steynsrus and Bloemhof.

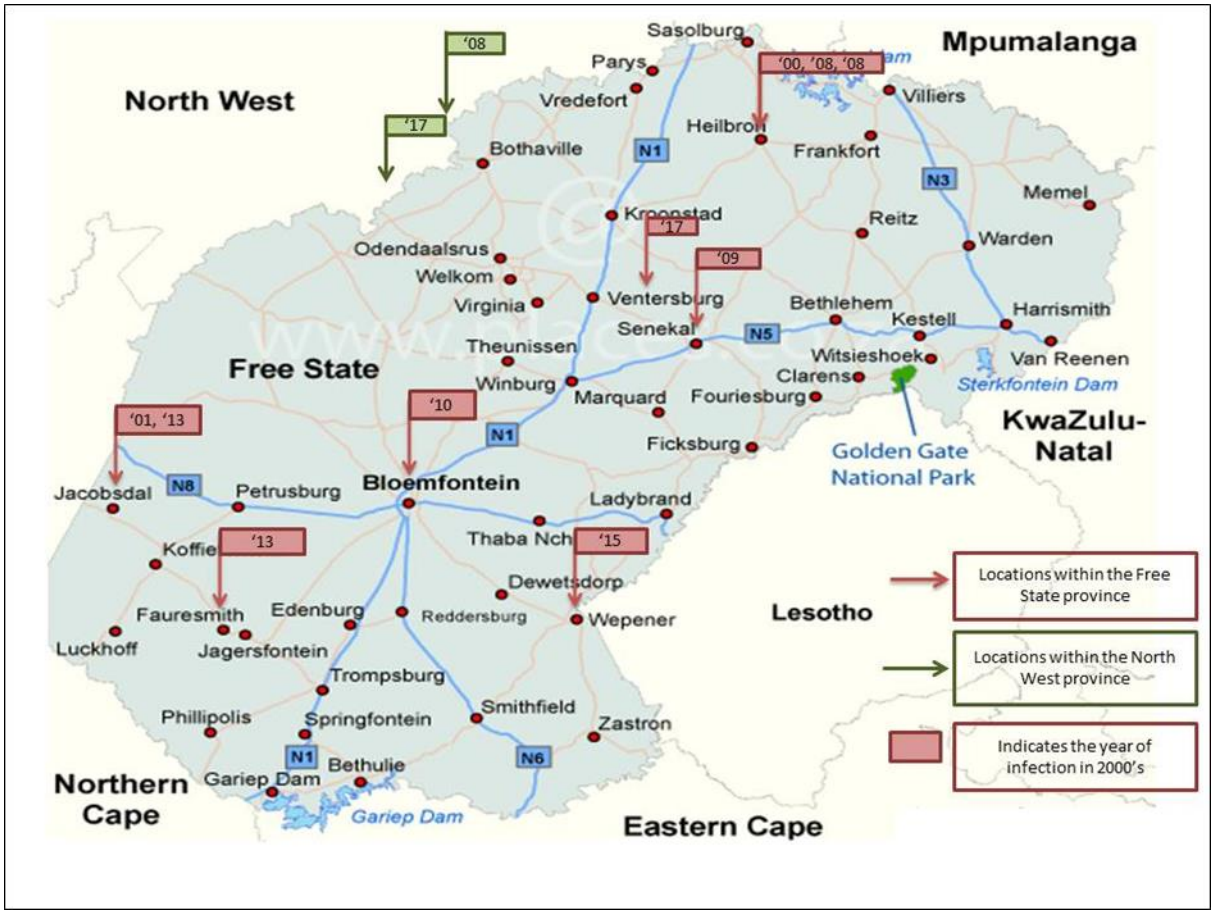


Figure 2.7. The approximate locations of infection for the twelve participants included in the study. The red and green arrows indicate the location of infection, and the year of infection is written in the blocks attached to the arrow. The red arrows highlight locations in the Free State and the green arrows highlight locations in the North West province.

Table 2.1. Participant details

Participant study number	Year of infection	Number of years since infection	Location of infection	Gender	Ethnicity
1	2013	6 years	Jacobsdal	Male	Caucasian
2	2015	4 years	Wepener	Male	Caucasian
3	2009	10 years	Senekal	Male	Caucasian
4	2001	18 years	Jacobsdal	Male	Caucasian
5	2010	9 years	Bloemfontein	Male	Caucasian
6	2000	19 years	Heilbron	Male	Caucasian
7	2008	11 years	Heilbron	Male	Caucasian
8	2008	11 years	Heilbron	Male	Caucasian
9	2013	6 years	Fauresmith	Male	African
10	2017	2 year	Steynsrus	Male	Caucasian
11	2008	11 years	Klerksdorp	Male	Caucasian
12	2017	2 year	Bloemhof	Male	Caucasian

Participants were given a study number from one to twelve to ensure anonymity. The infection profile of the participants in the study shows that the study included participants infected between the years 2000 to 2017. It was observed that all participants were male, and the majority were of Caucasian ethnicity.

2.3.2 IFN-γ ELISPOT

2.3.2.1 Visual observations of IFN-γ ELISpot plates

The figure on the following page depicts examples of the ELISpot results. The dots indicate a positive response therefore indicating IFN-γ stimulation. The dots were counted to determine the significance of the responses with a count of 50 SFC/10^6 or more indicating a positive response. The wells with no dots in them indicate that no IFN-γ was secreted; therefore, no stimulation occurred in the well. In cases where the spot counts were just above 50 SFC/10^6 or just below the 50 SFC/10^6, in the ranges 45 SFC/10^6 to 70 SFC/10^6 were considered as weak responses, and therefore the screening was repeated to confirm if the response was positive or non-specific.

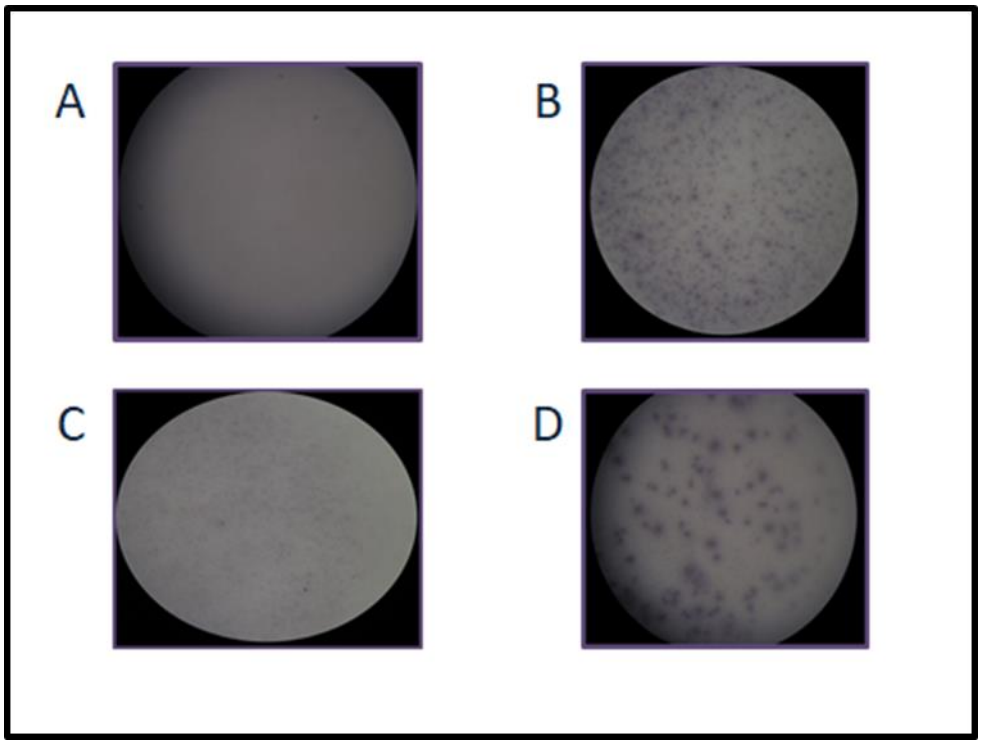


Figure 2.8. The appearance of IFN-γ ELISpot wells. Images A-D Depict an example of the assay outcome. A. Negative control. B. Positive control. C. Negative IFN-γ response. D. Positive IFN-γ response.

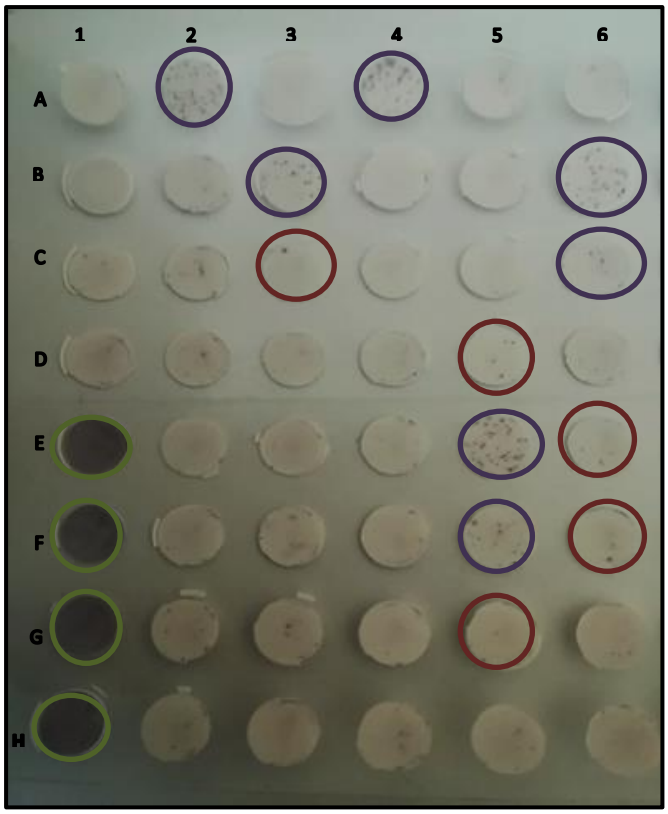


Figure 2.9. An example of a successful IFN-γ ELISpot. Column 1 contains the controls, wells A-D are the negative controls and wells E-H are the positive controls, with very strong responses highlighted with green circles. Columns 2-6 show the wells which were stimulated with peptides, where the wells with positive IFN-γ responses are highlighted with purple circles, while the red circles highlight weak IFN-γ responses which were not significant (<50 SFC/10^6). The rest of the wells had no responses and were thus negative for IFN-γ responses. The layout of the plate illustrated in Figure 2.5 was used for all experiments.

2.3.2.2 IFN-γ ELISpot screening results

For each participant, 24 pools as per appendix E were screened. The positive pools were identified, and the overlapping peptides were identified and tested individually in duplicate to confirm that T cells responses were stimulated. In some cases, individual screening of all peptides included in the positive pools was performed in order to ensure that no positive responses were missed, particularly where pool responses were relatively weak. Screening was repeated if high background was detected in the controls. The ELISpot results per participant are described below.

Participant 1

1st Pool screening	Results: too much background in the negative controls
2nd Pool screening	Results: too much background in the negative controls

High background cell counts were observed, including the negative control wells, despite using PBMC extracted on two separate occasions. Therefore, stimulation of CCHFV-specific T cell responses could not be evaluated and thus the participant was excluded from further analysis in the study.

Participant 2

Pool screening	Results: weakly positive responses against pool 7 and pool 15 Overlapping peptide: 66
Peptide screening	Peptides screened (all individual peptides in pool 7 and 15): 3, 15, 27, 39, 54, 66, 82, 94, 106, 118, 64, 65,66, 70, 71, 72, 73, 74, 75 and 76 Results: No individual responses ≥50 SFC/10^6

The participant had weak responses to two pools, while both positive and negative controls were satisfactory. Individual peptides from the two positive pools were screened to ensure that low level responses were not missed; however, no positive individual peptide responses were detected. This may indicate the possibility of non-specific responses or waning T cell immune responses.

Participant 3

Pool screening	Results: positive responses against pools 1, 4, 7, 13, 24 Overlapping peptides: 1, 36, 37, 64
1st peptide screening	Screened peptides 1, 7, 31, 36, 37, 64, 73, 110, 115 and 116 Results: No responses ≥50 SFC/10^6
2nd peptide screening	Screened peptides 1, 7, 31, 36, 37, 64, 73, 110, 115 and 116 Results: ≥50 SFC/10^6 for peptide 64
3rd peptide screening	Screened peptide 64 Results: ≥50 SFC/10^6 for peptide 64

Four overlapping peptides were identified from screening peptide pools. With the 1st peptide screening, no significant responses were detected. However, the controls were satisfactory. After observing strong responses for peptide 64 in the second peptide screening, the experiment was repeated to confirm significant responses for peptide 64.

Participant 4

1st pool screening	Results: No responses ≥50 SFC/10^6
2nd pool screening	Results: No responses ≥50 SFC/10^6. Weak responses against pool 5 and pool 21. Overlapping peptide: 46
Peptide screening	Screened peptides (all individual peptides in pool 5 and 21) 41, 43, 44, 45, 46, 47, 50, 51, 52, 53, 9, 21, 33, 46, 60, 75, 88, 100, 112 Results: No responses ≥50 SFC/10^6

Results showed weak responses against two peptide pools, while both positive and negative controls were acceptable. All the individual peptides from the two weakly positive pools were screened to ensure that low level responses were not missed; however, no positive responses against individual peptides were detected. This may indicate the possibility of non-specific responses or waning T cell related immune responses.

Participant 5

Pool screening	Results: positive responses for pool 7 and pool 15. Weak responses for pool 17 and pool 8. Overlapping peptides: 66, 71,82, 84
1st peptide screening	Screened peptides 66, 71, 82, 84 Results: too much background in the negative controls
2nd peptide screening	Screened peptides 66, 71, 82, 84 Results: ≥50 SFC/10^6 for peptide 66
3rd peptide screening	Screened peptide 66 Results: ≥50 SFC/10^6 for peptide 66

The pool screenings identified peptides 66, 71, 82 and 84 as the overlapping peptides. The peptide screening was repeated due to high background in 1st peptide screening. Peptide screening detected more than 50 SFC/10^6 for peptide 66.

Participant 6

Pool screening	Results: Positive response for pool 7
1st peptide screening	Screened peptides (all individuals in pool 7) 64, 65, 66, 70, 71, 72, 73, 74, 75, 76 Results: ≥50 SFC/10^6 for peptide 64
2nd peptide screening	Screened peptide 64 Results: ≥50 SFC/10^6 for peptide 64

A positive response was observed only for pool 7 meaning that an overlapping peptide could not be identified. Thus, all the individual peptides in pool 7 were screened, and significantly positive results were observed for peptide 64.

Participant 7

1st pool screening	Results: positive responses for pool 17 and pool 21. But significant background in the negative control.
2nd pool screening	Results: positive responses in pools 11, 13, 16, 17, 22, 24. Lower background in the negative control. Overlapping peptides 113, 115, 116
3rd pool screening	Results: positive responses in pools 11, 13, 21 and 23 Overlapping peptides 112, 114, 116,
1st peptide screening	Screened peptides 88, 90, 92, 93, 96, 100, 105, 104, 102, 108, 112, 114, 116, 117 Results: weak responses (≤50 SFC/10^6) for peptide 92
2nd peptide screening	Screened peptide 92 Results: ≤50 SFC/10^6 for peptide 92

Pools were screened three times, with different responses detected each time. However, there was significant background in the negative controls. The screens were repeated to ensure that responses were not missed. On repeated testing, weak insignificant responses were observed for peptide 92. Participant was excluded from further analysis in the study, as there was too much background in the negative controls.

Participant 8

Pool screening	Results: positive responses for pools 7, 10, 11, 13, 14, 21, 22, 24 Overlapping peptides 64, 65, 75, 76, 100, 101, 103, 104, 105, 112, 113, 116, 117
1st peptide screening	Screened peptides 64, 65, 75, 76, 100, 101, 103, 104, 105, 112, 113, 115, 116, 117 Results: ≥50 SFC/10^6 for peptides 65 and 113
2nd peptide screening	Screened peptides 65 and 113 Results: ≥50 SFC/10^6 for peptide 65 and peptide 113

From the pool screening, thirteen overlapping individual peptides were identified. All overlapping peptides were screened, and only peptides 65 and 113 stimulated significant positive responses.

Participant 9

Pool screening	Results: positive responses for pools 10, 13 and 22 Overlapping peptides: 101, 104
1st peptide screening	Screened peptides 10, 22, 34, 47, 61, 76, 89, 101, 104, 113 Results: no responses ≥50 SFC/10^6
2nd peptide screening	Screened peptides 10, 22, 34, 47, 61, 76, 89, 101, 104, 113 Results: ≥50 SFC/10^6 for peptide 101
3rd peptide screening	Screened peptide 101 Results: ≥50 SFC/10^6 for peptide 101

Peptides 101 and 104 were identified as the overlapping peptides after pool screenings. Since pool 22 had very strong responses, all individual peptides in the pool and peptide 104 were screened. Only peptide 101 stimulated significantly positive responses.

Participant 10

1st pool screening	Results: positive responses for pools 13, 14, 15, 16 and 17 Overlapping peptides: none
2nd pool screening	Results: positive responses for pool 9 and pool 13 Overlapping peptide: 92
1st peptide screening	Screened peptides (all individual peptides in pool 13):1, 13, 25, 37, 52, 64, 80, 92, 104, 116 Results: ≥50 SFC/10^6 for peptide 92
2nd peptide screening	Screened peptide 92 Results: ≥50 SFC/10^6 for peptide 92

No overlapping peptides were identified after the 1st pool screening, the screening with pools was repeated, and peptide 92 was identified as the overlapping peptide. The individual peptide testing confirmed peptide 92 to have significant positive responses.

Participant 11

Pool screening	Results: positive responses for pools 5, 7, 9, 11, 12, 13, 14, 15, 16, 17, 20, 21, 22, 23, 24 Overlapping peptides 41, 45, 46, 47, 50, 51, 52, 53, 64, 65, 66, 70, 71, 74, 75, 76, 88, 89, 90, 91, 92, 93, 94, 95, 96, 108, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120
1st peptide screening	Screened peptides 41, 45, 46, 47, 50, 51, 52, 53, 64, 65, 66, 70, 71, 74, 75, 76, 88, 89, 90, 91, 92, 93, 94, 95, 96, 108, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120 Results: ≥50 SFC/10^6 for peptides 41, 65, 88, 113, 114, 118, 119
2nd peptide screening	Screened peptides 41, 65, 88, 113, 114, 118, 119 Results: ≥50 SFC/10^6 for peptides 41, 65, 88, 113, 118

The pool screening identified thirty-six individual overlapping peptides in the pools. The controls in each screening were satisfactory, indicating the results were valid, although the pattern observed with this participant is very distinct. From the thirty-six individual peptides tested, five peptides stimulated significantly positive responses.

Participant 12

1st Pool screening	Result: positive results for pools 7, 11, 13, 14, 22 and 23 Overlapping peptides: 64, 65, 76, 113, 114, 116, 117
2nd pool screening	Results: positive results for pool 11, 13, 22, 23, 24 Overlapping peptides: 113, 114, 115, 116
1st peptide screening	Screened peptides 64, 65, 76, 113, 114, 116, 117 Results: Background in the negative controls
2nd peptide screening	Screened peptides 64, 65, 76, 113, 114, 116, 117 Results: Background in the negative controls
3rd peptide screening	Screened peptides 64, 65, 76, 113, 114, 116, 117 Results: ≥50 SFC/10^6 for peptide 65 and peptide 113
4th peptide screening	Screened peptide 65 and peptide 113 Results: ≥50 SFC/10^6 for peptide 65 and peptide 113

After the 1st pool screening, seven overlapping individual peptides were identified. However, when the individual peptides were screened, significant background was observed on two occasions. The pools were then repeated to confirm that the responses were not non-specific. A similar pattern was observed as previously and peptides 65 and 113 were identified with significant positive responses.

No non-specific responses were observed with the two participants recruited as negative controls. CCHFV-specific T cell responses were detected for eight of ten participants in the study. The positive results from the ELISpots are summarized in Table 2.2.

Table 2.2. The IFN-γ ELISpot results indicating positive individual peptides

Patient number	Peptide	Spot count 1 (SFC/10^6 cells)	Spot count 2 (SFC/10^6 cells)	Protein
3	64	95	65	NS_M
5	66	355	105	NS_M
6	64	230	60	NS_M
8	65	50	265	NS_M
	113	105	300	GP38
9	101	160	150	GP38
10	92	95	55	GP38
11	41	280	325	NS_M
	65	180	220	NS_M
	88	185	350	GP38
	113	215	275	GP38
	118	190	250	GP38
12	65	110	70	NS_M
	113	130	155	GP38

PBMCs isolated from eight participants had positive IFN-γ responses in the form of spot counts after stimulation with individual peptides. The peptide column refers to the number of the peptide, organised in numerical order, which stimulated a response. The spot count 1 and spot count 2 columns show the SFC/10^6 cells from the duplicate individual peptide screens. The protein column indicates from which protein the 15 mer peptide was derived.

In total, responses were detected against nine peptides within the NS_M and GP38 region. Four peptides, 41, 64, 65 and 66, were located in the NS_M, and five peptides, 88, 92, 101, 113 and 118, were located in the GP38 protein. The graph in Figure 2.10 shows the frequency of positive responses for each peptide. The results suggest peptides 65 and 113 represent epitopic regions that most frequently stimulated a response recognised by the cells isolated from survivors of CCHF infection. Other epitopic regions were represented by peptides 41, 64, 66, 88, 92, 101 and 118.

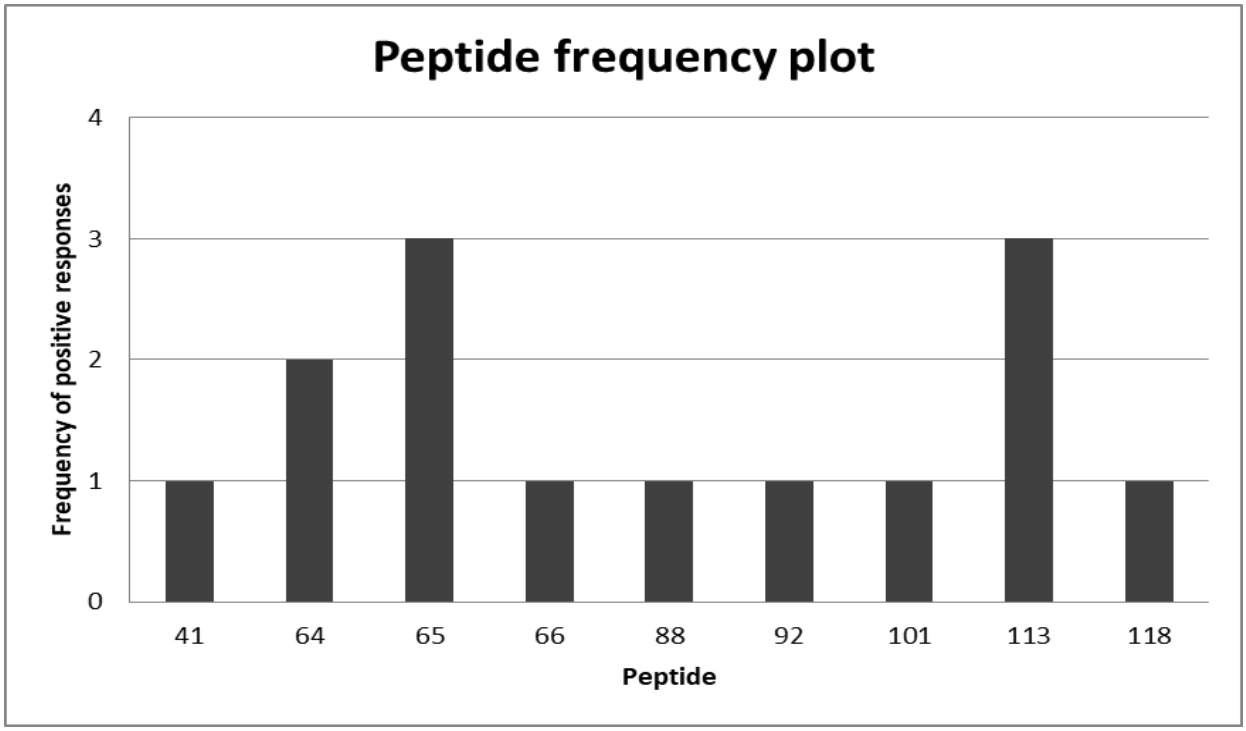
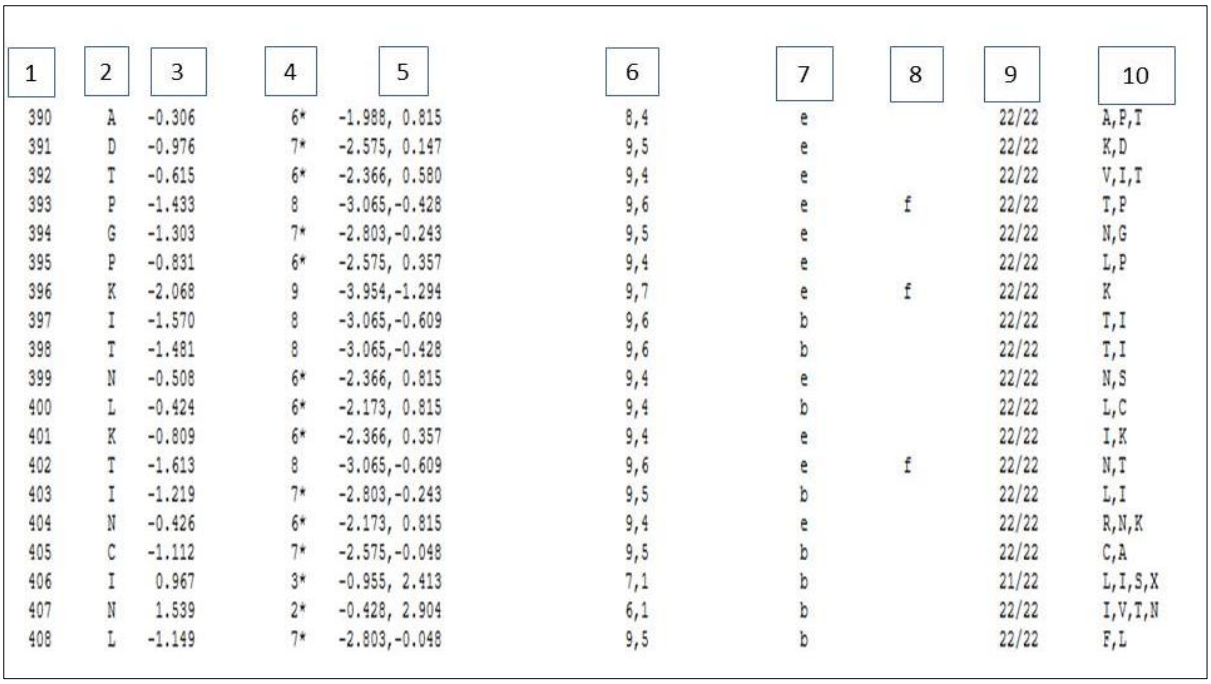


Figure 2.10. The peptide frequency plot illustrates how frequently a response was detected against specific peptides. Three participants reacted to stimulation from peptides 65 and 113; two participants reacted to peptide 64, and the remaining peptides stimulated a response from one participant each.

2.3.3 BIOINFORMATICS ANALYSIS TO DETERMINE AMINO ACID CONSERVATION

To determine if the epitopic regions identified using the peptide libraries were conserved amongst genetically and geographically distinct isolates of CCHFV, the predicted amino acid sequence data retrieved from GenBank for each of the nine regions was aligned and analysed using ConSurf software. In Figure 2.12, peptide 101 located at positions 392- 406 was used as an example. The complete analysis of all the nine peptides for global and SA strains can be found in appendix F, section B.



1	2	3	4	5	6	7	8	9	10
390	A	-0.306	6*	-1.988, 0.815	8,4	e		22/22	A,P,T
391	D	-0.976	7*	-2.575, 0.147	9,5	e		22/22	K,D
392	T	-0.615	6*	-2.366, 0.580	9,4	e		22/22	V,I,T
393	P	-1.433	8	-3.065,-0.428	9,6	e	f	22/22	T,P
394	G	-1.303	7*	-2.803,-0.243	9,5	e		22/22	N,G
395	P	-0.831	6*	-2.575, 0.357	9,4	e		22/22	L,P
396	K	-2.068	9	-3.954,-1.294	9,7	e	f	22/22	K
397	I	-1.570	8	-3.065,-0.609	9,6	b		22/22	T,I
398	T	-1.481	8	-3.065,-0.428	9,6	b		22/22	T,I
399	N	-0.508	6*	-2.366, 0.815	9,4	e		22/22	N,S
400	L	-0.424	6*	-2.173, 0.815	9,4	b		22/22	L,C
401	K	-0.809	6*	-2.366, 0.357	9,4	e		22/22	I,K
402	T	-1.613	8	-3.065,-0.609	9,6	e	f	22/22	N,T
403	I	-1.219	7*	-2.803,-0.243	9,5	b		22/22	L,I
404	N	-0.426	6*	-2.173, 0.815	9,4	e		22/22	R,N,K
405	C	-1.112	7*	-2.575,-0.048	9,5	b		22/22	C,A
406	I	0.967	3*	-0.955, 2.413	7,1	b		21/22	L,I,S,X
407	N	1.539	2*	-0.428, 2.904	6,1	b		22/22	I,V,T,N
408	L	-1.149	7*	-2.803,-0.048	9,5	b		22/22	F,L

Figure 2.11. An example of the ConSurf software analysis. The columns in order list: the position of the amino acids (1), the sequence of the reference strain (2), standard deviation score (3), amino acid conservation score (4), standard deviation with upper and lower intervals (5), the amino acid scores in upper and lower range (6), whether the amino acid is buried (b) or exposed (e) (7), whether the amino acid is proposed to be functional (f) or structural (s) (8), total number of strains analysed (9) and the variety of amino acids that can be found in the same location (10). The * indicates that the score was calculated in less than six non-gapped homologue sequences, thus the confidence interval for the score is equal to or more than four.

The ConSurf software results for all the strains and peptides are summarized in Table 2.3.

Table 2.3. ConSurf amino acid conservation scores

Peptide number	Score for global strains (138 sequences)*	Score for South African strains (21 sequences)
88	4	4
92	4	5
101	5	5
113	5	5
118	5	5
41	6	5
64,65,66	5	5

*The score is graded on a scale of 1 to 9, where 1 indicates that the amino acid at the specific location is highly variable, 5 indicates moderately variable and 9 indicates conserved.

Note that due to the overlap between peptides, peptides 64-66 were analysed as one region of 26 amino acids. The grades presented in the table are the average scores from the amino acids that constituted the peptides of interest.

The observation from both the global and SA strains is that the amino acid conservation scores range within 4- 6. However, the majority of peptides averaged to a score of grade 5. This indicates that the M segment is moderately diverse in these epitopic regions. This implies that the identified epitopes which are analysed are not strictly conserved.

An example of the alignment of amino acid sequence data using BioEdit is presented in Figure 2.13. Peptide 101 of the South African strains is highlighted at amino acids 397-411. The alignment of data representing all nine epitopic regions identified from the peptides is available in appendix F, section A.

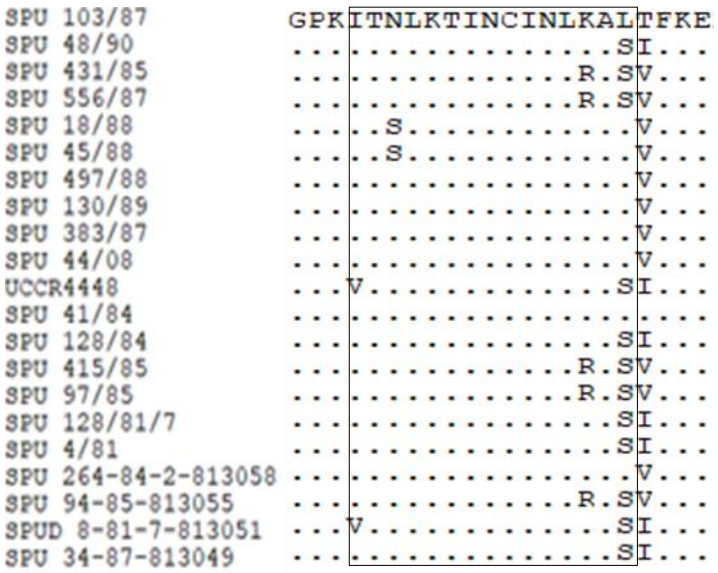


Figure 2.12. Alignment of amino acid data for South African strains using BioEdit. The strains were listed by the amino acid Genbank Accession number. GenBank Accession number ABB30034.1 was used as the reference strain. Dots indicate identical amino acids whereas the letter indicates that a different amino acid is present in the specific strain.

The results are summarized in Table 2.4 for the SA strains and Table 2.5 for the global strains of CCHFV. For a more comprehensive analysis the strains were grouped into three groups; strains that had an identical amino acid sequence, strains with less than five amino acid differences and strains that had a highly variable amino acid sequence compared to the reference strain.

Table 2.4. Analysis of alignments from BioEdit of South African strains of CCHFV

Peptide	88	92	101	113	118	41	64-66
Identical	5%	35%	30%	5%	5%	35%	35%
< 5 amino acid differences	95%	65%	70%	95%	95%	65%	65%
Highly diverse	0%	0%	0%	0%	0%	0%	0%

Table 2.5. Analysis of alignments from BioEdit of global strains of CCHFV

Peptide	88	92	101	113	118	41	64-66
Identical	0.7%	43%	5.8%	54%	0.7%	59.1%	10.2%
< 5 amino acid differences	86%	55.5%	92.7%	44.5%	97.8%	35.8%	80.3%
Highly diverse	13.3%	1.5%	1.5%	1.5%	1.5%	5.1%	9.5%

2.3.4 BIOINFORMATICS COMPARISON OF THE REGIONS OF INTEREST USING BIOEDIT

The three regions of interest in this study, specifically the highly variable mucin-like domain, GP38 and NS_M, were compared for amino acid conservation using BioEdit. Figure 2.14 provides examples of sections of the domains from the South African strains.

A. Highly variable mucin-like domain

B. The GP38 domain

C. The NS_M domain

Figure 2.13. The comparison of the highly variable mucin-like domain, GP38 and NS_M domain. A. Highly variable mucin-like domain. B. GP38 domain. C. NS_M domain. Dots indicate conserved amino acids.

The comparison of the three regions of interest showed that the highly variable mucin-like domain had a higher amino acid diversity compared to the GP38 and NS_M. In addition, there was variability in the length of the mucin-like domain. There was a much lower amino acid diversity demonstrated in the GP38 and NS_M regions, with the NS_M having the least amino acid diversity amongst the three regions.

2.4 SUMMARY

The aim of this chapter was to determine if there were T cell immune responses in survivors of CCHF against selected proteins, the GP38, NS_M and highly variable mucin-like domain of the virus. This was achieved by screening for the secretion of IFN-γ in samples that were stimulated with peptide libraries representing the proteins of interest. Immuno-dominant epitopes are likely conserved between different strains and hence the amino acid conservation of any peptides that stimulated a response and represent possible epitopic regions was investigated.

Twelve participants were recruited onto the study, all of whom were males and the majority were of Caucasian decent. A scattered distribution of CCHF cases was observed for the participants who were located in the Free State and North West provinces. The interval between confirmed CCHF and study participation ranged from 2 to 19 years. The ELISpot screenings showed that eight of the screened participants showed positive IFN-γ stimulation to one or more peptide.

In total nine epitopic peptides were identified as capable of stimulating IFN-γ secretion, of which peptides 65 and 113 were the peptides that most frequently stimulated positive responses. Five of the epitopic peptides were located on GP38 and four were located on NS_M, with no epitopes identified in the highly variable mucin-like domain. Two participants showed no positive responses to any of the CCHF peptides tested, while two participants were excluded from the final analysis due to significant background detected in the negative controls. There was no pattern observed between the ELISpot responses and the years post-infection. The responses varied from the number of peptides to the magnitude of responses.

Bioinformatics analysis was performed using ConSurf and BioEdit software to evaluate the level of conservation of the identified epitopic peptides amongst the global strains of CCHFV. The bioinformatics analysis of the identified epitopic regions demonstrated that the regions of interest are overall genetically moderately diverse. It was observed that the highly variable mucin-like domain had the highest amino acid diversity compared to the GP38 and NS_M regions.

CHAPTER 3
The characterization of T cell responses with flow cytometry

3.1 INTRODUCTION

During viral infection, antigen presenting cells (APCs) process the virus and present viral antigens that stimulate cells of the adaptive immune system, which include $CD4^+$ T cells and $CD8^+$ T cells. $CD4^+$ T cells, also known as helper cells, recognise antigens in association with major histocompatibility complex (MHC) class II molecules, release specific antiviral cytokines (including IL-2, -4, -5 and -6) and regulate the immune response towards either production of antibody or cytotoxic responses against the detected antigen (Gargani, 2012).

$CD8^+$ T cells, which are mostly cytotoxic lymphocytes, are stimulated through the endogenous or the ligand-ligand pathway (Betts *et al.*, 2003; Gargani, 2012). The endogenous pathway includes the internalization of the antigen by the APC, which will be processed and presented to $CD8^+$ T cells by MHC class I molecules (Chan *et al.*, 2007). Ligand-ligand induced cytotoxicity is triggered when a specific viral antigen interacts with a T cell receptor of the cytotoxic lymphocyte (Gargani, 2012). Once stimulated, the $CD8^+$ T cells attack the infected cell through the granule secretory pathway, which involves the secretion of pre-formed lysosomic granules in a cleft formed between the $CD8^+$ T cell and the infected cell. This process is known as degranulation, as illustrated in Figure 3.1 (Betts *et al.*, 2003; Dennert *et al.*, 1983; Peters *et al.*, 1991; Tschopp *et al.*, 1990).

The pre-formed lysosomal granules are membrane bound vesicles which have a large dense core with lysosomal enzymes such as perforins, granzymes and proteases, as illustrated in Figure 3.1 (Dennert *et al.*, 1983; Peters *et al.*, 1991; Tschopp *et al.*, 1990;). Once the $CD8^+$ T cells are stimulated either through the endogenous or the ligand-ligand pathway, the lysosomal granules move towards the cellular membranes, in preparation for the degranulation process (Betts *et al.*, 2004). During degranulation, the granule membrane merges with the cellular membrane of the cytotoxic $CD8^+$ T cells, which causes the CD107a, CD107b and CD63 to be

transiently expressed on the surface of cytotoxic $CD8^+$ T cells (Peters *et al.*, 1991; Tschopp *et al.*, 1990). The merging of the membranes causes the lysosomic core containing perforins, granzymes and proteases to be released to the target cell. This process is illustrated in Figure 3.1.

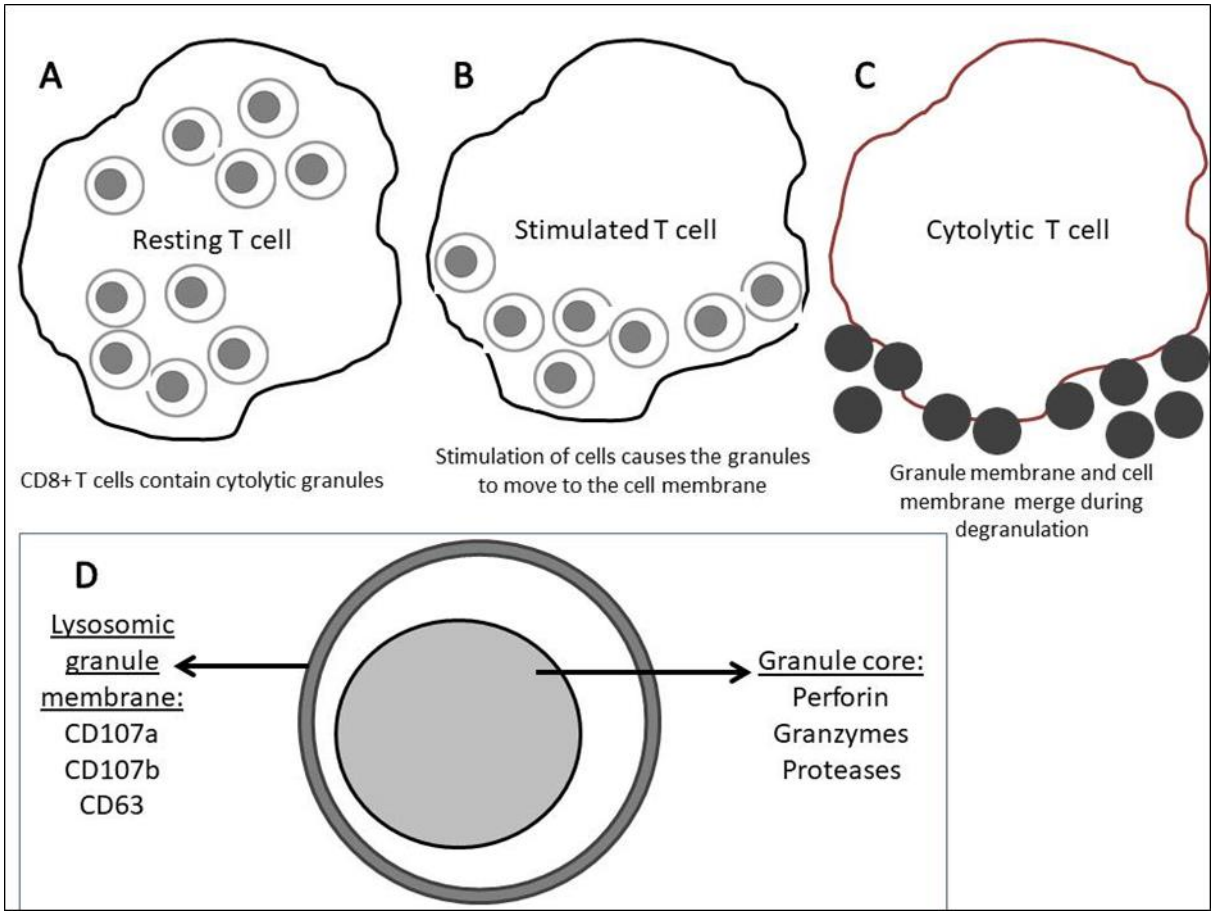


Figure 3.1. Illustration of the degranulation process. A. A resting $CD8^+$ T cell containing pre-formed lysosomic granules. B. Upon stimulation the lysosomic granules move towards the cellular membrane. C. The change in colour of the cellular membrane highlights the merging of the granule and cellular membranes. The granule cores are released to the target cell. D. Illustration of the lysosomic granule. Figure adapted from Betts *et al.*, 2003.

Lymphocyte cytotoxicity is investigated in viral infections, because $CD8^+$ T cells are known to have cytotoxic activity, while $CD4^+$ T cells are known to be inducers of such activity. Traditionally, $CD8^+$ T cell cytotoxicity has been investigated using the ^{51}Chromium (Cr) isotope release assay (Brunner *et al.*,1968). The assay involves the pre-labelling of the target cell with Cr, the cells were then stimulated and in the process the ^{51}Cr isotope is released and measured to determine the cytotoxicity of stimulated $CD8^+$ T cells (Brunner *et al*.,1968). Other techniques include granzyme B

or perforin enzyme-linked immunospot assays (ELISpots) (Chan *et al.*, 2007) and cytokine flow cytometry assays (Kuerten *et al.*, 2008; Maecker *et al.*, 2001). However, these techniques focus on the detection of secreted cytokines or enzymes by the cytotoxic T cells during degranulation (Betts *et al*., 2003; Kuerten *et al*., 2008).

The CD107a flow cytometry assay monitors degranulation of antigen activated $CD8^+$ T cells by measuring the cumulative exposure of granular membrane molecule CD107a on the cell surface of the activated cytotoxic cells (Betts *et al*., 2003). Therefore, CD107a is a surrogate marker for the cytotoxic activity of $CD8^+$ T cells and is proposed to protect against the leakage of lytic proteins from the granule vesicles (Betts *et al.*, 2003; Peters *et al.*, 1991). This assay is also performed to confirm cytolytic activity of IFN-γ secreting cells, thus simultaneously monitoring the secretion of IFN-γ and degranulation activity (Chan *et al.*, 2007). The CD107a assay is therefore a preferred technique for the quantitative measurement of degranulation. The assay involves a short-term activation of whole blood or PBMCs with an antigen, often with co-stimulatory antibodies and secretion inhibitors (Betts *et al*., 2003; Betts *et al.*, 2004). Activated cells are then fixed, permeabilized and stained for intracellular cytokines, which are analysed on a flow cytometer.

The flow cytometry technique is preferable because peptides, instead of the whole antigen, can be used for stimulation because the peptides bind directly to the MHC molecules. Therefore, the technique eliminates the need for APCs, of which the function easily deteriorates in transported blood or cryopreserved PBMCs (Maecker *et al.*, 2001). It was observed that 15 mer peptides were able to efficiently stimulate both $CD4^+$ T cells and $CD8^+$ T cells, when compared to 8-12 mer and 20 mer peptides. It was hypothesized that this is because the 15 mers bind efficiently to the MHC class II molecules, and to a certain degree to the MHC I binding grooves (Maecker *et al.*, 2001).

The characterization of T cells can be based on the expression of specific surface cell markers or on the secreted effector cytokines or enzymes. The secreted effector cytokines may include IFN-γ, TNF-α and interleukins, which are antiviral cytokines and therefore markers of effector T cells. The development of multicoloured flow cytometry has opened the path to analyse T cells with multiple functions, these cells are referred to as polyfunctional T cells (Almeida *et al.*, 2007; Boyd *et al.*, 2015;

Precopio *et al.*, 2007). Several studies use a score between one and five, based on the degranulation and the secretion of cytokines and chemokines, to group polyfunctional T cells (Almeida *et al.*, 2007; Boyd *et al*., 2015; Precopio *et al.*, 2007). In this study, analysis included the investigation of T cells with dual functionality as indicated by the secretion of both TNF-α and IFN-γ, which were referred to as polyfunctional cells. The cell surface markers can be antigen specific, such as the expression of CD107a, or receptors for certain chemokines, such as the CCR7. CD45RA is an isoform for CD45 and a receptor-linked tyrosine phosphatase (Hamann *et al.*, 1997). CCR7 binds to the CC chemokine receptor for lymph nodes (Campbell *et al.*, 1998).

Together, different combinations of CD45RA and CCR7 can be utilized to categorize T cell populations into four subsets of naïve T cells, terminally differentiated T cells, memory T cells, or effector T cells. Naïve T cells (T_N) are characterized by the expression of CD45RA and CCR7 ($CD45RA^+CCR7^+$), central memory T cells (T_{CM}) by the expression of CCR7 but not of CD45RA ($CD45RA^-CCR7^+$), effector memory T cells (T_{EM}) by the expression of neither CD45RA nor CCR7 ($CD45RA^-CCR7^-$) and the terminally differentiated memory T cells (T_{EMRA}) by the $CD45RA^+CCR7^-$ phenotype (Sallusto *et al.*, 1999; Sallusto *et al.*, 2004; Willinger *et al.*, 2005).

To date, few articles have been published with regards to adaptive immunity in CCHF survivors. Studies that have explored protective immunity have investigated humoral or cellular immunity in response to vaccination, but there is a lack of information regarding characterization of immune responses in survivors of CCHF (Dowall *et al.*, 2016a; Hinkula *et al.*, 2017).

The objective of this chapter was to characterize T cell responses by confirming functional cytotoxic activity against identified T cell epitopes. The 15 mer peptides that were able to stimulate a response in the IFN-γ ELISpot screening were used for the CD107a degranulation assay, characterization of cytokine responses and T cell subsets using flow cytometry. The CD107a degranulation assay was performed to determine if cytotoxic activity of the stimulated lymphocytes could be induced, through the detection of CD107a and other $CD8^+$ T cell specific markers.

3.2 METHODS

3.2.1 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) PROCESSING

Whole blood was collected from eight participants, selected based on positive IFN-γ stimulation with the IFN-γ ELISpot screening assay described in the previous chapter. PBMCs were isolated and stored in liquid nitrogen as per the protocol in chapter 2 (section 2.2.3). Stored cells were used in the CD107a degranulation assay. The frozen cells were thawed as follows. R1 and R10 media were prepared by mixing foetal bovine serum (FBS) (Gibco Life Sciences, South America) and Roswell Park Memorial Institute (RPMI) medium (Lonza, Belgium) supplemented with 1% penicillin-streptomycin (Lonza, Belgium) and 1% L-glutamine (Lonza, Belgium). To make the media, FBS and RPMI were mixed at the ratio 1:100 for R1 media and 1:10 for R10 media.

PBMCs were removed from liquid nitrogen and thawed in a water bath (Labotec, South Africa) heated to 37°C. The PBMCs were transferred into a 50 ml centrifuge tube (Sigma-Aldrich, USA), followed by the addition of R1 dropwise while swirling using a sterile pasteur pipette (Plastpro Scientific, South Africa) up to 15 ml, and topped up to 25 ml. The tube was centrifuged at 750 x*g* for 10 minutes. The cells were resuspended in 25 ml of R1 media and centrifuged at 750 x*g* for 10 minutes. Cells were resuspended in 2 ml R10 media, based on the volume of suspension required for the CD107a degranulation assay.

Equal volumes of 10 µl of cell suspension and 10 µl of trypan blue (Sigma-Aldrich, UK) were mixed. A cell count was performed on the Countess® II FL (Thermo Fisher Scientific, Massachusetts, USA) to determine the viability of cells in the suspension. Although this assay does not require a certain cell count to produce reliable results, it is of good practise to know the viability of the cells in the suspension.

3.2.2 SAMPLE PREPARATION

The protocol for the CD107a degranulation assay was adjusted from the manufacturer's protocol, BD FastImmune™ CD107a (H4A3). For each participant, 15 ml centrifuge tubes (Lasec, South Africa) were labelled as positive control, unstimulated control, and a stimulated tube with the peptide number. The number of tubes required per participant for stimulation was dependant on the number of peptides to be screened. For each of the tubes, 500 µl of cell suspension, 1 µl each

of the co-stimulants CD49d and CD28 (at 1 μg/ml) (BD Biosciences, USA) (Betts *et al.*, 2003; Betts *et al.*, 2004; Chan *et al.*, 2007), 0.5 μl GolgiStop protein transport inhibitor containing monensin (BD Biosciences, USA) (0.7 μg/ml final concentration) and 5 μl CD107a (BD Biosciences, USA) were added.

The co-stimulants CD49d and CD28 enhance antigen specific activation (Betts *et al.*, 2004). The GolgiStop protein transport inhibitor contains monensin which neutralises the pH of the endosomes and lysosomes, which thus prevents the degradation of re-internalized CD107a proteins, and also prevents the secretion of cytokines (Betts *et al.*, 2004; Lee *et al.*, 1990; Maecker *et al.*, 2001; Mollenhauer *et al.*, 1990). Since monensin is known to inhibit the fluorescence of IFN-γ and the other intracellular markers, the concentration was reduced as recommended in literature (Betts *et al.*, 2004). Although the manufacturer's instructions recommend that the assay be performed on fresh whole blood, experimental studies have observed that whole blood and cryopreserved PBMCs have similar levels of reproducibility, and background was dependent on the type of donor, not the type of sample used (Maecker *et al.*, 2005).

For the positive control, 6 μl of Staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB) (1 μg/ml final concentration) (Sigma-aldrich, USA) was added (Betts *et al.*, 2004). SEB is a super antigen, which means that it causes non-specific activation of T cells (Niedergang *et al.*, 1995). No stimulant was added to the negative control, while 10 μl of the relevant peptide (GenScript, USA) (at concentration 5 μg/ml) was added to the stimulated tube. A graphic presentation of this process is presented in Figure 3.2.

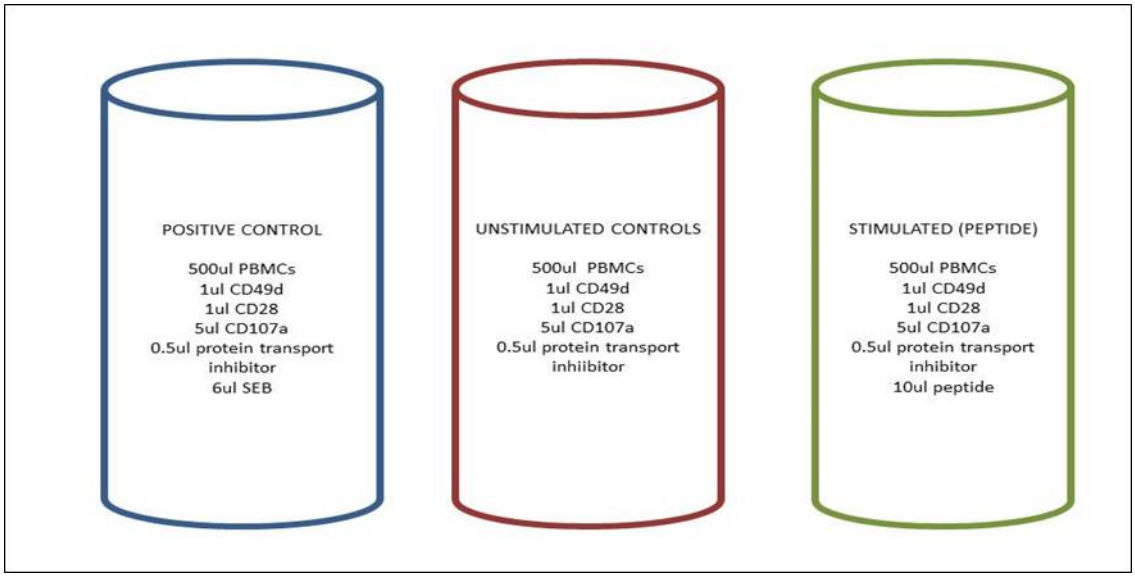


Figure 3.2. Graphic presentation of the first step of sample preparation for the CD107a degranulation assay.

The tubes were gently vortexed (VELP Scientifica, Italy) and incubated for one hour at 37°C in 5% CO_2 (Betts *et al.*, 2003; Betts *et al.*, 2004; Chan *et al.*, 2007). Brefeldin A (BFA) (BD Biosciences, USA) was prepared at 1:10 with PBS (Sigma-Aldrich, USA), according to manufacturer's recommendation and 5 µl BFA was added to each tube. The tubes were gently vortexed and incubated for a further five hours at 37°C in 5% CO_2 (Betts *et al.*, 2003; Betts *et al.*, 2004). The BFA prevents the exocytosis of cytokine containing vesicles following stimulation (Maecker *et al.*, 2001).

After stimulation, 50 µl ethylenediaminetetraacetic acid (EDTA) (BD Biosciences, USA) was added to each tube according to the manufacturer's instructions. The EDTA was added to remove cells that adhered to the surface of the tube. The tubes were vortexed vigorously and incubated in the dark at room temperature for 15 minutes. Tubes were vortexed again on high for 10 seconds. The markers used in this assay are fluorescent markers, thus it was of importance to ensure that a dark environment was simulated as recommended. This was done by using a cardboard box that would not expose the samples to direct light. For the subsequent steps, the lights in the BSL II cabinet were switched off.

The BD FACS lysing solution (BD Biosciences, USA) was diluted at 1:10 with distilled water as per manufacturer's instructions and 2 ml of the working solution

was added to each tube and incubated in the dark at room temperature for 10 minutes. The lysing solution was added to fix the white blood cells and lyse erythrocytes. To each tube, 1 ml of wash buffer was added and centrifuged at 750 x*g* for five minutes. The BD FACS permeabilizing solution 2 (BD Biosciences, USA) was prepared at 1:10 with distilled water as per manufacturer's instructions. The pellet was re-suspended in 1 ml of permeabilizing solution 2 by vortexing. Tubes were incubated in the dark at room temperature for 10 minutes.

The permeabilizing solution allows for the pores of the cells to open so that the fluorochrome-labelled antibodies can be internalized and stain intracellular markers accordingly. The supernatant was decanted instead of aspirated after centrifugation, as per the manufacturer's recommendation, because permeabilized cells are known to be buoyant. Wash buffer consisted of 0.5% bovine serum albumin (BSA) and 0.1% sodium azide (NaN_3) in PBS, to make up 500 ml of wash buffer. Cells were washed by adding 2 ml of wash buffer to each tube and centrifuged at 750 x*g* for five minutes at room temperature. The supernatant was decanted, and 2 ml of wash buffer was added to each tube to re-suspend the pellet.

Two 15 ml centrifuge tubes were labelled reaction 1 and 2 for the positive control, unstimulated and each of the stimulated tubes. The resuspension in 2 ml of the wash buffer was divided between the two tubes of reaction 1 and 2, for each sample. Tubes were centrifuged at 750 x*g* for five minutes at room temperature. The supernatant was decanted, and the appropriate monoclonal antibodies were added as indicated in Table 3.1. The tubes were incubated in the dark at room temperature for one hour.

Table 3.1. The list of antibodies and conjugated fluorochrome added for each reaction.

Reaction 1		Reaction 2	
Antibody	Fluorochrome	Antibody	Fluorochrome
CD107a	APC	CD3	APC-H7
CD3	APC-H7	CD8	PE-Cy7
CD8	PE-Cy7	CD4	PacB
CD4	PacB	IFN-γ	FITC
IFN-γ	FITC	CD45RA	PE
TNF-α	PE	CCR7	Per CP-Cy5.5

APC-Allophycocyanin. PE-Phycoerythrin. FITC-Fluorescein Isothiocyanate. PerCP-Peridinin Chlorophyll Protein. PacB-Pacific Blue. Cy-Cyanine.

After incubation, 2 ml of wash buffer was added to each tube and centrifuged at 750 x*g* for five minutes at room temperature. The washing step was repeated. The pellet was resuspended in 200 µl of 1% paraformaldehyde (Sigma Aldrich, USA) by vortexing. Tubes were stored at 4°C in the dark overnight.

3.2.3 CD107a DEGRANULATION FLOW CYTOMETRY ASSAY

The samples were transferred to the Human Molecular Biology unit, Department of Haematology and Cell Biology, University of the Free State, to be assayed by Dr. Walter Janse van Rensburg. The samples were transferred to 5ml polystyrene FACS tubes and a phosphate buffer was added to increase the volume of the samples to 1 ml. The samples were assayed on a BD FACSCanto II (BD Biosciences, USA) and analysed using the BD FACSDiva software (BD Biosciences, USA).

The BD FACSCanto II used in this study was routinely used for diagnostics, and quality control was performed based on EuroFlow protocols. Compensation was performed once a month using BD CompBeads (BD Biosciences, USA), which are a negative control compensation particle set used to set compensation levels for multicolour flow cytometry assays. Immuno-Troll cells (BD Biosciences, USA) quality control was performed weekly, and the Sphero™ rainbow calibration particles (Spherotech Inc, Biocom Africa, South Africa) were run daily. Both the Immuno-Troll cells and the rainbow calibration particles are positive controls.

A live/dead marker was not used. However, considering that for the assay the focus was on secreted cytokines and expressed molecules, the analysis confirmed that the majority of the cells were viable. The absence of a live/dead marker may have resulted in lower reported percentages of functional T cells as indicated by the secretion of cytokines or degranulation. The antibodies were titrated on permeabilized cells. Doublets were not gated out, as it would have significantly affected the already low cell numbers within the heterogeneous population of lymphocytes. However, a low flow rate was selected to limit excess doublets.

A minimum of 50 000 lymphocytes were acquired for each reaction, this was adjusted from manufacturer's instruction, BD FastImmune™ CD107a (H4A3), which recommended a minimum of 30 000-40 000 lymphocytes. By acquiring the same number of events for the control, unstimulated and stimulated samples, the variation associated with different cell counts was ruled out.

The lymphocyte population was gated from a forward and side scatter dot plot and subsequently, $CD3^+$ T cells were further gated from the lymphocyte population. Backgating was performed on the $CD3^+$ population to determine if the correct population of lymphocytes were initially selected. The $CD3^+CD8^+$ and $CD3^+CD4^+$ populations were gated from the $CD3^+$ T cell population. The $CD3^+CD4^+$ T cell population and $CD3^+CD8^+$ T cell population were further analysed for the markers of interest such as IFN-γ, TNF-α and CD107a. The gating strategy is depicted in Figure 3.3.

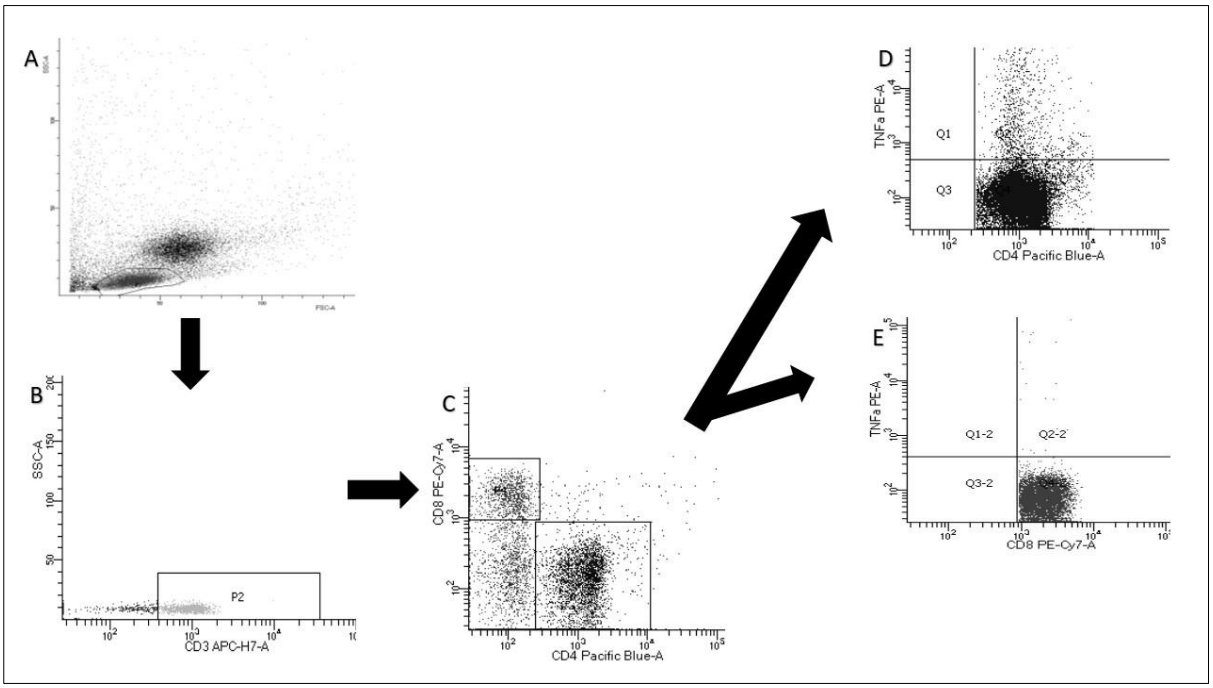


Figure 3.3. The flow cytometry gating strategy for the analysis of T cell responses. A. 50 000 events were acquired, and lymphocyte population was identified. B. Gating of $CD3^+$ T cell population. C. Gating of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell population. D. Analysis if $CD4^+$ T cell populations for the markers of interest. E. Analysis of the $CD8^+$ T cell population for the markers of interest.

In order to account for background cytokine expression in the absence of antigen stimulation, the presence of dead cells or debris, the percentage of cytokine expressing events in the unstimulated sample was subtracted from the percentage of cytokine expressing events in the stimulated sample. A positive response was defined as any response which is 0,01% or more. The results from the BD FACSDiva software were analysed using Microsoft Excel to characterise T cell responses per participant and peptide.

3.3 RESULTS

The results were analysed for the characterization of CD4 and CD8 populations, including the secretion of the antiviral cytokines IFN-γ and TNF-α. The CD8 population was analysed for the expression of CD107a, which is a marker for cytotoxic function and further characterized based on the expression of CD45RA and CCR7, which indicates the presence of naïve (T_N), effector memory (T_{EM}), central memory (T_{CM}) or terminally differentiated (T_{EMRA}) T cells. The details of the flow cytometry analysis are shown in sections 3.3.1-3.3.5. For the purposes of the analysis, the samples were labelled as participant-peptide, for example 11-41, indicates participant 11 peptide 41.

The gated populations are presented as dot plot graphs as in Figure 3.4. Each plot is divided into four quadrants, indicated by Q1, Q2, Q3 and Q4. The x and y axes are the parameters of interest. In the example in Figure 3.4, the x-axis is CD8 and y-axis is TNF-α. Events in Q1 are positive for y-axis and negative for x-axis parameters. Events in Q2 are positive for both parameters, while events in Q3 are negative for both parameters and events in Q4 are positive for the parameter on the x-axis, but negative for the parameter on the y-axis. The percentage of each response of interest was indicated in the appropriate quadrant, for example, in Figure 3.4 the Q2 population which is $CD8^+TNF\text{-}\alpha^+$ is 1,3%, which is implies that 1,3% of the $CD8^+$ T cell population is TNF-α positive.

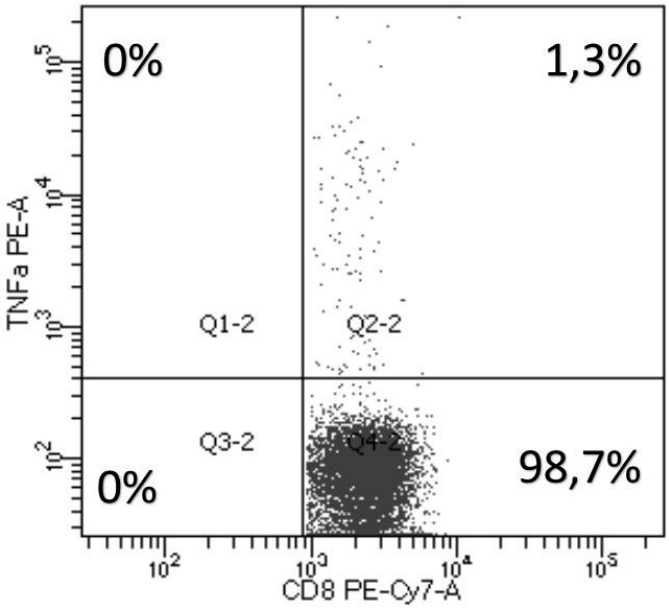


Figure 3.4. Dot plot presentation of flow cytometry results.

Any negative responses after the subtraction of background were recorded as zero (0%), as this indicated that the sample had a high background. Responses that were 0.01% or more were recorded as positive. A high background was observed in sample 6-64, throughout the analysis, including the negative controls. The unstimulated samples are added in appendix H. In all the tested samples, the level of responses in the positive control were lower than expected but were detectable at significant levels which were greater than 0.01%.

3.3.1 INDUCTION OF IFN-γ RESPONSES

For the analysis of IFN-γ production by CD4 T cells, the population used was the $CD3^+CD4^+$ population gated from the $CD3^+$ and $CD4^+$ dot plots. The range for $CD4^+IFN\text{-}\gamma^+$ responses for the positive and negative controls were 0,01%-4,08% and 0,18%-2,40% respectively. The results for the peptide stimulated samples are indicated in Figure 3.5. The unstimulated sample can be seen in appendix H.

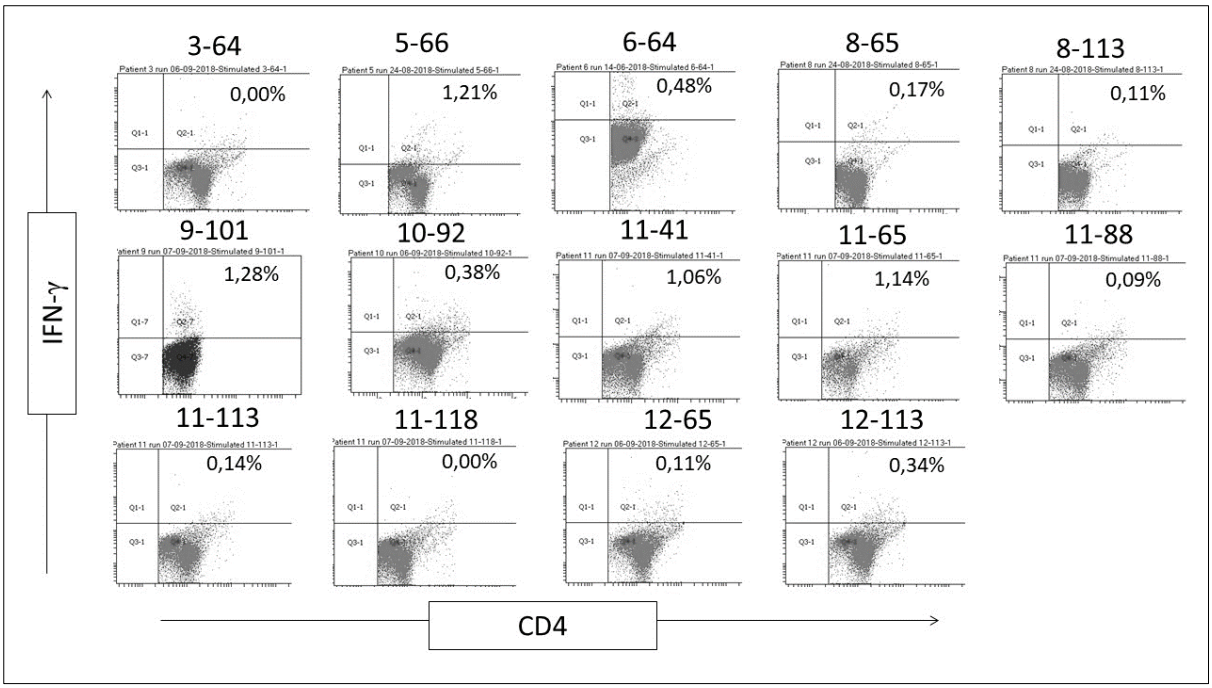


Figure 3.5. Flow cytometry results for $CD4^+$ T cell population secretion of IFN-γ. Positive responses were detected for samples 5-66, 6-64, 8-65, 8-113, 9-101, 10-92, 11-41, 11-65, 11-88, 11-113, 12-65 and 12-113.

To evaluate IFN-γ production by CD8 T cells, the population used in this analysis was gated from the CD3 CD8 plot and thus was $CD3^+CD8^+$. The ranges for $CD8^+IFN\text{-}\gamma^+$ responses for the positive control and negative control were 0,01%-8,39% and 0,04%-1,23%. The responses for the peptide stimulated samples are illustrated in Figure 3.6.

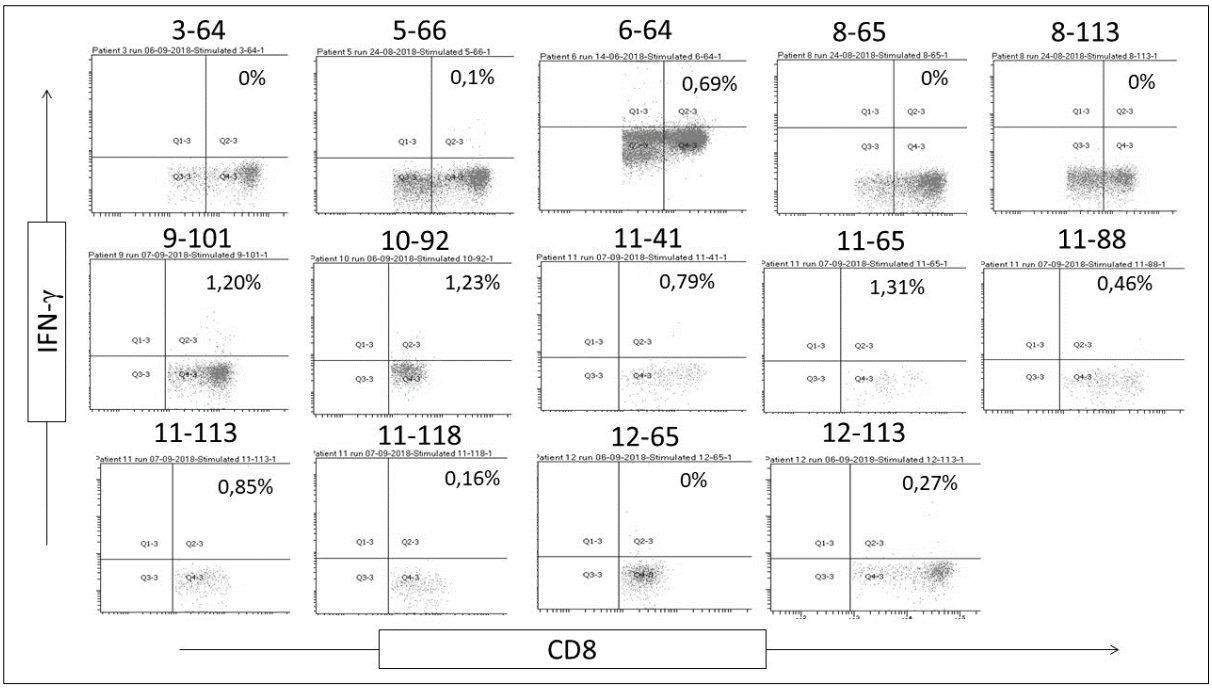


Figure 3.6. Flow cytometry results for secretion of IFN-γ by CD8 T cells. Positive responses were detected for samples 5-66, 6-64, 9-101, 10-92, 11-41, 11-65, 11-88, 11-113, 11-118 and 12-113.

Secretion of IFN-γ was detected in thirteen samples with positive responses for $CD4^+$ T cells ranging from 0,09%-1,21% and $CD8^+$ responses ranging from 0,16%-1,32%. Samples 5-66, 6-64, 9-101, 10-92, 11-41, 11-65, 11-88, 11-113 and 12-113 were positive for IFN-γ stimulation in both the CD4 and CD8 populations. Samples 8-65, 8-113 and 12-65 was only positive for IFN-γ^+ in the $CD4^+$ population and sample 11-118 was only positive in the $CD8^+$ population. The results are summarized in Figure 3.7.

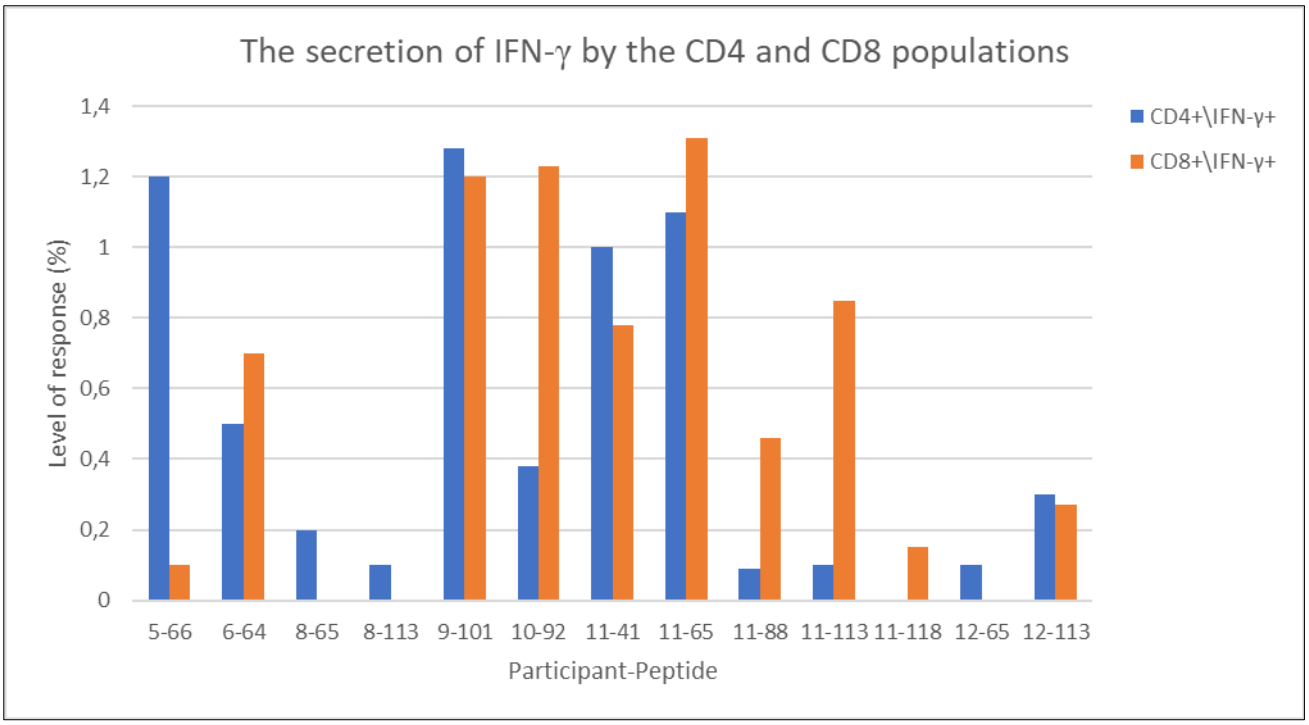


Figure 3.7. Level of IFN-γ secretion for $CD4^+$ and $CD8^+$ T cell populations for samples with positive responses.

3.3.2. INDUCTION OF TNF-α RESPONSES

The $CD3^+CD4^+$ T cell population was gated and analysed for the secretion of TNF-α. The ranges of responses for the $CD4^+TNF\text{-}\alpha^+$ positive control and negative control were 0,64%-11,3% and 1,09%-6,04%. The responses for the peptide stimulated samples are illustrated in Figure 3.8. The unstimulated plots are attached in appendix H.

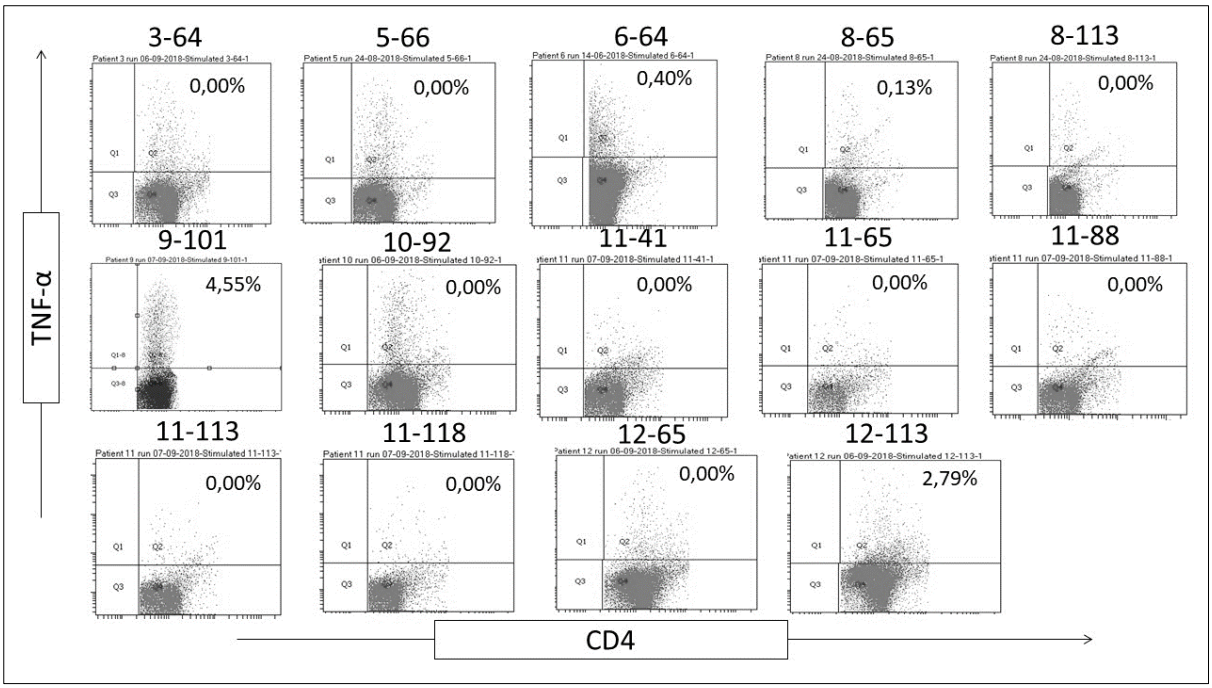


Figure 3.8. Flow cytometry results for $CD4^+$ T cells secreting TNF-α. Positive responses were detected in samples 6-64, 8-65, 9-101 and 12-113.

The $CD3^+CD8^+$ population was gated and analysed for TNF-α secretion. The ranges of responses for the $CD8^+TNF\text{-}\alpha^+$ positive control and negative control were 0,10%-6,99% and 0,04%-4,76%. The responses for the stimulated samples are illustrated in Figure 3.9. The plots for the unstimulated samples are in appendix H.

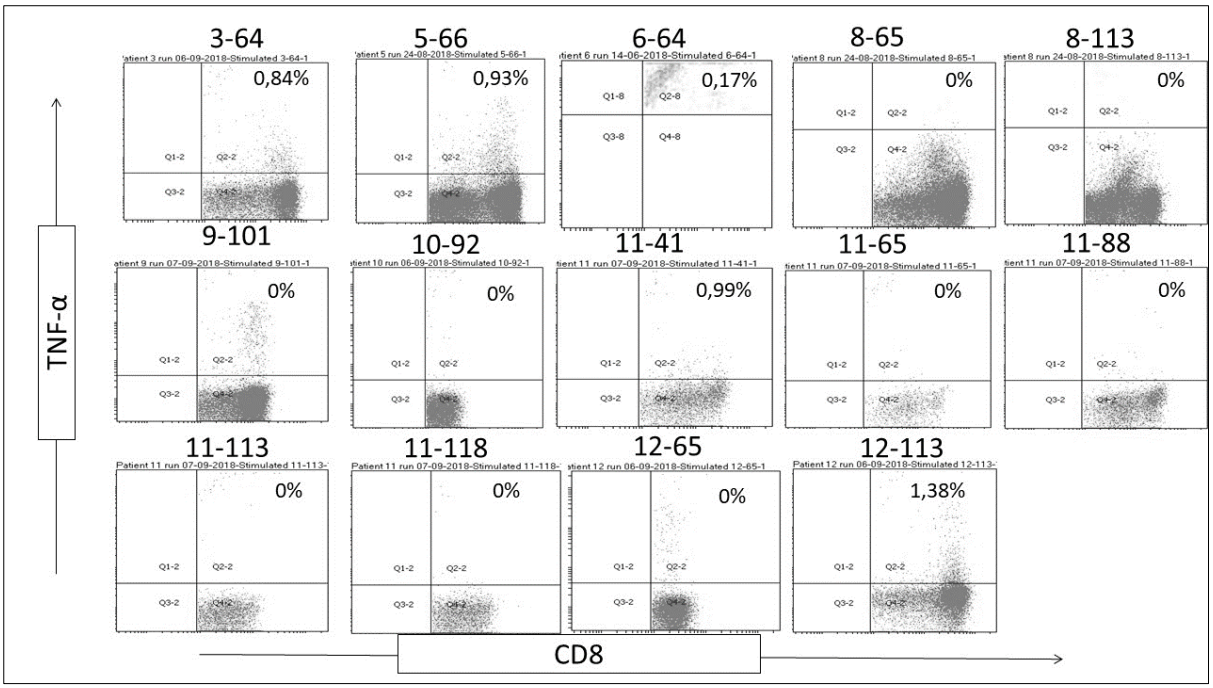


Figure 3.9. Flow cytometry results for $CD8^+$ T cells secreting TNF-α. Positive responses were detected in samples 3-64, 5-66, 6-64, 11-41 and 12-113.

Seven samples were positive for TNF-α within the $CD4^+$ and/or $CD8^+$ populations. Samples 6-64 and 12-113 were positive for TNF-α in both CD4 and CD8 populations. While samples 8-65 and 9-101 were only positive for $CD4^+TNF\text{-}\alpha^+$ and samples 3-64, 5-66 and 11-41 were positive only in the $CD8^+$ T cell population, as shown in Figure 3.10 below.

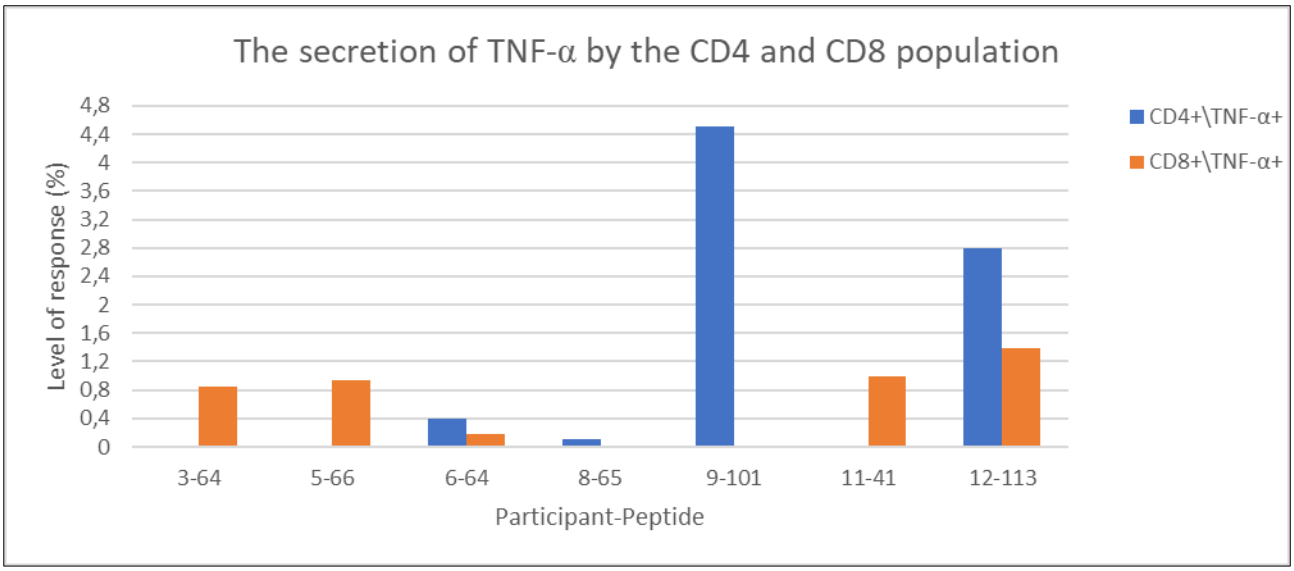


Figure 3.10. Levels of TNF-α secretion by the $CD4^+$ and $CD8^+$ T cell populations for samples with positive responses.

3.3.3 IDENTIFICATION OF POLYFUNCTIONAL T CELLS

The $CD3^+CD4^+$ population was analysed for the dual secretion of IFN-γ and TNF-α, which is an indication of polyfunctional T cells. The range for the $CD4^+$ cell population secreting both IFN-γ and TNF-α for the positive and negative controls were 0,12%-5,00% and 0,01%-4,81% respectively. The responses for the antigen stimulated samples are depicted in Figure 3.11. The unstimulated plots are in appendix H.

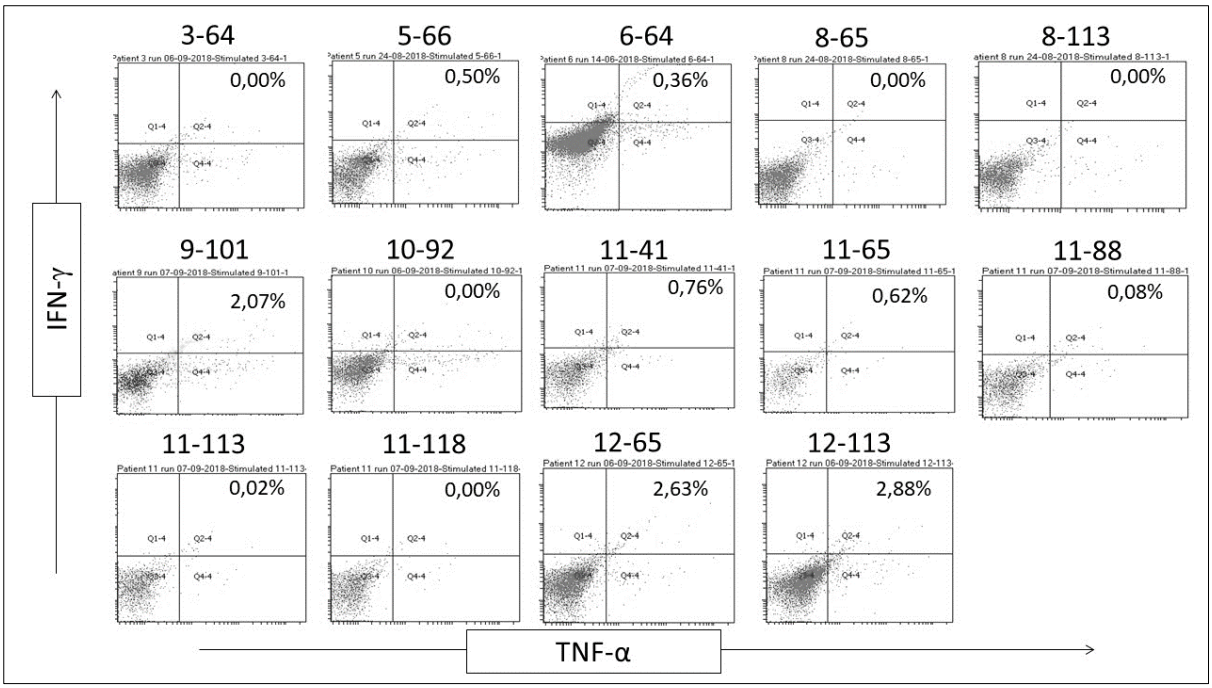


Figure 3.11. Flow cytometry results for IFN-γ TNF-α in the $CD4^+$ T cell population. Positive responses were detected in samples 5-66, 6-64, 9-101, 11-41, 11-65, 11-88, 11-113, 12-65 and 12-113.

The $CD3^+CD8^+$ population was also analysed for the secretion of IFN-γ and TNF-α. The range for the $CD8^+$ population secreting IFN-γ and TNF-α was 0,04%-6,8% and 0,05%-1,85% for the positive and negative controls respectively. The ranges for the responses of the stimulated samples are indicated in Figure 3.12 below.

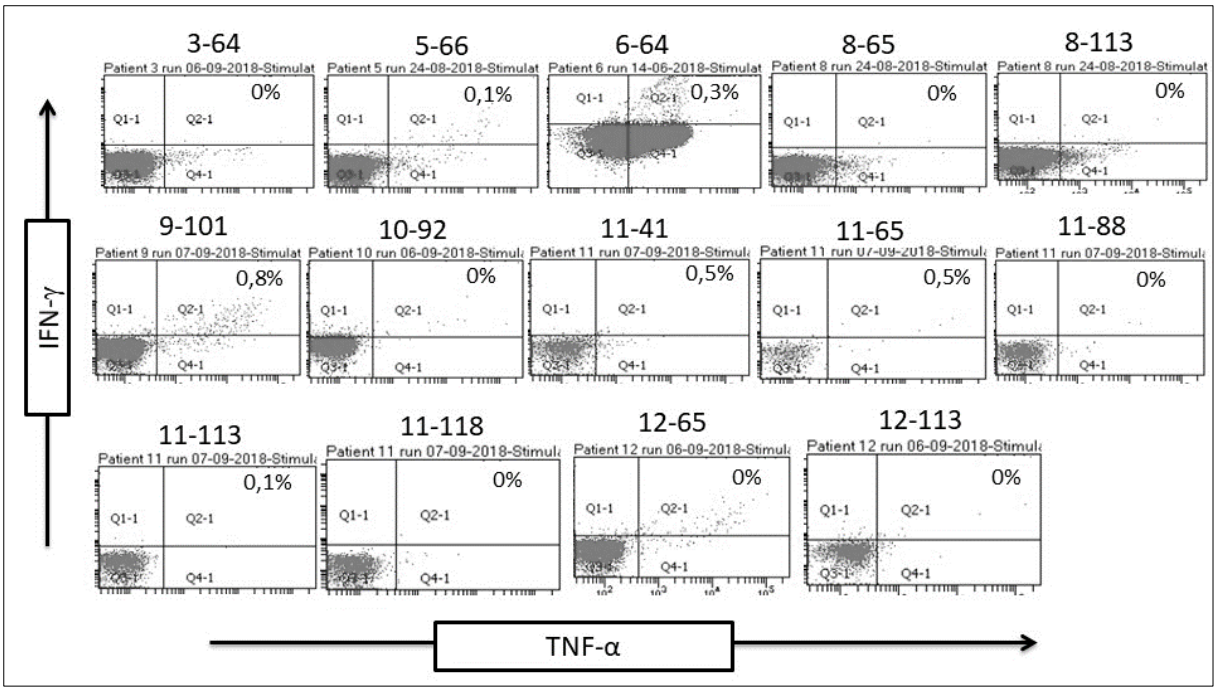


Figure 3.12. Flow cytometry results for IFN-γ TNF-α in the $CD8^+$ T cell population. Positive responses were observed in samples 5-66, 6-64, 9-101, 11-41, 11-65 and 11-113.

For the analysis, only samples with positive responses for polyfunctional T cells, with the IFN-γ^+ TNF-α^+ phenotype was included. This was done to compare the proportion of the polyfunctional cells to the proportion of cells secreting only TNF-α or IFN-γ within a selected population. Samples 3-64,8-65, 8-113, 10-92 and 11-118 were excluded only for the analysis of the CD4 population, as there were no polyfunctional T cell responses detected. Samples 3-64, 8-65, 8-113, 10-92, 11-88, 11-118, 12-65 and 12-113 were excluded for the analysis of the CD8 population, as there were no polyfunctional T cell responses detected.

There were more samples secreting IFN-γ than TNF-α in both the CD4 and CD8 populations. Seven samples with positive responses secreted IFN-γ in the CD4 population, and only three samples secreted IFN-γ in the CD8 population. Polyfunctional T cells were demonstrated in nine samples within the CD4 population, and in six samples within the CD8 population. Samples 5-66 and 11-65 showed 100% of polyfunctional T cells in the CD8 population, and sample 12-65 presented 100% polyfunctional T cells in the CD4 population. Only samples 6-64 and 9-101 showed a significant presence of all the three cytokine expressing population, that is IFN-γ+, TNF-α+ and IFN-γ+ TNF-α+.

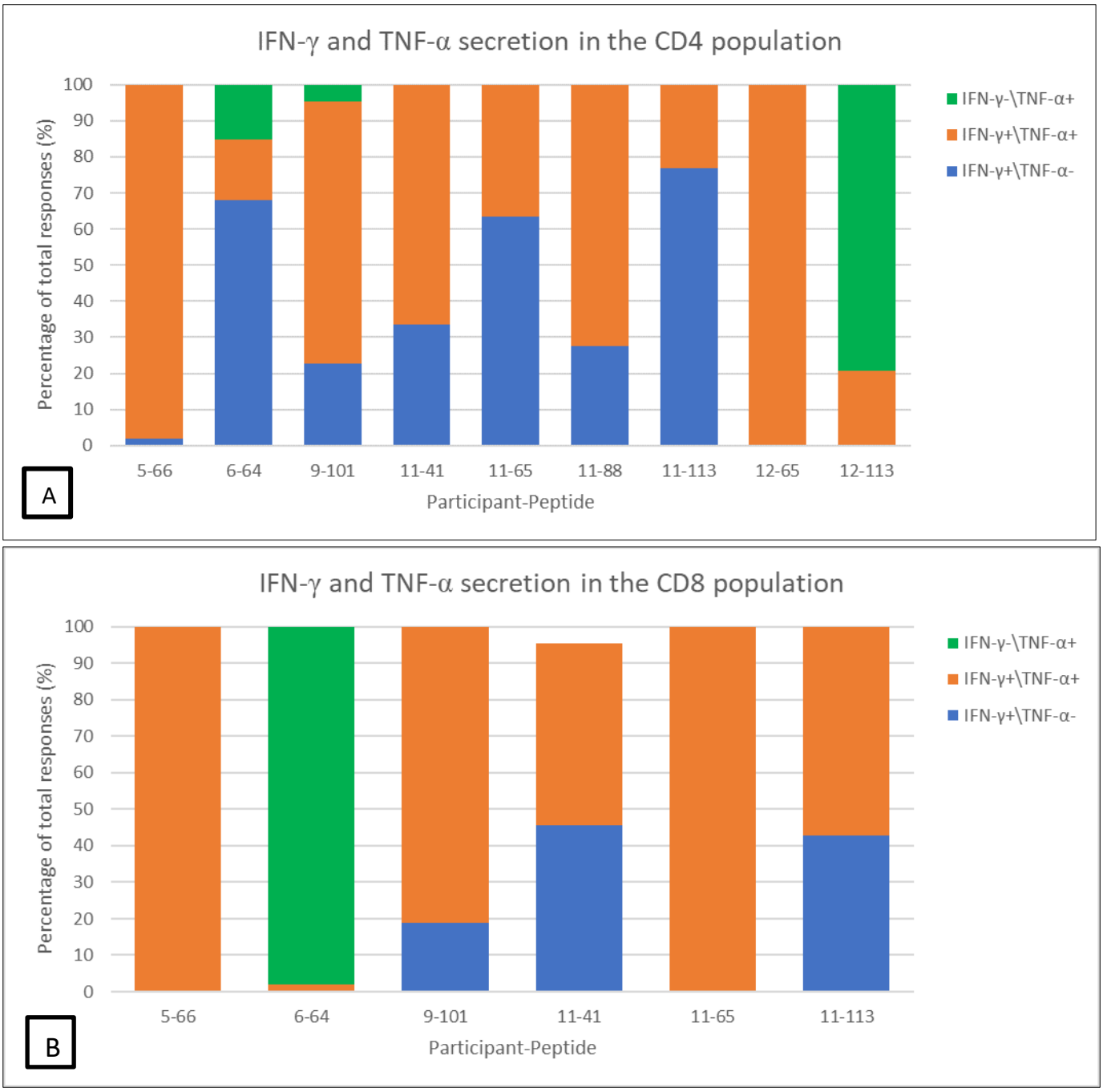


Figure 3.13. The analysis of IFN-γ and TNF-α secretion by the $CD4^+$ and $CD8^+$ T cell populations for samples with positive responses. A. The secretion of IFN-γ^+ and TNF-α^+ by the $CD4^+$ population. B. The secretion of IFN-γ^+ and TNF-α^+ by the $CD8^+$ population.

3.3.4 CYTOTOXIC $CD8^+$ T CELLS

The $CD3^+CD8^+$ population was gated and analysed for the expression of CD107a and concomitant secretion of IFN-γ. The ranges of the positive control and negative control were 0,03%-7,69% and 0,00%-1,18%. The responses for the peptide stimulated samples are illustrated in Figure 3.14 below.

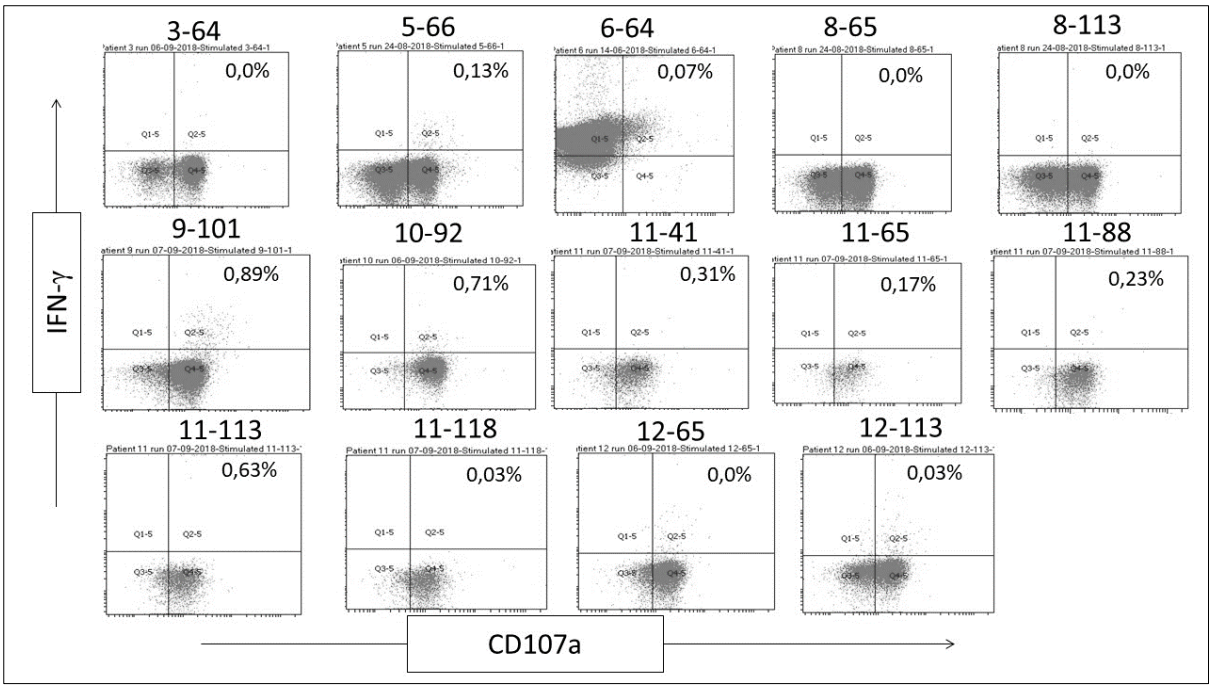


Figure 3.14. Flow cytometry results for IFN-γ^{+}CD107a^{+} within the CD8^{+} T cell population. Positive responses were observed for samples 5-66, 6-64, 9-101, 10-92, 11-41, 11-65, 11-88, 11-113, 11-118 and 12-113.

Ten samples had concurrent detectable IFN-γ and CD107a expression. This shows that the immunogenic peptides have the potential to stimulate the secretion of IFN-γ and expression of CD107a. The results for this analysis are depicted in Figures 3.15.

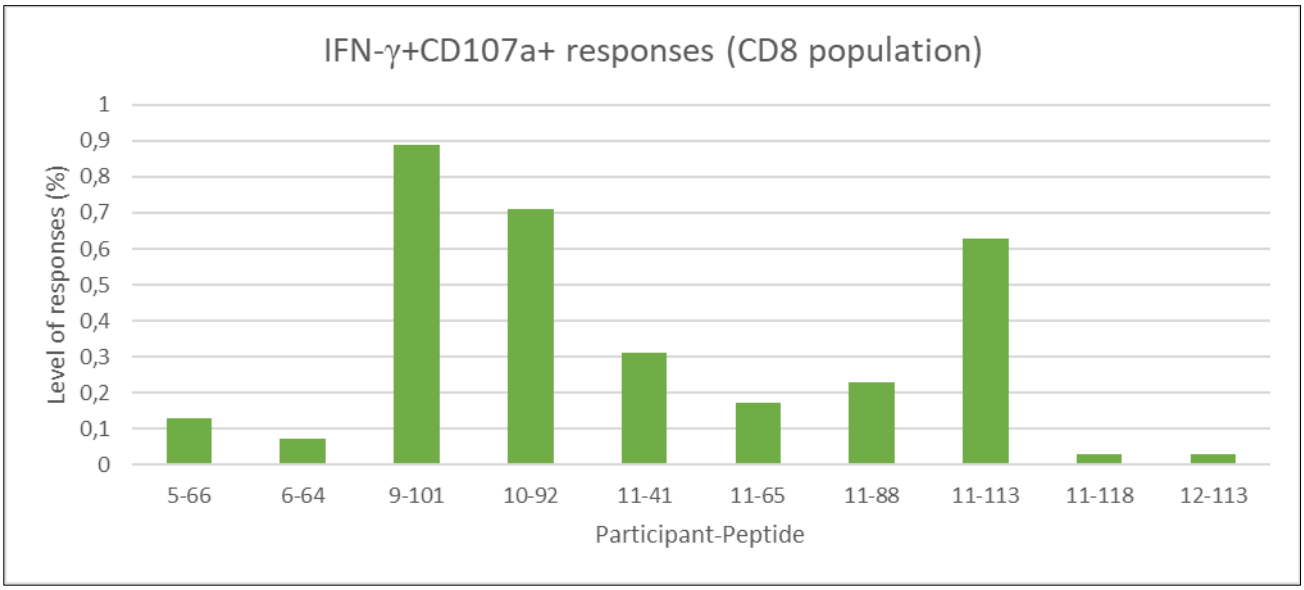


Figure 3.15. Analysis of IFN-γ and CD107a expression for the CD8^{+} T cell population for samples with positive responses.

3.3.5 CHARACTERIZATION OF IFN-γ^+ CD8 T CELLS

The characterization of $CD8^+IFN\text{-}\gamma^+$ population into the four T cell memory subsets was performed using reaction 2 for each sample. The population $CD8^+IFN\text{-}\gamma^+$ was gated from the $CD3^+CD8^+$ population and analysed for the expression of CD45RA and CCR7. Only the ten samples that were positive in reaction 2 for $CD8^+IFN\text{-}\gamma^+$ responses were characterized for the memory subsets. The samples positive for $CD8^+IFN\text{-}\gamma^+$ in reaction 2 were 6-64, 8-65, 9-101, 11-41, 11-65, 11-88, 11-113, 11-118, 12-65 and 12-113. Although there were positive IFN-γ responses for sample 8-65, no cells were detected in the CD45RA CCR7 analysis. This could be due to the small population size in this sample and therefore it may have been possible to miss some events in the gating process.

Antigen specific $CD8^+$ T cells with detectable IFN-γ responses were predominantly of the T_{EMRA} phenotype (range 26,3-100%), followed by T_N cells which were demonstrated in seven subjects (range 10,7-73,7%). Cells of the T_{EM} phenotype were detected in four subjects (range 3,2-40%) and T_{CM} cells at low levels in only two subjects (range 3,2-4,6%). It is important to note that this analysis included only the $CD8^+IFN\text{-}\gamma^+$ population, therefore percentages were calculated based on a low number of isolated events that differ between individuals. The number of events in this population varied from 22 to 154 events. The analyses for the T cell memory subsets for the stimulated samples are illustrated in figure 3.16 below.

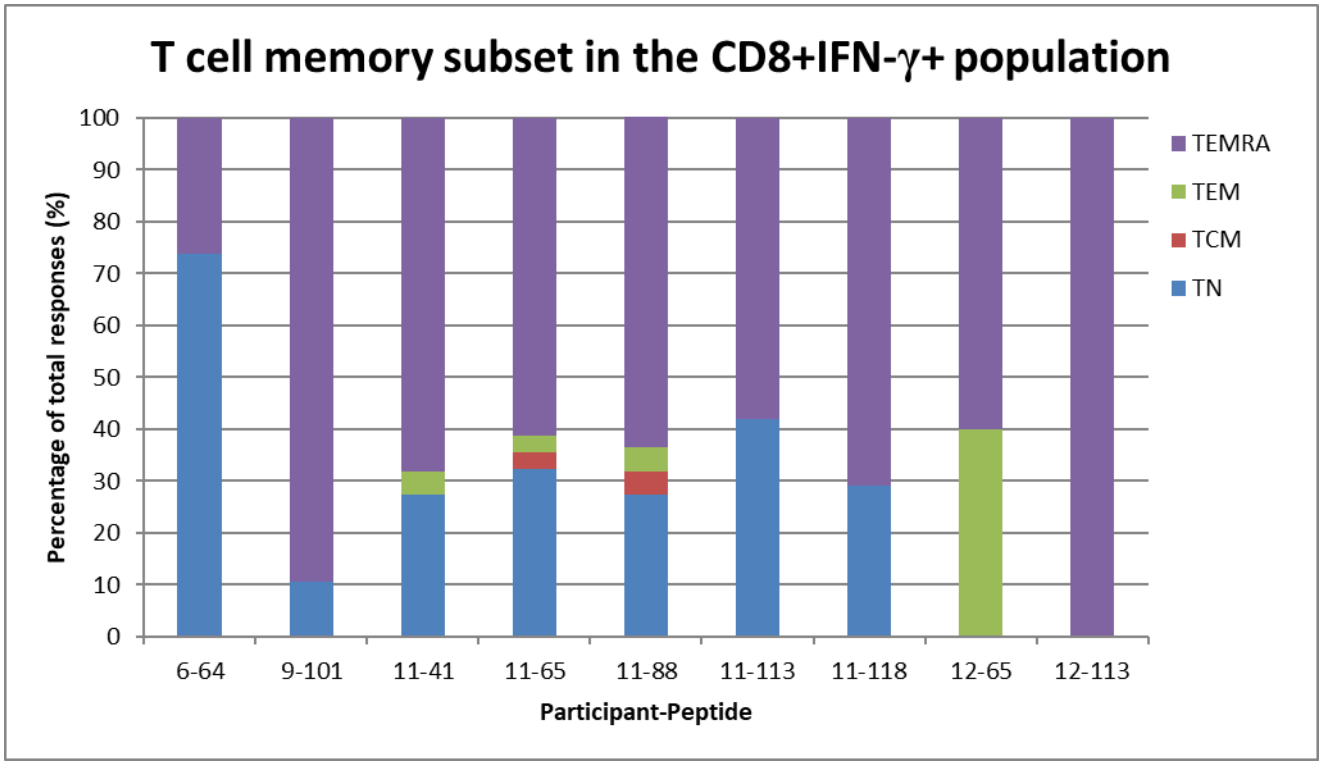


Figure 3.16. The analysis of the expression of CD45RA and CCR7 within the $CD8^{+}IFN\text{-}\gamma^{+}$ T cell population.

3.4 SUMMARY

T cell responses for individual participants were characterized by means of flow cytometry for each of the peptides identified by IFN-γ ELISpot testing (41, 64, 65, 66, 88, 92, 101, 113 and 118). The T cell responses were characterized by the expression of CD45RA and CCR7 which are markers that can be used to identify the presence of T_N, T_{EM}, T_{CM} or T_{EMRA} T cells. The analysis also investigated the evidence of cytotoxic activity in $CD8^+$ T cells by examining the expression of CD107a and secretion of IFN-γ. Finally, T cells were characterized by investigating the secretion of cytokines, namely IFN-γ and TNF-α.

The results for the secretion of IFN-γ and TNF-α showed that there was a higher number of participants PBMCs secreting IFN-γ than TNF-α in both the $CD4^+$ and $CD8^+$ populations. Detection of polyfunctional T cells, secreting both IFN-γ and TNF-α, was comparable in the $CD4^+$ and $CD8^+$ T cell populations. The secretion of IFN-γ and expression of CD107a was detected in ten samples, indicating the presence of cytotoxic $CD8^+$ T cells that secrete IFN-γ. These polyfunctional T cells have been shown to provide a more effective pathogen specific response than monofunctional cells (Almeida *et al.*, 2007; Precopio *et al.*, 2007). Results further indicated that responses may be participant and peptide dependent, in other words, the same peptide may stimulate different responses in different individuals.

The high TNF-α secretion noted in participant 6 may be linked to a number of medical conditions that are associated with elevated levels of circulating TNF-α, such as rheumatoid arthritis, diabetes, and Crohn's disease (De *et al.*, 2002; Feldmann *et al.*, 2001; Vasanthi *et al.*, 2007). However, the precise cause could not be confirmed as the medical histories were not available for study participants.

T cells exist as a heterogeneous cocktail of immune cell subsets. In this study, $CD8^+$ T cells were characterized into the four subsets, T_{EMRA}, T_{CM}, T_N and T_{EM}, based on the expression of CD45RA and CCR7. Antigen specific $CD8^+$ T cells were, in order of decreasing frequency, predominantly T_{EMRA} cells followed by T_N, T_{EM} and finally T_{CM}. This shows that within the $CD8^+IFN\text{-}\gamma^+$ population, there is a more dominant stimulation of the T_{EMRA} cells, while the T_{CM} is the least represented memory cell

subset in this population. The samples showed variable distribution of the subsets, thus there was no peptide specific link associated with the specific subset, but instead the heterogeneous nature of the memory T cells was observed.

CHAPTER 4
Discussion

The alarming rate of expansion of the distribution of CCHF is the motive behind the need to develop interventions. Research is one tool that can be used to understand CCHF and further develop intervention strategies. Existing interventions that have been implemented in CCHF prone areas during outbreaks include the use of personal protective equipment (PPE), targeted education regarding the disease, removal of ticks and precaution measures one should take to avoid contracting the disease, and in rare cases the use of insecticides for tick control (Akuffo *et al.*, 2016; Gozel *et al.*, 2013; Hoogstraal, 1978; Swanepoel *et al.*, 1998).

One major intervention strategy is the development of CCHFV vaccines as a preventative measure for the spread of disease. An inactivated vaccine is used in Bulgaria but is not approved for use in other countries and lacks efficacy and safety studies (Mousavi-Jazi *et al.*, 2012). There is currently no internationally approved CCHF vaccine, but there are a number of vaccines under development with variable approaches and targets (Buttigieg *et al.*, 2014; Canakoglu *et al.*, 2015; Dowall *et al.*, 2016b; Ghiasi *et al.*, 2011; Hinkula *et al.*, 2017; Spik *et al.*, 2006).

The approaches to vaccine development include the use of a transcriptionally competent virus-like particle (tc-VPLs), recombinant DNA vaccines, transgenic plant vaccines and a modified vaccinia virus Ankara (MVA) vector amongst others (Buttigieg *et al.*, 2014; Canakoglu *et al.*, 2015; Dowall *et al.*, 2016b; Ghiasi *et al.*, 2011; Hinkula *et al.*, 2017; Spik *et al.*, 2006). The different vaccines being developed target the proteins or complete open reading frames encoded by the S and/or M segments of CCHFV (Buttigieg *et al.*, 2014; Canakoglu *et al.*, 2015; Dowall *et al.*, 2016b; Ghiasi *et al.*, 2011; Hinkula *et al.*, 2017; Spik *et al*., 2006).

Another significant area of research is the understanding of the immunology associated with CCHFV infection, which may aid in the development of vaccines and therapeutic interventions. Thus far, challenge studies in mice have indicated that both arms of the immune system are required for protective immunity against infection (Dowall *et al.*, 2016a; Hinkula *et al.*, 2017). In a study by Buttigieg *et al.,*

mice were vaccinated with MVA-GP vaccine which was composed of the open reading frame of CCHFV M segment (Buttigieg *et al.*, 2014), and strong T cell immune responses were induced against NS_M, variable mucin-like domain, GP38 and the N-terminus of the G_C (Buttigieg *et al.*, 2014). The strong T cell responses detected against these regions of the virus revealed the immunogenic potential of these domains of the virus. A study in survivors of CCHF tested for T cell responses against the NP and structural glycoproteins G_N and G_C, identified positive T cell responses were detected against peptides from the NP and G_C regions (Goedhals *et al.*, 2017).

However, there is limited information about immunity in patients following a natural CCHFV infection. The adaptive immunity is crucial in the immune response to viruses as it is more specific for clearing intracellular parasites and possesses a feature of immunological memory. However, the role of the adaptive immune response in protection and clearance is unknown. Conserved immunogenic B cell epitopes and T cell epitopes potentially play a role in protection. Conserved B cell epitopes have previously been identified using peptide libraries (Fritzen *et al.*, 2018; Goedhals *et al.*, 2015).

Briefly, peptide libraries and immune sera from survivors were used to identify epitopes on the glycoprotein precursor (Fritzen *et al.*, 2018). Epitopic regions were identified in the NS_M, G_N and highly variable mucin-like domain (Fritzen *et al.*, 2018). Similarly, Goedhals *et al.*, also screened for B cell epitopes in the nucleoprotein (NP), mature G_N and G_C proteins. Responses were detected towards two peptides within the G_C protein (Goedhals *et al.*, 2015). B cell mapping using monoclonal antibodies identified epitopic regions recognised by multiple isolates of CCHFV, thus further indicating the immunogenic potential of G_N and G_C regions of CCHFV (Ahmed *et al.*, 2005).

It is important to identify viral epitopes that stimulate T cells, which are an important component of the adaptive immune system. Viral epitopes, usually in the form of peptides, are preferred because they are biologically safer and much easier to work with than the whole virus. Epitopes can theoretically stimulate a more potent response compared to the whole virus, due to the smaller size of the epitope as

compared to the whole virus, and do not require processing by antigen processing cells, thus simplifying *in vitro* studies.

The importance of selecting the correct length of peptide for constructing peptide libraries has been proven, as 15 mers bound more efficiently to MHC I and MHC II complexes compared to 20 mer or 8 mer peptides (Maecker *et al.*, 2001). The efficient binding ability of the 15 mers was believed, amongst other reasons, to be due to the ability to fit properly to the MHC II complexes, and although it may be too long to bind properly to MHC I complexes, some degree of binding occurs which is sufficient to produce a detectable response (Maecker *et al.*, 2001).

In this study, the aim was to characterize T cell responses specific to the non-structural proteins of the M segment, which are the highly variable mucin-like domain, GP38 and NS_M of CCHFV in survivors of infection. The first step was to identify immunogenic epitopes and to determine the level of conservation of the identified epitopes across all strains of CCHFV. The final step was to investigate the presence of the four subsets of memory T cells, the secretion of IFN-γ and TNF-α, and expression of CD107a in response to stimulation with the immunogenic peptides.

The study was based in the Free State and North West provinces. An average of five cases of CCHFV are reported in South Africa annually (Msimang *et al.*, 2013), therefore a limited number of survivors were available to be included in the study. Twelve participants were recruited for the study, based on the history of laboratory confirmed CCHFV infection. All participants were male, with the majority being of Caucasian descent. The high occurrence of CCHF cases in males is likely due to the possibility that these individuals are at a higher risk of being exposed to the virus through the vector or reservoir, due to occupations involving work outdoors which may bring them into close proximity to animals that could harbour CCHFV vectors (Akuffo *et al.*, 2016; Hoogstraal, 1978; Orkun *et al.*, 2017; Shepherd *et al.*, 1989a; Swanepoel *et al.*, 1998).

There was no pattern observed with the geographical distribution of the time of infection of the participants included in the study, and this agrees with the sporadic nature of the disease observed in the country (Msimang *et al.*, 2013). The wide range of 2-19 years since infection of the participants in the study highlights the

continuous presence of the disease in the country and hence supports the need to investigate interventions (Swanepoel *et al.*, 1998; van Eeden *et al.*,1985).

To identify the immunogenic regions on the CCHFV M segment, overlapping 15 mer peptides were designed covering the GP38, NS_M and highly variable mucin-like domain, and tested for the ability to induce interferon gamma (IFN-γ) secretion. IFN-γ is an antiviral cytokine and marker of T cell stimulation. The PBMCs of twelve participants were screened by utilizing an interferon gamma enzyme linked immunospot (IFN-γ ELISpot) assay.

Significant T cell responses were detected towards five peptides in GP38 (peptides 88, 92, 101, 113 and 118) and four peptides in the NS_M region (peptides 41, 64, 65 and 66). Three peptides 64, 65 and 66 were overlapping within the same location. These regions can therefore be identified as immunogenic viral epitopic regions. There were no T cell responses detected to the highly variable mucin-like domain, possibly due to the high genetic diversity, which was also evidenced with the BioEdit alignment. Additionally, the region has been observed to be heavily O-glycosylated, thus it is expected that the region has high amino acid diversity, as this region is where the majority of mutations are accumulated within the M segment of CCHFV (Deyde *et al.*, 2006).

The identified epitopic regions are moderately variable across all CCHFV strains globally. This is not a surprise since CCHFV is labelled as one of the most genetically diverse viruses, with the M segment harbouring the highest genetic diversity (Carroll *et al.*, 2010; Deyde *et al.*, 2006). It is suspected that the diversity is due to the need for the virus to constantly adapt to host, reservoir and vector environments and body systems (Holland *et al.*, 1998). The high genetic diversity is also influenced by the lack of proof reading RNA dependent RNA polymerase (RdRp), which has resulted in the accumulation of mutations in the genome of the virus (Holland *et al.*, 1998). The functions of the regions of interest are not yet confirmed, but it is possible that the domains of interest need not be conserved for their function, and this state is the most efficient for the survival of the virus (Altamura *et al.*, 2007; Deyde *et al.*, 2006; Holland *et al.*, 1998).

The T cell population is mainly composed of CD8 and CD4 cells, therefore the analysis and characterization of T cell responses was focused on these two

populations. T cell responses were characterized based on the cell markers and secreted antiviral cytokines, upon stimulation with the nine identified CCHFV epitopes.

The $CD8^+IFN\text{-}\gamma^+$ population was analysed to determine the memory/naïve phenotype of the IFN-γ secreting cells. The results showed a high frequency of T_{EMRA}, followed by T_N, T_{EM} and T_{CM}. This indicated the dominant and abundant presence of T_{EMRA} memory cells in survivors of infection, which are readily activated upon stimulation with a CCHFV epitope. Most importantly, the characterization of CD8 cells into the four T cell memory subsets showed the heterogeneous functionality of T cells.

The four memory T cell subsets are not only differentiated phenotypically, but also functionally. The T_N and T_{CM} are known to have a greater proliferative ability than the T_{EM} and T_{EMRA} subsets (Geginat *et al.*, 2013). Interestingly, the T_{CM} cells were observed to generate other heterogeneous phenotypes, including T_N, T_{EM} and T_{EMRA}, upon antigenic stimulation and even at homeostatic conditions (Geginat *et al.*, 2013). This could explain why even in this current study the T_{CM} subset had the lowest frequency of events, while T_{EMRA} was more dominant despite having such a limited proliferative capability. It was observed that with regards to cytolytic functionality, immediate degranulation is a feature of antigen exposed $CD8^+$ T cells, including the T_{EMRA}, T_{EM} and T_{CM} subsets, but is not in found in T_N subsets of $CD8^+$ T cells (Wolint *et al.*, 2004).

Cytotoxic activity was tested and confirmed in the CD8 population, by the detection of CD107a. Cytotoxic $CD8^+$ T cells were detected in ten participants in response to peptide stimulation. The CD4 and CD8 populations were further investigated for the secretion of IFN-γ and TNF-α. IFN-γ secretion was detected in twelve participants and the secretion of TNF-α was detected in seven participants, within either the CD4 or CD8 T cell populations. IFN-γ and TNF-α secretion were observed at similar frequencies in both the CD4 and CD8 populations. IFN-γ secretion ($IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^-$) was more frequent than the secretion of TNF-α ($IFN\text{-}\gamma^-TNF\text{-}\alpha^+$) alone. However, polyfunctional T cells secreting both IFN-γ and TNF-α ($IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$) were detected in five of the eight participants.

The results show that although $CD8^+$ T cells are known to be predominantly cytotoxic T cells (also known as killer cells), these cells are still capable of cytokine secretion.

The results also confirm the function of CD4 T cells as antiviral cytokine secreting cells (Betts *et al.*, 2003; Gargani, 2012). Polyfunctional T cells have been found to be an indication of pathogen specific responses (Almeida *et al.*, 2007; Precopio *et al.*, 2007), which might further confirm the presence of CCHFV specific immune responses in survivors of infection.

There was no correlation between the time after infection and the magnitude of responses observed with the IFN-γ ELISpot. Similarly, the immune responses were participant and peptide dependent with the flow cytometry assay. This means that when the same peptide was used to stimulate PBMCs from different participants, the responses differed, and therefore a specific peptide could not be linked to stimulating specific immune responses. This was also observed in a study where persistent viral infections elicited distinct T cell responses with different phenotypic characteristics (Appay *et al.*, 2002). As such, both the IFN-γ ELISpot and the flow cytometry assay indicate that there is no correlation between time after infection and immune responses in survivors of CCHF.

The IFN-γ ELISpot and flow cytometry assay detect responses at a single cell level after stimulation of the PBMCs with an antigen. In both experiments the secretion of IFN-γ was tested, and although the responses from the two techniques cannot be directly compared, the sensitivity can be used to compare the reliability of the techniques. The results of the ELISpot were calculated as spot forming cells per million PBMC, while the flow cytometry results are recorded as the percentage of the parent population ($CD4^+$ or $CD8^+$) showing a detectable response. The secretion of IFN-γ was therefore used as a basis for comparing the sensitivity of the two techniques.

The ELISpot assay identified eight participants with significant IFN-γ responses (>50 SFC/10^6 PBMC). When tested with the flow cytometry assay, IFN-γ was not detected in either the CD4 or the CD8 populations for one of the eight participants (sample 3-64). Interestingly, the analysis of the IFN-γ secretion with the IFN-γ^+ TNF-α^- phenotype, IFN-γ secretion was observed in all participants, indicating that with different gating strategies it is possible to increase the sensitivity of the flow cytometry assay. For example, the CD4 IFN-γ and CD8 IFN-γ was focused on one

parameter of cytokine, but when analysing the IFN-γ TNF-α phenotypes the focus is two cytokine parameters combined.

The results were comparable between the IFN-γ ELISpot assay and the flow cytometry assay. Therefore, while flow cytometry allows further characterization of cells and the evaluation of multiple parameters concomitantly, ELISpot assays remain a useful screening tool suggesting that a combination of assays is beneficial.

The limitations to the study include a small sample size of twelve participants, however this was influenced by the small number of CCHF cases recorded in the Free State and North West provinces. Regarding the flow cytometry assays, the positive control, Staphylococcal enterotoxin B (SEB), did not stimulate as strong responses as expected. Optimization of testing concentrations of the SEB with serial dilutions may have been beneficial. Future studies may also acquire more events with the flow cytometry assay, which would allow for the presence of more events when analysing certain populations such as the $CD8^{+}IFN\text{-}\gamma^{+}$ population.

While the number of events captured and lack of a live/dead cell marker are limitations to the study which may have resulted in lower reported percentages of functional T cells as indicated by the secretion of cytokines or degranulation, this does not detract from the finding that functional cytotoxic T cells are present in survivors of CCHF many years after infection. This is in line with the study objective which was to confirm the presence of cytolytic activity against identified T cell epitopes, rather than to accurately quantify these responses.

No patterns or correlations were observed between the time after survival of infection, immune responses or number of peptides that stimulate T cell responses. Amongst many other factors, the different HLA types of participants could influence the responses a participant produces, although this was not tested in this study. The study by Akinci *et al.* identified HLA-A*23 as a genetic risk factor for severe CCHF disease progression (Akinci *et al.*, 2013). This example illustrates the possibility that the HLA types could influence the immune defences a host develops against a virus.

The high diversity of CCHFV is one of the stumbling blocks for the development of disease interventions across the globe. This information is useful for the development of not just diagnostic tools, but also for vaccine development. The

implication could be that CCHFV vaccines may have to include immunogenic epitopes derived from multiple CCHFV strains covering a wide geographic region, as there is limited sequence conservation globally (Ahmed *et al.*, 2005). Further studies involving wider geographic areas and diverse patient populations, as well as larger patient cohorts would therefore provide a more robust understanding of immune responses to CCHFV. This would be useful to consider as CCHF is a widely distributed disease globally, is re-emerging and is predicted to have the potential to continue to emerge in more countries across the globe (Messina *et al.*, 2015; Negredo *et al.*, 2017; Papa *et al.*, 2010;).

The results in this project have confirmed the presence of cytotoxic $CD8^+$ T cells at significant levels, and more interesting the presence of polyfunctional T cells with the phenotypes $CD107a^+IFN\text{-}\gamma^+$ and $IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$. These T cells are known to be more effective than the monofunctional T cells. Although the presence of heterogeneous T cell memory subsets was confirmed, the T_{ERMA} subset was more dominant in survivors of CCHF. This possibly implies that there is a potential for robust and effective T cell immune responses in survivors of infection.

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APPENDIX A

CONSENT DOCUMENT

PROJECT TITLE: Characterization of T cell responses to the nonstructural proteins of the M segment in survivors of Crimean-Congo haemorrhagic fever virus (CCHFV) infection

You have been asked to participate in a research study. You have been informed about the study by the study investigator or other healthcare worker.

You may contact Dr Dominique Goedhals at 051 4053162 at any time if you have questions about the research or if you are injured as a result of the research. You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number 051 4052812 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate.

1. I will donate blood on up to 3 separate occasions as described in the information document
2. My blood, including the cells from my blood, may be stored for future research
3. Research data obtained from the study may be published anonymously in a scientific journal

Name of participant Telephone no

Email address

Approximate date of CCHF infection

_____ _____
Signature of Participant Date

_____ _____
Signature of Witness Date
(Where applicable)

_____ _____
Signature of Translator Date
(Where applicable)

INFORMATION DOCUMENT

Study title: Characterization of T cell responses to the non structural proteins of the M segment in survivors of Crimean-Congo haemorrhagic fever virus (CCHFV) infection

Good morning
We, the Department of Virology, are doing research to identify how the human body responds to infections of CCHFV. In this project we want to study which cells in your immune response contributed to your recovery from the infection

Invitation to participate: We are inviting you to participate in this research study

What is involved in the study – We are inviting patients with a confirmed history of infection with CCHFV to participate in the study. We will request blood from you on up to 3 occasions, consisting of 5 x 10 ml blood tubes on each occasion. We will contact you telephonically to arrange to collect the samples from you at a time and place which is convenient to you. This may include the laboratory at Universitas Academic, a pathology laboratory in your own area of residence, or your home/work. Different cells in the body are activated when we have a viral infection and these cells all play a role in clearing the virus from our body. We will isolate blood cells from your sample and try to identify which cells were activated by the virus. We request permission to store these cells indefinitely and use them for related projects to study CCHFV.

Risks There are no foreseeable risks of being involved in the study.

Benefits Participating in this study will not directly benefit you as patient.

Participation is voluntary, and refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled; the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

Reimbursements All procedures will be performed at no extra cost to you. You will not receive any reimbursement for participating in the study.

Confidentiality: Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Ethics Committee for Medical Research.

Contact details of researcher– Dr Dominique Goedhals, Department of Medical Microbiology and Virology, University of the Free State, Bloemfontein. Tel 051 4053162, gnvrdg@ufs.ac.za

Contact details of Research Ethics Committee – For reporting complaints or problems please contact :Ethics Committee of the Faculty of Health Sciences, University of the Free State
☎(051) 4052812

APPENDIX B

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

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

27 September 2016

MS MG MAOTOANA
DEPT OF MEDICAL MICROBIOLOGY/VIROLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Ms Maotoana

ECUFS 195/2015B
PROJECT TITLE: CHARACTERIZATION OF T CELL RESPONSES TO THE NON-STRUCTURAL PROTEINS OF M SEGMENT OF SURVIVORS OF CRIMEAN-CONGO HAEMORRHAGIC FEVER

1. You are hereby kindly informed that, at the meeting held on 20 September 2016, the Health Sciences Research Ethics Committee (HSREC) approved the above project after all conditions were met.
2. The Committee must be informed of any serious adverse event and/or termination of the study.
3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.
4. A progress report should be submitted within one year of approval and annually for long term studies.
5. A final report should be submitted at the completion of the study.
6. Kindly use the **ECUFS NR** as reference in correspondence to the HSREC Secretariat.
7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Cc DR D GOEDHALS
PROF FJ BURT

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | F: +27 (0)51 444 4359 | E: ethicsfhs@ufs.ac.za
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

APPENDIX C

The amino acid sequence of the South African isolate SPU103/87 used in the project for the design of the peptide library.

>ABB30034.1 glycoprotein precursor [Crimean-Congo hemorrhagic fever orthonairovirus]

MHISLMYAVLCLQLCGLGGAHGSHNGTRYNNTGALTTLGDGQSSEPPVSTAPSTTHNPSTVTSTTPASGS
EGSGEVYTASPVTTEGLSPPGTTPELPATTGIDTSGASDADPSTQAAGDTSALTVRTSLPSSPSTLPTSQ
DTHHPVRNLLSVTSPRPEETTAPSGSGIESSATSSPHPASDRPPTPPAAAQGPTESNSHNATEHLESLTQ
SATPDLVTSPAQTVHPQSATSIAAPDTHPSPTNRSKRNLEVEIILTLSQGLKKYYGKILRLLHLTLEEDT
EGLLEWCKRNLGLDCDDTFFQKRIEEFFITGEGHFNEVLQFRTPGTVSTTESTHAGPPTAEPFKSYFAKG
FLSIDSGYYSAKCYSGTSNSGLQLINITRHSTRIADTPGPKITNLKTINCINLKALTFKEHREVEINVLL
PQVAVNLSNCHVVIKSHVCDYSLDIDGTVRLPHIHHEGIFIPGTYKIVIDKKNKLNDRCTLFTDCVIKGR
EVRKGQSVLKQYKTEIRIGKASTGSRRLLSEESSDDCISRTQLLRTETAEIHGDNYGGPGDKITICNGST
IVDQRLGSELGCYTINRVKTFKLCENSATGKSCEIDSVPVKCRQGYCLKITQEGRGHVKLSRGSEVVLDA
CDTSCEVMIPKGTGDILVDCSGGQQHFLKDNLIDLGCPKIPLLGKMAIYICRMSNHPKTTMAFLFWFSFG
YVITCVLCKAIFYLLIIVGTLGKRLKQYRELKPQTCTICETTPVNAIDAEMHDLNCSYNICPYCASRLTS
DGLARHVTQCPKRKEKVEETELYLNLERIPWVVRRLLQVSESTGVALKRSSWLIVLLVLFTVSLSPVQSA
PISHGKTVEAYQAREGYTGMCLFVLGSILFLVSCLMKGLVDSVGNTFFPGLSICKTCSISSINGFEIESH
RCYCSLFCCPYCRHCSANKEIHQLHLSICKKRKTGSNVMLAVCKRMCFRATMEVSNRALFIRSIINTTFV
LCILILAVCVVSTSAVEMESLPAGTWEREEDLTNFCHQECQVTDTECLCPYEALVLRRPLFLDNIVKGMK
NLLNSTSLETSLSIEAPWGAINVQSTYKPTVSTANIALSWSSVEHRGNKILVSGRSESIMKLEERTGISW
DLGVEDASESKLLTVSVMDLSQMYSPVFEYLSGDRQVEEWPKATCTGDCPERCGCTSSTCLHKEWPHSRN
WRCNPTWCWGVGTGCTCCGLDVKDLFTDYMFVKWKIEYIKTEAIVCVELTSQERQCSLIEAGTRFNLGPV
TITLSEPRNIQQKLPSEIITLHPRIEEGFFDLMHVQKVLSASTVCKLQSCTHGVPGDLQVYHIGNLLKGD
KVNGHLVHKIEPHFNTSWMSWDGCDLDYYCNMGDWPSCTYTGVTQHNHASFVNLLNIETDYTKNFHFHSK
RVTAHGDTPQLDLKARPTYGAGEITVLVEVADMELHTKKIEISGLKFTSLTCTGCYACSSGISCKVRIHV
DEPDELTVHVKSDDPDVVAASSSLMARKLEFGTDSTFKAFSAMPKTSLCFYIVEREYCKSCSEEDTQKCV
NTKLEQPQSILIEHKGTIIGKQNNTCKARAGCWLESVKSFFYGLKNMLSGIFGNVFIGIFLFLAPFILLI
LFFMFGWRILFCFKCCRRTRGLFKYRHLKDDEETGYRKIIERLNNKKGKNKLLDGERLADRRIAELFSTK
THIG

The regions highlighted indicate the domains screened in the project. In order is the highly variable mucin domain, GP38 and NS_M.

APPENDIX D

peptide number	Peptide name	Amino acid sequence
1	Mucin-1	MHISLMYAVLCLQLC
2	Mucin-2	YAVLCLQLCGLGGAH
3	Mucin-3	QLCGLGGAHGSHNGT
4	Mucin-4	GAHGSHNGTRYNNTG
5	Mucin-5	NGTRYNNTGALTTLG
6	Mucin-6	NTGALTTLGDGQSSE
7	Mucin-7	TLGDGQSSEPPVSTA
8	Mucin-8	SSEPPVSTAPSTTHN
9	Mucin-9	STAPSTTHNPSTVTS
10	Mucin-10	THNPSTVTSTTPASG
11	Mucin-11	VTSTTPASGSEGSGE
12	Mucin-12	ASGSEGSGEVYTASP
13	Mucin-13	SGEVYTASPVTTEGL
14	Mucin-14	ASPVTTEGLSPPGTT
15	Mucin-15	EGLSPPGTTPELPAT
16	Mucin-16	GTTPELPATTGIDTS
17	Mucin-17	PATTGIDTSGASDAD
18	Mucin-18	DTSGASDADPSTQAA
19	Mucin-19	DADPSTQAAGDTSAL
20	Mucin-20	QAAGDTSALTVRTSL
21	Mucin-21	SALTVRTSLPSSPST
22	Mucin-22	TSLPSSPSTLPTSQD
23	Mucin-23	PSTLPTSQDTHHPVR
24	Mucin-24	SQDTHHPVRNLLSVT
25	Mucin-25	PVRNLLSVTSPRPEE
26	Mucin-26	SVTSPRPEETTAPSG
27	Mucin-27	PEETTAPSGSGIESS
28	Mucin-28	PSGSGIESSATSSPH
29	Mucin-29	ESSATSSPHPASDRP
30	Mucin-30	SPHPASDRPPTPPAA
31	Mucin-31	DRPPTPPAAAQGPTE
32	Mucin-32	PAAAQGPTESNSHNA
33	Mucin-33	PTESNSHNATEHLES
34	Mucin-34	HNATEHLESLTQSAT
35	Mucin-35	LESLTQSATPDLVTS
36	Mucin-36	SATPDLVTSPAQTVH
37	Mucin-37	VTSPAQTVHPQSATS
38	Mucin-38	TVHPQSATSIAAPDT
39	Mucin-39	ATSIAAPDTHPSPTN

40	NSM-1	QVSESTGVALKRSSW
41	NSM-2	GVALKRSSWLIVLLV
42	NSM-3	SSWLIVLLVLFTVSL
43	NSM-4	LLVLFTVSLSPVQSA
44	NSM-5	VSLSPVQSAPISHGK
45	NSM-6	QSAPISHGKTVEAYQ
46	NSM-7	HGKTVEAYQAREGYT
47	NSM-8	AYQAREGYTGMCLFV
48	NSM-9	GYTGMCLFVLGSILF
49	NSM-10	LFVLGSILFLVSCLM
50	NSM-11	ILFLVSCLMKGLVDS
51	NSM-12	CLMKGLVDSVGNTFF
52	NSM-13	VDSVGNTFFPGLSIC
53	NSM-14	TFFPGLSICKTCSIS
54	NSM-15	SICKTCSISSINGFE
55	NSM-16	SISSINGFEIESHRC
56	NSM-17	GFEIESHRCYCSLFC
57	NSM-18	HRCYCSLFCCPYCRH
58	NSM-19	LFCCPYCRHCSANKE
59	NSM-20	CRHCSANKEIHQLHL
60	NSM-21	NKEIHQLHLSICKKR
61	NSM-22	LHLSICKKRKTGSNV
62	NSM-23	KKRKTGSNVMLAVCK
63	NSM-24	SNVMLAVCKRMCFRA
64	NSM-25	VCKRMCFRATMEVSN
65	NSM-26	FRATMEVSNRALFIR
66	NSM-27	VSNRALFIRSIINTT
67	NSM-28	FIRSIINTTFVLCIL
68	NSM-29	NTTFVLCILILAVCV
69	NSM-30	CILILAVCVVSTSAV
70	NSM-31	VCVVSTSAVEMESLP
71	NSM-32	SAVEMESLPAGTWER
72	NSM-33	SLPAGTWEREEDLTN
73	NSM-34	WEREEDLTNFCHQEC
74	NSM-35	LTNFCHQECQVTDTE
75	NSM-36	QECQVTDTECLCPYE
76	NSM-37	DTECLCPYEALVL

77	GP38-1	NLEVEIILTLSQGLK
78	GP38-2	ILTLSQGLKKYYGKI
79	GP38-3	GLKKYYGKILRLLHL
80	GP38-4	GKILRLLHLTLEEDT
81	GP38-5	LHLTLEEDTEGLLEW
82	GP38-6	EDTEGLLEWCKRNLG
83	GP38-7	LEWCKRNLGLDCDDT
84	GP38-8	NLGLDCDDTFFQKRI
85	GP38-9	DDTFFQKRIEEFFIT
86	GP38-10	KRIEEFFITGEGHFN
87	GP38-11	FITGEGHFNEVLQFR
88	GP38-12	HFNEVLQFRTPGTVS
89	GP38-13	QFRTPGTVSTTESTH
90	GP38-14	TVSTTESTHAGPPTA
91	GP38-15	STHAGPPTAEPFKSY
92	GP38-16	PTAEPFKSYFAKGFL
93	GP38-17	KSYFAKGFLSIDSGY
94	GP38-18	GFLSIDSGYYSAKCY
95	GP38-19	SGYYSAKCYSGTSNS
96	GP38-20	KCYSGTSNSGLQLIN
97	GP38-21	SNSGLQLINITRHST
98	GP38-22	LINITRHSTRIADTP
99	GP38-23	HSTRIADTPGPKITN
100	GP38-24	DTPGPKITNLKTINC
101	GP38-25	ITNLKTINCINLKAL
102	GP38-26	INCINLKALTFKEHR
103	GP38-27	KALTFKEHREVEINV
104	GP38-28	EHREVEINVLLPQVA
105	GP38-29	INVLLPQVAVNLSNC
106	GP38-30	QVAVNLSNCHVVIKS
107	GP38-31	SNCHVVIKSHVCDYS
108	GP38-32	IKSHVCDYSLDIDGT
109	GP38-33	DYSLDIDGTVRLPHI
110	GP38-34	DGTVRLPHIHHEGIF
111	GP38-35	PHIHHEGIFIPGTYK
112	GP38-36	GIFIPGTYKIVIDKK
113	GP38-37	TYKIVIDKKNKLNDR
114	GP38-38	DKKNKLNDRCTLFTD
115	GP38-39	NDRCTLFTDCVIKGR
116	GP38-40	FTDCVIKGREVRKGQ
117	GP38-41	KGREVRKGQSVLKQY
118	GP38-42	KGQSVLKQYKTEIRI
119	GP38-43	KQYKTEIRIGKASTG
120	GP38-44	IRIGKASTGS

APPENDIX E

The table shows the 24 peptide pools used in the study, as well as the individuals included in the study.

POOL 1	POOL 2	POOL 3	POOL 4	POOL 5	POOL 6	POOL 7	POOL 8	POOL 9	POOL 10	POOL 11	POOL 12
1	11	21	31	41	54	64	78	88	98	108	118
2	12	22	32	43	55	65	79	89	99	109	119
3	13	23	33	44	56	66	80	90	100	110	120
4	14	24	34	45	57	70	81	91	101	111	
5	15	25	35	46	58	71	82	92	102	112	
6	16	26	36	47	59	72	83	93	103	113	
7	17	27	37	50	60	73	84	94	104	114	
8	18	28	38	51	61	74	85	95	105	115	
9	19	29	39	52	62	75	86	96	106	116	
10	20	30	40	53	63	76	87	97	107	117	

POOL 13	POOL 14	POOL 15	POOL 16	POOL 17	POOL 18	POOL 19	POOL 20	POOL 21	POOL 22	POOL 23	POOL 24
1	2	3	4	5	6	7	8	9	10	11	12
13	14	15	16	17	18	19	20	21	22	23	24
25	26	27	28	29	30	31	32	33	34	35	36
37	38	39	40	41	43	44	45	46	47	50	51
52	53	54	55	56	57	58	59	60	61	62	63
64	65	66	70	71	72	73	74	75	76	78	79
80	81	82	83	84	85	86	87	88	89	90	91
92	93	94	95	96	97	98	99	100	101	102	103
104	105	106	107	108	109	110	111	112	113	114	115
116	117	118	119	120							

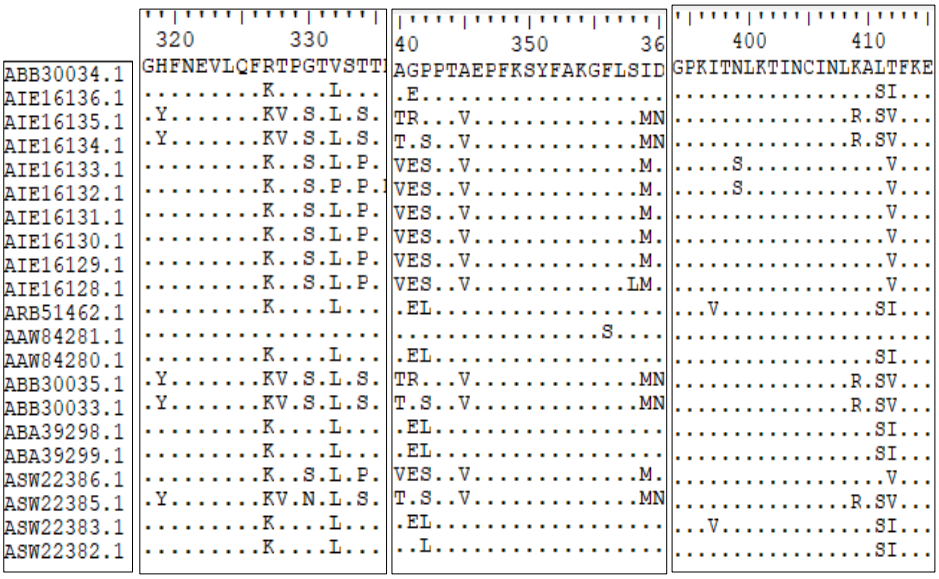
APPENDIX F

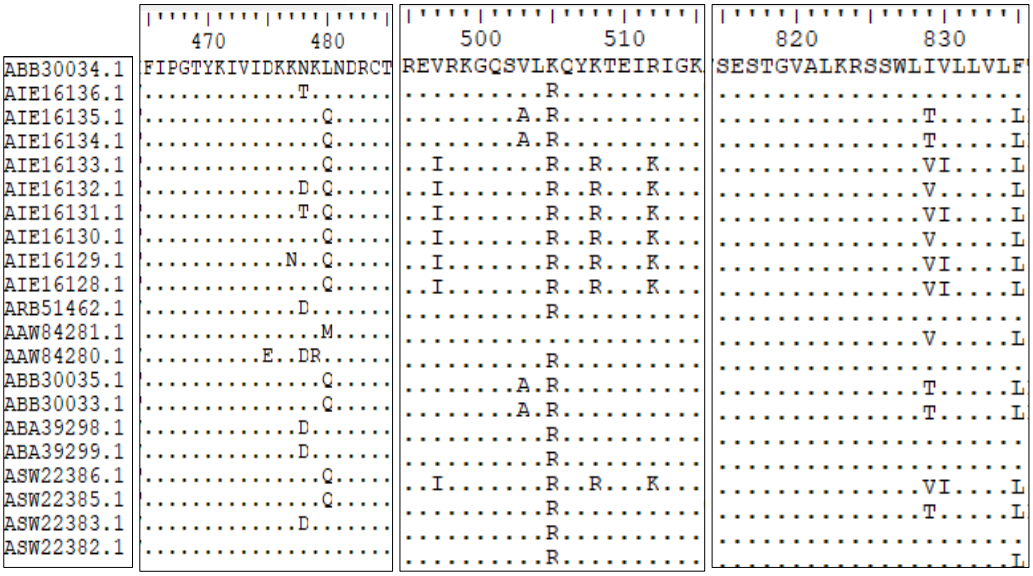
SECTION A: BioEdit Sequence Alignment Editor software analysis of amino acid sequence conservation

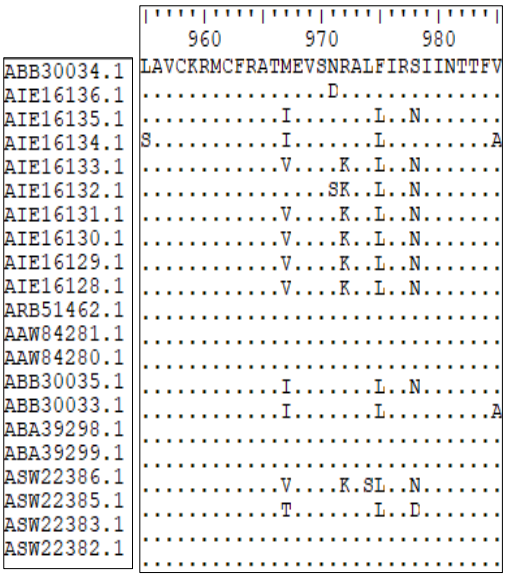
The strains were listed by the amino acid Genbank Accesion number. The ruler on top of the sequences indicates the position of the amino acid. The reference strain is listed first and the complete sequence is written out in full. The dots indicate that the amino acid of a certain strain is identical to the amino acid of the reference strain, whereas the letter indicates that a different amino acid is present in the specific strain. The letters are the standard letters used for presentation of amino acids.

A.1 SOUTH AFRICAN STRAINS (21 strains)

In order peptides 88, 92, 101, 113, 118, 41, 64-66

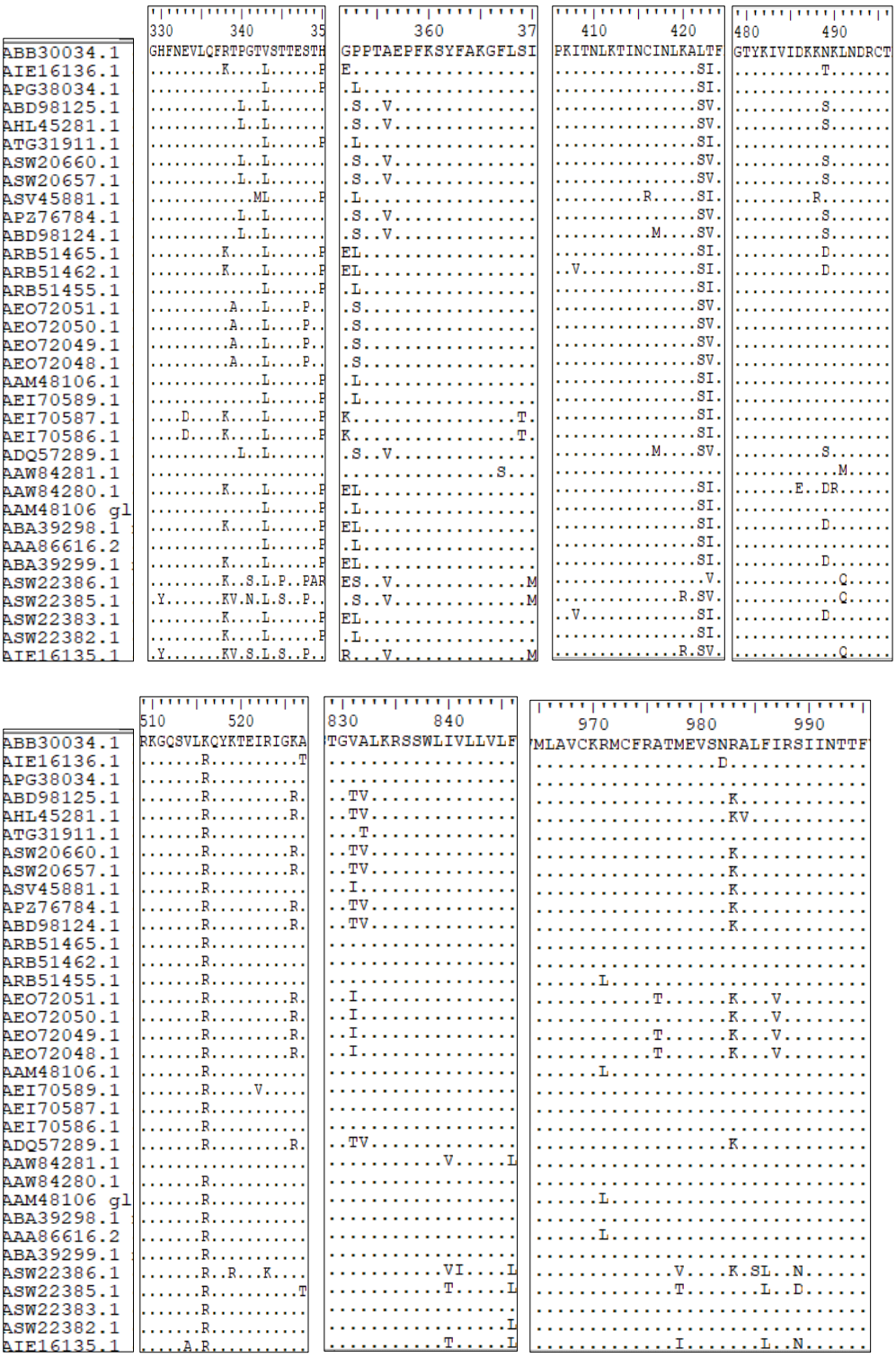


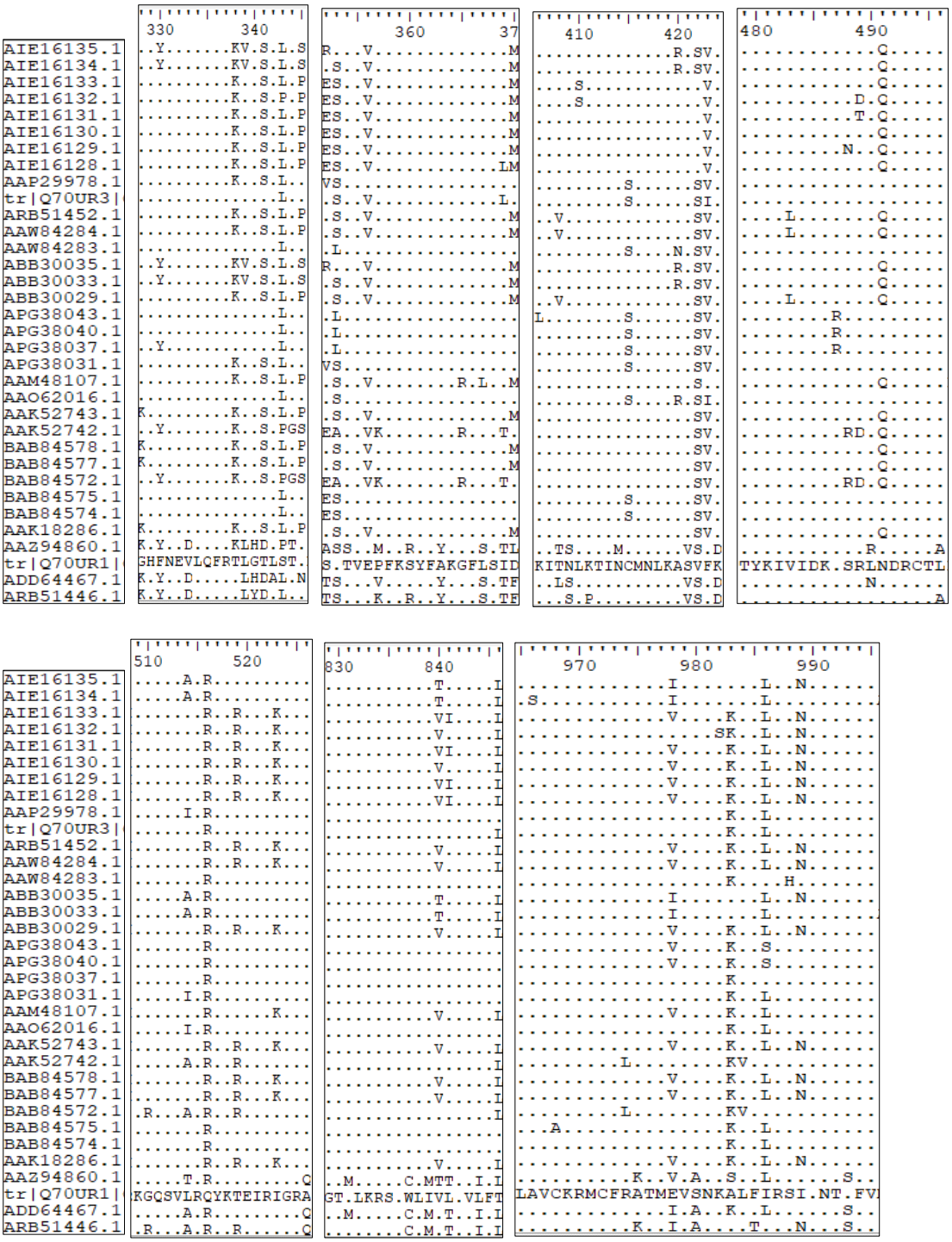



960
970
980
ABB30034.1 LAVCKRMCFRATMEVSNRALFIRSIINTTFV
AIE16136.1 .....D.....
AIE16135.1 .....I.....L..N.....
AIE16134.1 S.....I.....L.....A
AIE16133.1 .....V....K..L..N.....
AIE16132.1 .....SK..L..N.....
AIE16131.1 .....V....K..L..N.....
AIE16130.1 .....V....K..L..N.....
AIE16129.1 .....V....K..L..N.....
AIE16128.1 .....V....K..L..N.....
ARB51462.1 .....
AAW84281.1 .....
AAW84280.1 .....
ABB30035.1 .....I.....L..N.....
ABB30033.1 .....I.....L.....A
ABA39298.1 .....
ABA39299.1 .....
ASW22386.1 .....V....K.SL..N.....
ASW22385.1 .....T.....L..D.....
ASW22383.1 .....
ASW22382.1 .....

A.2 GLOBAL STRAINS (138 strains)

Peptides put in the order 88, 92, 101, 113, 118, 41, 64-66




AIE16135.1
AIE16134.1
AIE16133.1
AIE16132.1
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AAP29978.1
tr|Q70UR3|
ARB51452.1
AAW84284.1
AAW84283.1
ABB30035.1
ABB30033.1
ABB30029.1
APG38043.1
APG38040.1
APG38037.1
APG38031.1
AAM48107.1
AAO62016.1
AAK52743.1
AAK52742.1
BAB84578.1
BAB84577.1
BAB84572.1
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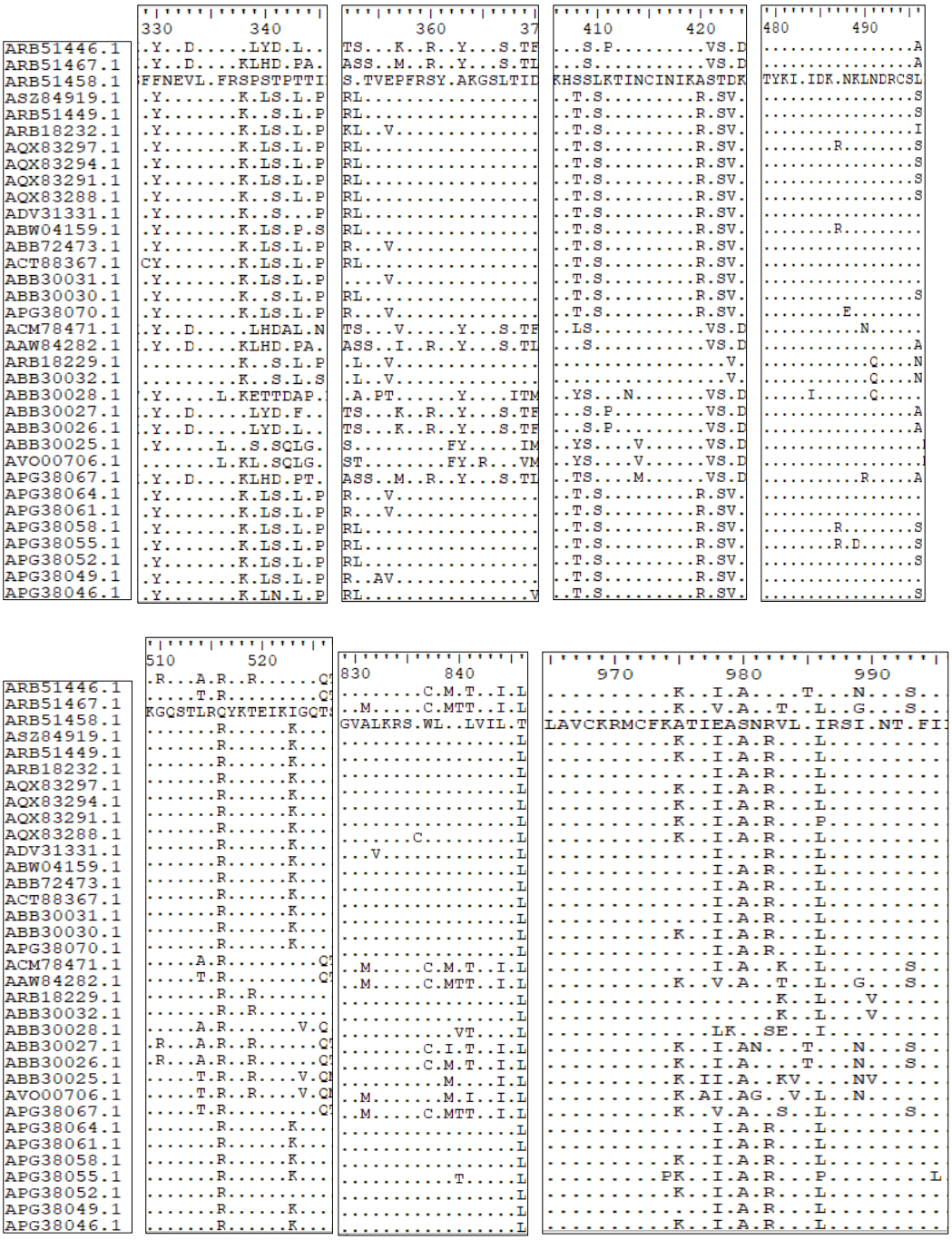
BAB84574.1
AAK18286.1
AAZ94860.1
tr|Q70UR1|
ADD64467.1
ARB51446.1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510 520 830 840 970 980 990
ARB51446.1
ARB51467.1
ARB51458.1
ASZ84919.1
ARB51449.1
ARB18232.1
AQX83297.1
AQX83294.1
AQX83291.1
AQX83288.1
ADV31331.1
ABW04159.1
ABB72473.1
ACT88367.1
ABB30031.1
ABB30030.1
APG38070.1
ACM78471.1
AAW84282.1
ARB18229.1
ABB30032.1
ABB30028.1
ABB30027.1
ABB30026.1
ABB30025.1
AVO00706.1
APG38067.1
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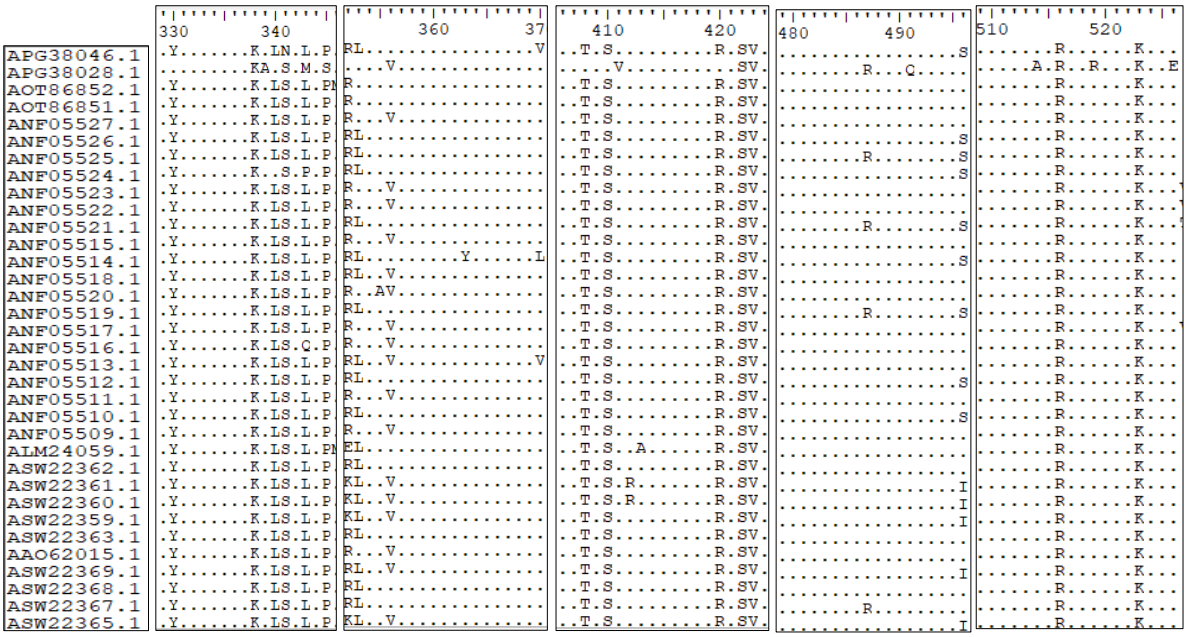
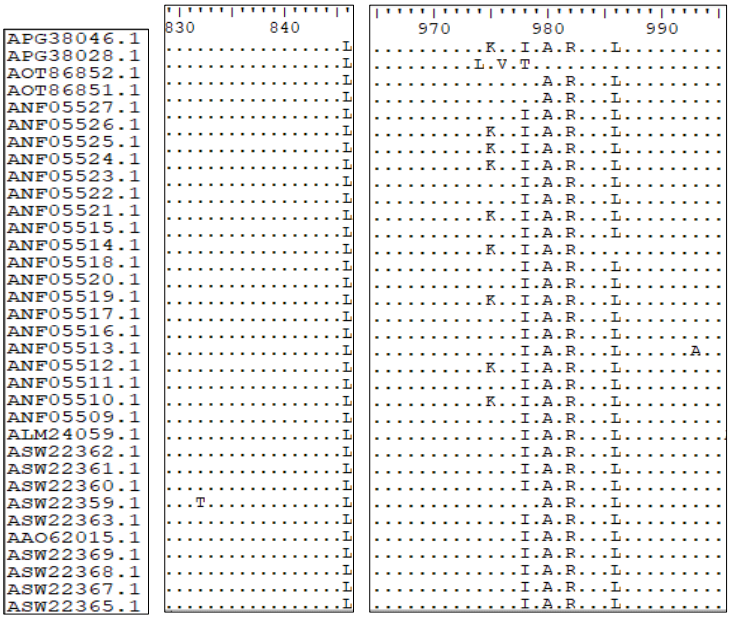
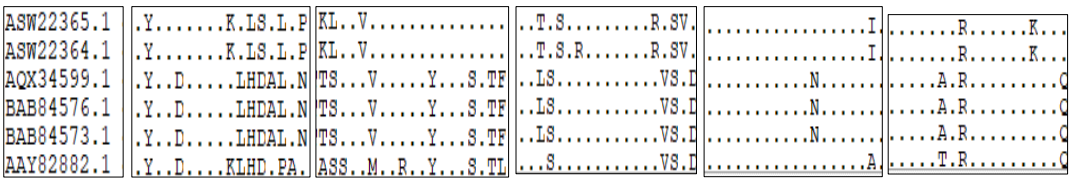
APG38052.1
APG38049.1
APG38046.1


330 340 360 370 410 420 480 490 510 520
APG38046.1 .Y.....K.LN.L.P RL.....V ..T.S.....R.SV. .....S .....R.....K...
APG38028.1 .....KA.S.M.S ....V..... .....V.....SV. .....R...Q..... .....A.R..R...K..E
AOT86852.1 .Y.....K.LS.L.PN R..... ..T.S.....R.SV. .....R.....K...
AOT86851.1 .Y.....K.LS.L.P R..... ..T.S.....R.SV. .....R.....K...
ANF05527.1 .Y.....K.LS.L.P R...V..... ..T.S.....R.SV. .....R.....K...
ANF05526.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....S .....R.....K...
ANF05525.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....R.....S .....R.....K...
ANF05524.1 .Y.....K..S.P.P RL..... ..T.S.....R.SV. .....S .....R.....K...
ANF05523.1 .Y.....K.LS.L.P R...V..... ..T.S.....R.SV. .....R.....K...
ANF05522.1 .Y.....K.LS.L.P R...V..... ..T.S.....R.SV. .....R.....K...
ANF05521.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....R.....S .....R.....K...
ANF05515.1 .Y.....K.LS.L.P R...V..... ..T.S.....R.SV. .....R.....K...
ANF05514.1 .Y.....K.LS.L.P RL.....Y.....L ..T.S.....R.SV. .....S .....R.....K...
ANF05518.1 .Y.....K.LS.L.P RL..V..... ..T.S.....R.SV. .....R.....K...
ANF05520.1 .Y.....K.LS.L.P R..AV..... ..T.S.....R.SV. .....R.....K...
ANF05519.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....R.....S .....R.....K...
ANF05517.1 .Y.....K.LS.L.P R...V..... ..T.S.....R.SV. .....R.....K...
ANF05516.1 .Y.....K.LS.Q.P R...V..... ..T.S.....R.SV. .....R.....K...
ANF05513.1 .Y.....K.LS.L.P RL..V.....V ..T.S.....R.SV. .....R.....K...
ANF05512.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....S .....R.....K...
ANF05511.1 .Y.....K.LS.L.P R...V..... ..T.S.....R.SV. .....R.....K...
ANF05510.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....S .....R.....K...
ANF05509.1 .Y.....K.LS.L.P R...V..... ..T.S.....R.SV. .....R.....K...
ALM24059.1 .Y.....K.LS.L.PN EL..... ..T.S..A.....R.SV. .....R.....K...
ASW22362.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....R.....K...
ASW22361.1 .Y.....K.LS.L.P KL..V..... ..T.S.R.....R.SV. .....I .....R.....K...
ASW22360.1 .Y.....K.LS.L.P KL..V..... ..T.S.R.....R.SV. .....I .....R.....K...
ASW22359.1 .Y.....K.LS.L.P KL..V..... ..T.S.....R.SV. .....I .....R.....K...
ASW22363.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....R.....K...
AAO62015.1 .Y.....K.LS.L.P R...V..... ..T.S.....R.SV. .....R.....K...
ASW22369.1 .Y.....K.LS.L.P RL..V..... ..T.S.....R.SV. .....I .....R.....K...
ASW22368.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....R.....K...
ASW22367.1 .Y.....K.LS.L.P RL..... ..T.S.....R.SV. .....R.....K...
ASW22365.1 .Y.....K.LS.L.P KL..V..... ..T.S.....R.SV. .....I .....R.....K...


830 840 970 980 990
APG38046.1 .....L .....K..I.A.R...L.....
APG38028.1 .....L .....L.V.T.....
AOT86852.1 .....L .....A.R...L.....
AOT86851.1 .....L .....A.R...L.....
ANF05527.1 .....L .....I.A.R...L.....
ANF05526.1 .....L .....K..I.A.R...L.....
ANF05525.1 .....L .....K..I.A.R...L.....
ANF05524.1 .....L .....K..I.A.R...L.....
ANF05523.1 .....L .....I.A.R...L.....
ANF05522.1 .....L .....I.A.R...L.....
ANF05521.1 .....L .....K..I.A.R...L.....
ANF05515.1 .....L .....I.A.R...L.....
ANF05514.1 .....L .....K..I.A.R.....
ANF05518.1 .....L .....I.A.R...L.....
ANF05520.1 .....L .....I.A.R...L.....
ANF05519.1 .....L .....K..I.A.R...L.....
ANF05517.1 .....L .....I.A.R...L.....
ANF05516.1 .....L .....I.A.R...L.....
ANF05513.1 .....L .....I.A.R...L.....A..
ANF05512.1 .....L .....K..I.A.R...L.....
ANF05511.1 .....L .....I.A.R...L.....
ANF05510.1 .....L .....K..I.A.R...L.....
ANF05509.1 .....L .....I.A.R...L.....
ALM24059.1 .....L .....I.A.R...L.....
ASW22362.1 .....L .....I.A.R...L.....
ASW22361.1 .....L .....I.A.R...L.....
ASW22360.1 .....L .....I.A.R...L.....
ASW22359.1 ...T.....L .....A.R...L.....
ASW22363.1 .....L .....I.A.R...L.....
AAO62015.1 .....L .....I.A.R...L.....
ASW22369.1 .....L .....I.A.R...L.....
ASW22368.1 .....L .....I.A.R...L.....
ASW22367.1 .....L .....I.A.R...L.....
ASW22365.1 .....L .....I.A.R...L.....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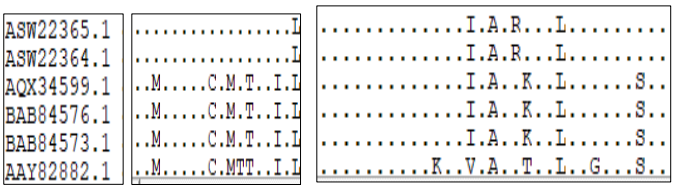
ASW22364.1 .Y.....K.LS.L.P KL..V..... ..T.S.R.....R.SV. .....I .....R.....K...
AQX34599.1 .Y..D.....LHDAL.N TS...V.....Y...S.TF ..LS.....VS.I .....N..... .....A.R.....C
BAB84576.1 .Y..D.....LHDAL.N TS...V.....Y...S.TF ..LS.....VS.I .....N..... .....A.R.....C
BAB84573.1 .Y..D.....LHDAL.N TS...V.....Y...S.TF ..LS.....VS.I .....N..... .....A.R.....C
AAY82882.1 .Y..D....KLHD.PA. ASS..M..R..Y...S.TL ...S.....VS.I .....A .....T.R.....C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ASW22364.1 .....L .....I.A.R...L.....
AQX34599.1 ..M.....C.M.T..I.L .....I.A..K..L.....S..
BAB84576.1 ..M.....C.M.T..I.L .....I.A..K..L.....S..
BAB84573.1 ..M.....C.M.T..I.L .....I.A..K..L.....S..
AAY82882.1 ..M.....C.MTT..I.L .....K..V.A..T..L..G...S..

SECTION B

CONSURF RESULTS

Amino Acid Conservation Scores

- POS: The position of the AA in the SEQRES derived sequence.
- SEQ: The SEQRES derived sequence in one letter code.
- SCORE: The normalized conservation scores.
- COLOR: The color scale representing the conservation scores (9 - conserved, 1 - variable).
- CONFIDENCE INTERVAL: When using the bayesian method for calculating rates, a confidence interval is assigned to each of the inferred evolutionary conservation scores.
- CONFIDENCE INTERVAL COLORS: When using the bayesian method for calculating rates. The color scale representing the lower and upper bounds of the confidence interval.
- B/E: Burried (b) or Exposed (e) residue.
- FUNCTION: functional (f) or structural (s) residue (f - highly conserved and exposed, s - highly conserved and burried).
- MSA DATA: The number of aligned sequences having an amino acid (non-gapped) from the overall number of sequences at each position.
- RESIDUE VARIETY: The residues variety at each position of the multiple sequence alignment.

B.1 South African strains

link: http://consurf.tau.ac.il/results/1528278751/output.php

peptide 88 (position 314-328)

314	H	0.662	4*	-1.121, 1.911	7,1	b		21/21	Y,H,L
315	F	-0.166	5*	-1.777, 1.001	9,3	b		21/21	F,T
316	N	-0.620	6*	-2.308, 0.524	9,4	e		21/21	N,G
317	E	-1.559	8	-3.280,-0.618	9,6	e	f	21/21	E
318	V	-0.344	6*	-1.947, 0.754	9,3	b		21/21	V,G
319	L	0.691	3*	-1.121, 1.911	7,1	b		21/21	Y,H,L
320	Q	0.011	5*	-1.611, 1.273	9,2	e		21/21	F,Q
321	F	-0.083	5*	-1.777, 1.001	9,3	e		21/21	F,N
322	R	0.274	4*	-1.611, 1.569	9,2	e		21/21	K,R,E
323	T	0.192	5*	-1.611, 1.569	9,2	e		21/21	V,T
324	P	-0.420	6*	-2.123, 0.754	9,3	e		21/21	L,P
325	G	1.446	2*	-0.449, 2.786	6,1	e		21/21	Q,S,N,G
326	T	-0.238	6*	-1.947, 1.001	9,3	e		21/21	F,T
327	V	0.944	3*	-0.956, 2.303	7,1	e		21/21	L,K,V
328	S	0.113	5*	-1.777, 1.273	9,2	e		21/21	S,V,T

Peptide 92 (position 338-352)

338	P	0.264	4*	-1.611, 1.569	9,2	e		21/21	T,P,A
339	T	0.398	4*	-1.446, 1.569	8,2	e		21/21	R,T,H
340	A	0.988	3*	-0.956, 2.303	7,1	e		21/21	A,V,T
341	E	0.944	3*	-1.121, 2.303	7,1	e		21/21	G,E,R
342	P	0.148	5*	-1.777, 1.569	9,2	e		21/21	P,S
343	F	-0.049	5*	-1.777, 1.273	9,2	e		21/21	P,F
344	K	-0.559	6*	-2.308, 0.524	9,4	e		21/21	K,T
345	S	-0.647	6*	-2.308, 0.524	9,4	e		21/21	V,S
346	Y	0.088	5*	-1.611, 1.273	9,2	e		21/21	Y,E
347	F	-0.049	5*	-1.777, 1.273	9,2	b		21/21	P,F
348	A	-0.179	5*	-1.777, 1.001	9,3	b		21/21	F,A
349	K	-1.597	8	-3.280,-0.618	9,6	e	f	21/21	K
350	G	-0.741	7*	-2.505, 0.311	9,4	b		21/21	S,G
351	F	-0.165	5*	-1.947, 1.001	9,3	b		21/21	Y,F,S
352	L	-0.772	7*	-2.505, 0.311	9,4	b		21/21	L,F

Peptide 101 (position 392-406)

392	I	0.082	5*	-1.777, 1.273	9,2	e	21/21	T,I,V
393	T	-0.759	7*	-2.308, 0.311	9,4	e	21/21	P,T
394	N	-0.620	6*	-2.308, 0.524	9,4	e	21/21	G,N
395	L	-0.413	6*	-2.123, 0.754	9,3	e	21/21	L,P
396	K	-1.597	8	-3.280,-0.618	9,6	e f	21/21	K
397	T	-0.891	7*	-2.505, 0.109	9,5	b	21/21	I,T
398	I	-0.885	7*	-2.505, 0.109	9,5	b	21/21	I,T
399	N	0.626	4*	-1.446, 2.303	8,1	e	21/21	S,N
400	C	-0.022	5*	-1.777, 1.273	9,2	b	21/21	L,C
401	I	-0.207	5*	-1.947, 1.001	9,3	e	21/21	K,I
402	N	-0.917	7*	-2.505, 0.109	9,5	e	21/21	N,T
403	L	-0.858	7*	-2.505, 0.109	9,5	b	21/21	I,L
404	K	0.182	5*	-1.611, 1.569	9,2	e	21/21	N,K,R
405	A	-0.469	6*	-2.123, 0.754	9,3	b	21/21	A,C
406	L	1.194	2*	-0.792, 2.786	7,1	b	21/21	I,L,S

Peptide 113 (position 464-478)

464	T	-1.559	8	-3.280,-0.449	9,6	e f	21/21	T
465	Y	-1.064	7*	-2.722,-0.083	9,5	b	21/21	F,Y
466	K	-0.169	5*	-1.777, 1.001	9,3	e	21/21	I,K
467	I	-0.316	6*	-1.947, 0.754	9,3	b	21/21	I,P
468	V	-0.344	6*	-1.947, 0.754	9,3	b	21/21	V,G
469	I	-0.885	7*	-2.505, 0.109	9,5	b	21/21	I,T
470	D	0.748	3*	-1.121, 2.303	7,1	e	21/21	Y,E,D
471	K	-1.597	8	-3.280,-0.618	9,6	e f	21/21	K
472	K	-0.169	5*	-1.777, 1.001	9,3	e	21/21	K,I
473	N	1.419	2*	-0.449, 2.786	6,1	e	21/21	N,D,V,T
474	K	0.750	3*	-1.121, 1.911	7,1	e	21/21	R,I,K
475	L	1.426	2*	-0.618, 2.786	6,1	e	21/21	D,Q,M,L
476	N	-0.859	7*	-2.505, 0.311	9,4	e	21/21	N,K
477	D	0.385	4*	-1.446, 1.569	8,2	e	21/21	D,K,N
478	R	1.242	2*	-0.618, 2.786	6,1	e	21/21	D,R,T,N

Peptide 118 (position 494-508)

494	K	-0.315	6*	-1.947, 0.754	9,3	e	21/21	K,G
495	G	-0.534	6*	-2.308, 0.524	9,4	e	21/21	R,G
496	Q	-0.918	7*	-2.505, 0.109	9,5	e	21/21	Q,E
497	S	0.281	4*	-1.446, 1.569	8,2	e	21/21	I,V,S
498	V	-0.250	6*	-1.947, 1.001	9,3	e	21/21	V,R
499	L	0.009	5*	-1.777, 1.273	9,2	e	21/21	K,L
500	K	0.433	4*	-1.446, 1.911	8,1	e	21/21	G,K,R
501	Q	-1.775	9	-3.802,-0.792	9,7	e f	21/21	Q
502	Y	-0.369	6*	-2.123, 0.754	9,3	e	21/21	S,Y
503	K	1.598	2*	-0.449, 3.409	6,1	e	21/21	R,K,V,A
504	T	-0.438	6*	-2.123, 0.754	9,3	e	21/21	L,T
505	E	-0.486	6*	-2.123, 0.524	9,4	e	21/21	E,R
506	I	-0.233	6*	-1.947, 1.001	9,3	b	21/21	I,Q
507	R	0.774	3*	-1.121, 2.303	7,1	e	21/21	Y,K,R
508	I	0.682	4*	-1.121, 1.911	7,1	e	21/21	R,K,I

Peptide 41 (position 814-828)

814	G	-0.282	6*	-1.947, 1.001	9,3	b	21/21	V,G
815	V	-0.627	6*	-2.308, 0.524	9,4	b	21/21	V,S
816	A	-0.586	6*	-2.308, 0.524	9,4	e	21/21	A,E
817	L	-0.358	6*	-2.123, 0.754	9,3	b	21/21	S,L
818	K	-0.559	6*	-2.308, 0.524	9,4	e	21/21	T,K
819	R	-0.574	6*	-2.308, 0.524	9,4	e	21/21	R,G
820	S	-0.647	6*	-2.308, 0.524	9,4	b	21/21	V,S
821	S	-1.068	7*	-2.722,-0.083	9,5	b	21/21	S,A
822	W	-0.089	5*	-1.947, 1.273	9,2	b	21/21	W,L
823	L	0.009	5*	-1.777, 1.273	9,2	e	21/21	K,L
824	I	2.497	1	0.311, 4.321	4,1	e	21/21	V,I,R,T
825	V	0.325	4*	-1.446, 1.569	8,2	b	21/21	V,I,S
826	L	-0.358	6*	-2.123, 0.754	9,3	b	21/21	S,L
827	L	-0.130	5*	-1.947, 1.001	9,3	b	21/21	L,W
828	V	-0.839	7*	-2.505, 0.311	9,4	b	21/21	L,V

Peptide 64-66 (position 952-978)

952	V	-0.409	6*	-2.123, 0.754	9,3	b		21/21	N,V
953	C	-0.285	6*	-1.947, 1.001	9,3	b		21/21	C,V
954	K	-0.332	6*	-2.123, 0.754	9,3	e		21/21	M,K
955	R	0.777	3*	-1.121, 2.303	7,1	e		21/21	R,L,S
956	M	-0.628	6*	-2.308, 0.524	9,4	b		21/21	A,M
957	C	-0.285	6*	-1.947, 1.001	9,3	b		21/21	C,V
958	F	-0.404	6*	-2.123, 0.754	9,3	b		21/21	F,C
959	R	-1.230	8	-2.970,-0.269	9,6	e	f	21/21	R,K
960	A	-0.511	6*	-2.123, 0.524	9,4	e		21/21	A,R
961	T	-0.848	7*	-2.505, 0.311	9,4	b		21/21	M,T
962	M	1.708	1*	-0.269, 3.409	6,1	b		21/21	M,T,V,C
963	E	0.289	4*	-1.446, 1.569	8,2	e		21/21	F,E
964	V	-0.250	6*	-1.947, 1.001	9,3	e		21/21	R,V
965	S	-1.068	7*	-2.722,-0.083	9,5	b		21/21	A,S
966	N	0.065	5*	-1.777, 1.273	9,2	e		21/21	N,T,D
967	R	2.526	1	0.524, 4.321	4,1	e		21/21	V,K,I,R,M
968	A	-0.586	6*	-2.308, 0.524	9,4	e		21/21	A,E
969	L	0.183	5*	-1.611, 1.569	9,2	b		21/21	V,S,L
970	F	0.613	4*	-1.284, 1.911	8,1	b		21/21	L,S,F
971	I	0.497	4*	-1.284, 1.911	8,1	b		21/21	I,S,N
972	R	-0.387	6*	-2.123, 0.754	9,3	e		21/21	K,R
973	S	1.111	3*	-0.792, 2.786	7,1	b		21/21	D,N,S,A
974	I	-0.913	7*	-2.505, 0.109	9,5	b		21/21	L,I
975	I	-0.913	7*	-2.505, 0.109	9,5	b		21/21	I,L
976	N	-0.429	6*	-2.123, 0.754	9,3	b		21/21	I,N
977	T	-0.579	6*	-2.308, 0.524	9,4	e		21/21	R,T
978	T	0.071	5*	-1.777, 1.273	9,2	b		21/21	N,S,T

*Below the confidence cut-off. The calculations for this site were performed on less than 6 non-gaped homologue sequences, or the confidence interval for the estimated score is equal to or larger than 4 color grades.

B.2 GLOBAL STRAINS

Peptide 88 (position 314-328)

314	H	-0.019	5	-0.897, 0.653	7,4	e	138/138	S,T,N,Y,A,G,I,H,R,L,D
315	F	-0.025	5	-0.795, 0.653	6,4	b	138/138	F,Y,K,I,T,E,G
316	N	-0.424	6	-1.200, 0.155	7,5	e	138/138	E,T,G,R,N,K,F
317	E	0.141	5	-0.795, 0.862	6,4	e	138/138	C,N,K,Y,E,F,D,G,I,H
318	V	0.262	5	-0.584, 0.862	6,4	e	138/138	V,Y,E,F,G,I,H,R
319	L	0.663	4	-0.242, 1.396	5,3	b	138/138	E,T,N,Y,I,H,R,L,F
320	Q	0.402	4	-0.474, 1.104	6,3	e	138/138	F,G,L,I,Q,K,N,E
321	F	-0.543	6	-1.302, 0.015	7,5	e	138/138	F,N,V,T,E
322	R	0.425	4	-0.474, 1.104	6,3	e	138/138	G,L,R,I,F,E,V,K
323	T	-0.097	5	-0.897, 0.471	7,4	b	138/138	V,Q,F,E,T,A,L
324	P	-0.084	5	-0.897, 0.471	7,4	e	138/138	Q,P,F,G,L,I
325	G	0.702	4	-0.358, 1.396	6,3	e	138/138	Y,Q,K,N,T,S,F,H,I,G
326	T	-0.480	6	-1.302, 0.155	7,5	e	138/138	T,E,G,F,K,M
327	V	1.256	3*	0.155, 2.330	5,1	e	138/138	N,K,V,P,Y,T,A,G,L,R
328	S	0.712	4	-0.358, 1.396	6,3	e	138/138	F,L,G,P,K,V,T,E,S

Peptide 92 (position 338-352)

338	P	-0.085	5	-0.999, 0.471	7,4	e	138/138	A,H,L,R,D,T,V,P
339	T	1.083	3	0.015, 1.773	5,2	e	138/138	H,R,L,I,G,A,T,P,V
340	A	2.679	1	1.396, 3.692	3,1	e	138/138	D,A,G,R,L,K,V,Y,E,T
341	E	2.443	1	1.104, 3.692	3,1	e	138/138	G,L,R,D,T,S,E,V,P
342	P	-0.734	6	-1.511,-0.242	8,5	e	138/138	T,A,S,G,L,N,P
343	F	0.772	4*	-0.358, 1.773	6,2	e	138/138	F,P,V,L,A,S,T
344	K	-0.696	6	-1.620, 0.015	8,5	e	138/138	A,S,E,T,P,V,K
345	S	-0.469	6	-1.302, 0.015	7,5	e	138/138	H,L,A,G,T,S,E,P,V
346	Y	0.619	4	-0.474, 1.396	6,3	e	138/138	F,A,K,V,P,Y,E,T,S
347	F	-0.173	5	-1.098, 0.471	7,4	e	138/138	K,Q,F,P,E,S,G
348	A	-1.413	7	-2.150,-0.999	9,7	e	138/138	K,F,P,A,S,T
349	K	-0.865	6	-1.620,-0.358	8,6	e	138/138	T,S,L,K,V,P,Y
350	G	-0.474	6	-1.406, 0.155	7,5	e	138/138	E,T,S,Q,P,Y,A,G,F
351	F	0.409	4	-0.584, 1.104	6,3	b	138/138	N,F,Y,P,T,S,A
352	L	-0.600	6	-1.406, 0.015	7,5	b	138/138	F,K,L,E,S,T,A

Peptide 101 (position 392-406)

392	I	-0.707	6	-1.406,-0.242	7,5	e	138/138	S,T,E,P,Q,V,N,I,G
393	T	-0.641	6	-1.406,-0.118	7,5	e	138/138	I,H,A,G,S,T,P,V
394	N	-0.960	7	-1.620,-0.474	8,6	e	138/138	Q,K,N,P,G,T,A
395	L	0.842	4	-0.242, 1.773	5,2	e	138/138	I,H,L,T,N,K,Q,M,V,P
396	K	-0.527	6	-1.302, 0.015	7,5	e	138/138	H,I,T,P,V,K
397	T	-0.988	7	-1.735,-0.474	8,6	e	138/138	S,T,I,M,V,N,P
398	I	-1.143	7	-1.859,-0.691	8,6	e	138/138	N,K,V,T,S,I,L,A,G
399	N	-0.255	5	-1.098, 0.307	7,4	e	138/138	S,I,L,K,N,V,P
400	C	0.019	5	-0.897, 0.653	7,4	e	138/138	G,A,L,R,I,C,K,T
401	I	0.165	5	-0.691, 0.862	6,4	e	138/138	T,M,N,K,P,A,H,I
402	N	-2.012	8	-2.679,-1.735	9,8	b	138/138	K,N,I,G,T,S
403	L	-0.874	6	-1.620,-0.358	8,6	b	138/138	L,I,N,C,Y,P,S,T
404	K	-0.095	5	-0.999, 0.471	7,4	e	138/138	S,N,C,K,P,G,I,R,L
405	A	-0.868	6	-1.620,-0.358	8,6	b	138/138	P,C,N,K,I,L,A,G
406	L	-0.604	6	-1.406, 0.015	7,5	e	138/138	T,S,L,I,N,K,P

Peptide 113 (position 464-478)

464	T	-1.130	7	-1.859,-0.584	8,6	e	138/138	Q,F,T,S,L,I
465	Y	0.239	5	-0.584, 0.862	6,4	b	138/138	V,M,Y,P,H,R,I,F
466	K	-0.749	6	-1.511,-0.242	8,5	e	138/138	K,P,E,T,G,R,L,I
467	I	-0.443	6	-1.200, 0.155	7,5	b	138/138	T,G,I,L,H,V,P
468	V	-0.966	7	-1.735,-0.474	8,6	e	138/138	Y,P,K,V,E,T,A,G
469	I	-0.371	6	-1.098, 0.155	7,5	b	138/138	F,G,H,I,V,K,Y,T
470	D	-0.578	6	-1.406, 0.015	7,5	e	138/138	D,A,I,K,N,Y,T,E
471	K	0.257	5	-0.584, 0.862	6,4	e	138/138	H,I,G,A,F,T,Y,P,V,K
472	K	-0.785	6	-1.511,-0.242	8,5	e	138/138	K,N,V,G,I,H,R,L
473	N	0.236	5	-0.691, 0.862	6,4	e	138/138	F,D,I,G,P,N,V,T,S
474	K	0.488	4	-0.474, 1.104	6,3	e	138/138	I,R,G,Y,D,K,V
475	L	1.536	2	0.307, 2.330	4,1	e	138/138	T,P,Q,M,K,L,R,A,D,F
476	N	0.318	4	-0.584, 1.104	6,3	e	138/138	E,Y,K,N,M,I,R,G,F
477	D	-0.083	5	-0.999, 0.471	7,4	e	138/138	N,K,D,P,T,I,R
478	R	0.693	4	-0.242, 1.396	5,3	e	138/138	Y,P,Q,K,N,I,D,L,R,I,G

Peptide 118 (position 494-508)

494	K	-0.945	7	-1.735,-0.474	8,6	e	138/138	C,K,R,L,E,T,G
495	G	-0.531	6	-1.302, 0.015	7,5	e	138/138	R,I,G,A,V,C,N,E,T
496	Q	-0.361	6	-1.200, 0.155	7,5	e	138/138	L,R,I,A,Q,V,C,E,T
497	S	-0.615	6	-1.302,-0.118	7,5	e	138/138	S,I,L,R,N,K,V
498	V	-0.561	6	-1.302, 0.015	7,5	e	138/138	K,C,V,T,F,I,R,G
499	L	-0.576	6	-1.406, 0.015	7,5	e	138/138	G,L,R,K,N,V,Q,T
500	K	-0.486	6	-1.302, 0.015	7,5	e	138/138	R,I,G,Q,C,N,K,S,E
501	Q	-0.562	6	-1.302, 0.015	7,5	e	138/138	S,R,L,Q,V,C,K
502	Y	-0.254	5	-1.098, 0.307	7,4	e	138/138	A,G,I,R,L,V,Y,S,E
503	K	0.436	4	-0.584, 1.104	6,3	e	138/138	R,L,I,A,V,K
504	T	-1.209	7	-1.859,-0.795	8,6	e	138/138	L,R,G,T,E,Q,K
505	E	-1.203	7	-1.859,-0.795	8,6	e	138/138	V,Q,K,Y,G,E,R
506	I	-0.462	6	-1.302, 0.155	7,5	e	138/138	K,V,Q,Y,E,S,G,I,R
507	R	-0.406	6	-1.200, 0.155	7,5	e	138/138	R,E,T,Y,K,V,Q
508	I	0.099	5	-0.795, 0.653	6,4	e	138/138	V,K,S,T,E,R,L,I,G

Peptide 41 (position 814-828)

814	G	-0.519	6	-1.302, 0.015	7,5	e	138/138	S,E,K,V,I,R,G,W
815	V	0.406	4	-0.474, 1.104	6,3	e	138/138	R,L,I,P,V,E,S,T
816	A	0.174	5	-0.691, 0.862	6,4	e	138/138	L,A,W,S,T,E,P,V,K
817	L	-0.660	6	-1.406,-0.118	7,5	e	138/138	G,S,T,R,L,Q,W
818	K	-0.967	7	-1.735,-0.474	8,6	e	138/138	K,V,L,T,G
819	R	-0.775	6	-1.511,-0.242	8,5	e	138/138	A,S,G,L,R,Q,V
820	S	-0.359	6	-1.098, 0.155	7,5	b	138/138	R,L,T,A,E,S,V
821	S	-1.231	7	-1.995,-0.691	8,6	b	138/138	Q,K,A,S,L
822	W	-0.357	6	-1.200, 0.307	7,4	e	138/138	K,V,W,T,E,R,L
823	L	-0.950	7	-1.735,-0.358	8,6	e	138/138	Q,K,R,L,G,S
824	I	0.126	5	-0.795, 0.862	6,4	e	138/138	Q,V,C,E,T,S,R,I
825	V	-1.545	8	-2.345,-1.098	9,7	b	138/138	V,W,G,A,S,I
826	L	-0.424	6	-1.200, 0.155	7,5	b	138/138	M,V,W,T,E,S,L
827	L	-0.105	5	-0.999, 0.471	7,4	b	138/138	I,L,A,G,W,E,S,K
828	V	-1.135	7	-1.735,-0.691	8,6	b	138/138	V,M,I,R,L,T,S

Peptide 64-66 (position 952-978)

952	V	-0.177	5	-0.999, 0.471	7,4	b	138/138	R,L,M,V,C,K,N,S
953	C	-0.389	6	-1.200, 0.155	7,5	b	138/138	V,M,N,C,K,A,R,L,I
954	K	-0.611	6	-1.406,-0.118	7,5	e	138/138	L,G,A,V,M,C,K
955	R	0.022	5	-0.897, 0.653	7,4	e	138/138	T,S,K,C,V,A,L,R
956	M	-0.661	6	-1.406,-0.118	7,5	b	138/138	G,A,R,V,M,K,C,N
957	C	-0.365	6	-1.200, 0.155	7,5	b	138/138	C,K,M,V,A,S,R
958	F	-1.017	7	-1.735,-0.584	8,6	e	138/138	M,K,N,C,F,G,R
959	R	-0.775	6	-1.511,-0.242	8,5	e	138/138	L,R,A,S,M,V,K
960	A	-0.769	6	-1.511,-0.242	8,5	e	138/138	T,V,M,C,N,R,G,A
961	T	-0.945	7	-1.620,-0.474	8,6	b	138/138	C,M,V,S,T,F,G,L
962	M	0.815	4	-0.242, 1.396	5,3	b	138/138	F,A,R,L,K,N,C,V,M,P,T,S
963	E	0.206	5	-0.691, 0.862	6,4	e	138/138	R,L,A,F,E,K,N,V
964	V	-0.975	7	-1.735,-0.474	8,6	b	138/138	V,M,C,R,A,T
965	S	-1.582	8	-2.150,-1.200	9,7	b	138/138	A,L,I,M,V,K,S,T
966	N	0.173	5	-0.691, 0.862	6,4	b	138/138	T,E,C,N,M,A,I,L,R,D
967	R	2.073	1	0.862, 3.692	4,1	e	138/138	A,R,I,F,E,V,M,K
968	A	-0.241	5	-1.098, 0.307	7,4	e	138/138	V,K,C,S,A,E,R
969	L	0.116	5	-0.795, 0.653	6,4	b	138/138	F,A,R,L,N,C,K,V,M,S
970	F	-0.124	5	-1.098, 0.471	7,4	e	138/138	L,R,F,T,S,N,C,K
971	I	-0.401	6	-1.200, 0.155	7,5	e	138/138	S,N,V,M,A,I,R,F
972	R	0.104	5	-0.795, 0.862	6,4	e	138/138	M,C,K,T,E,L,R,I,A
973	S	0.342	4	-0.584, 1.104	6,3	b	138/138	A,L,I,D,F,S,V,C,N
974	I	0.872	4	-0.118, 1.773	5,2	b	138/138	E,T,S,P,K,I,L,F
975	I	-0.012	5	-0.897, 0.653	7,4	b	138/138	A,R,L,I,F,S,V,K,N
976	N	-1.131	7	-1.859,-0.691	8,6	e	138/138	I,R,A,G,N,T,S,E
977	T	-0.972	7	-1.735,-0.474	8,6	b	138/138	N,V,T,S,I,R,H,A
978	T	0.314	4	-0.584, 1.104	6,3	b	138/138	E,S,T,N,K,L,R,I

*Below the confidence cut-off. The calculations for this site were performed on less than 6 non-gaped homologue sequences, or the confidence interval for the estimated score is equal to- or larger than-4 colour grades.

Link: http://consurf.tau.ac.il/results/1528278990/output.php

APPENDIX G

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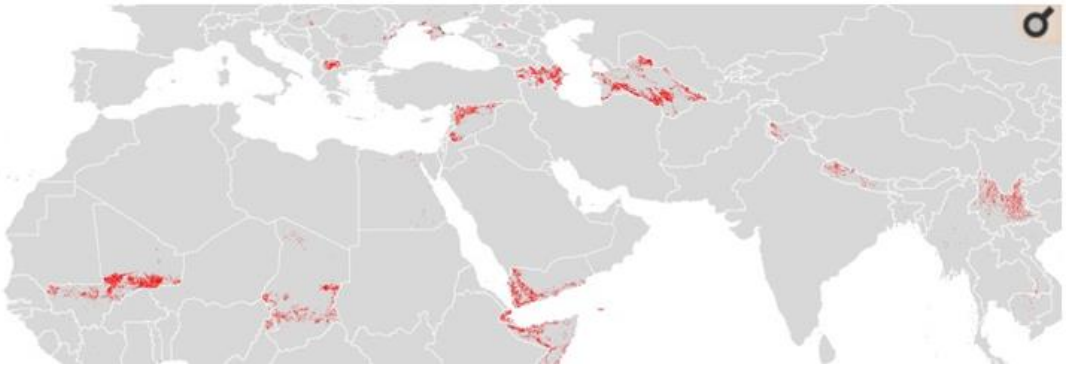
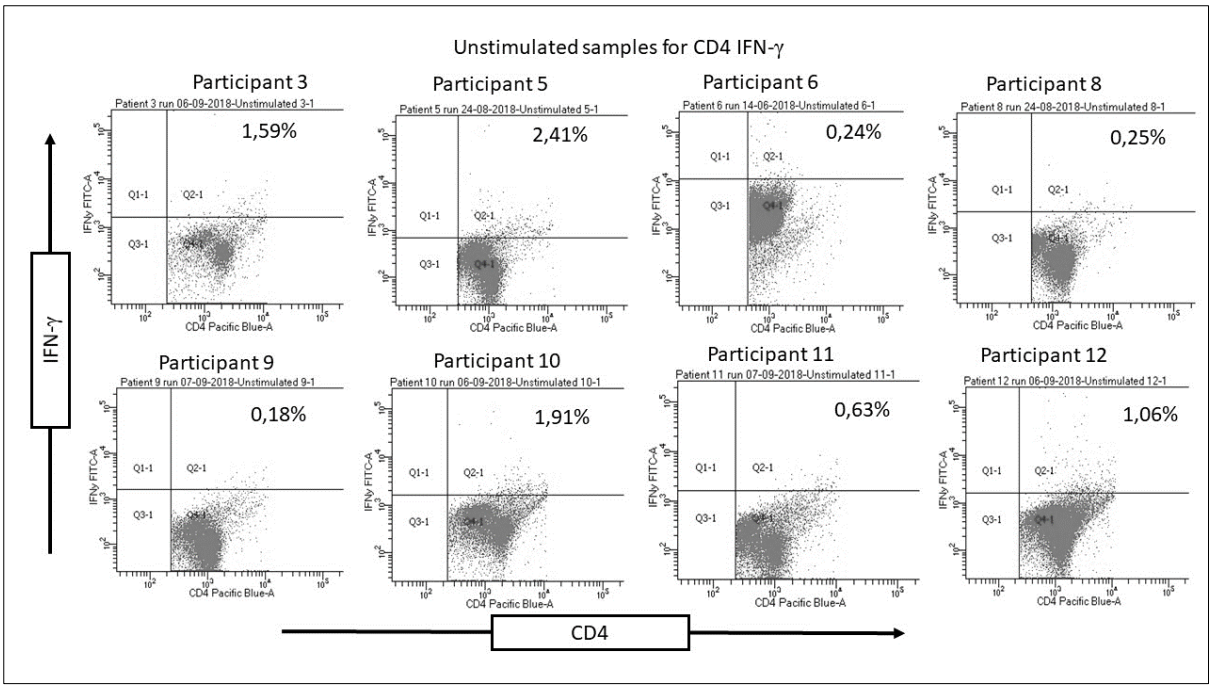
« Prev Figure 5.

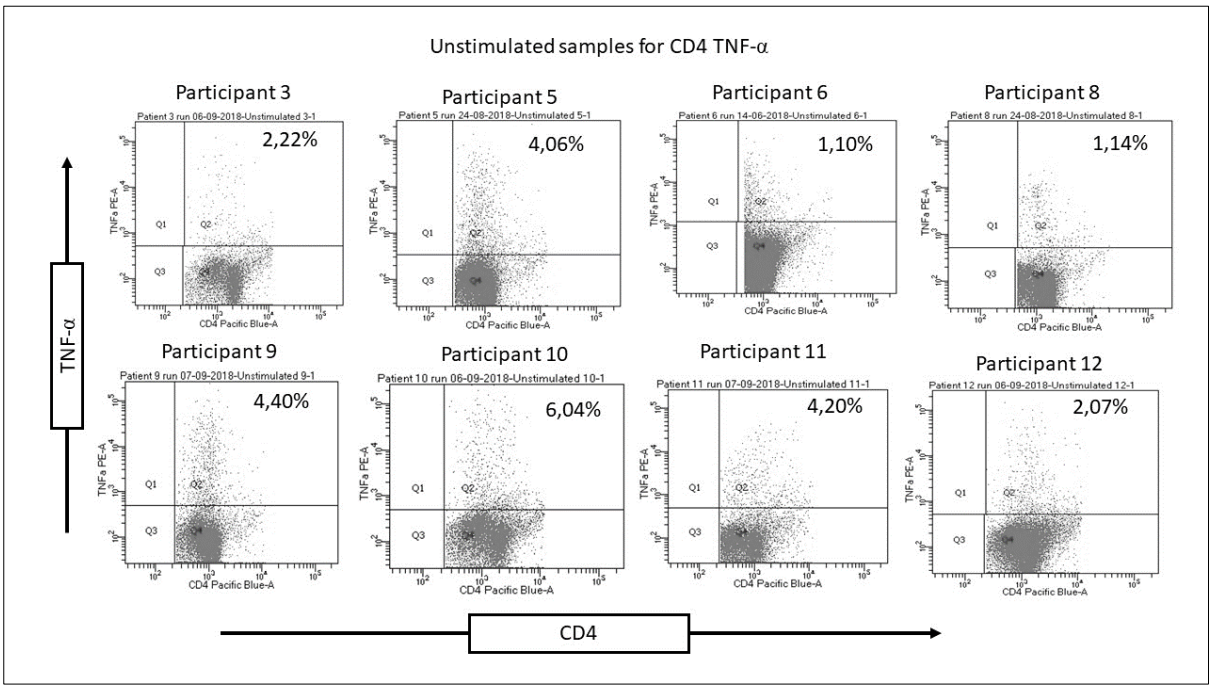
Figure 5.

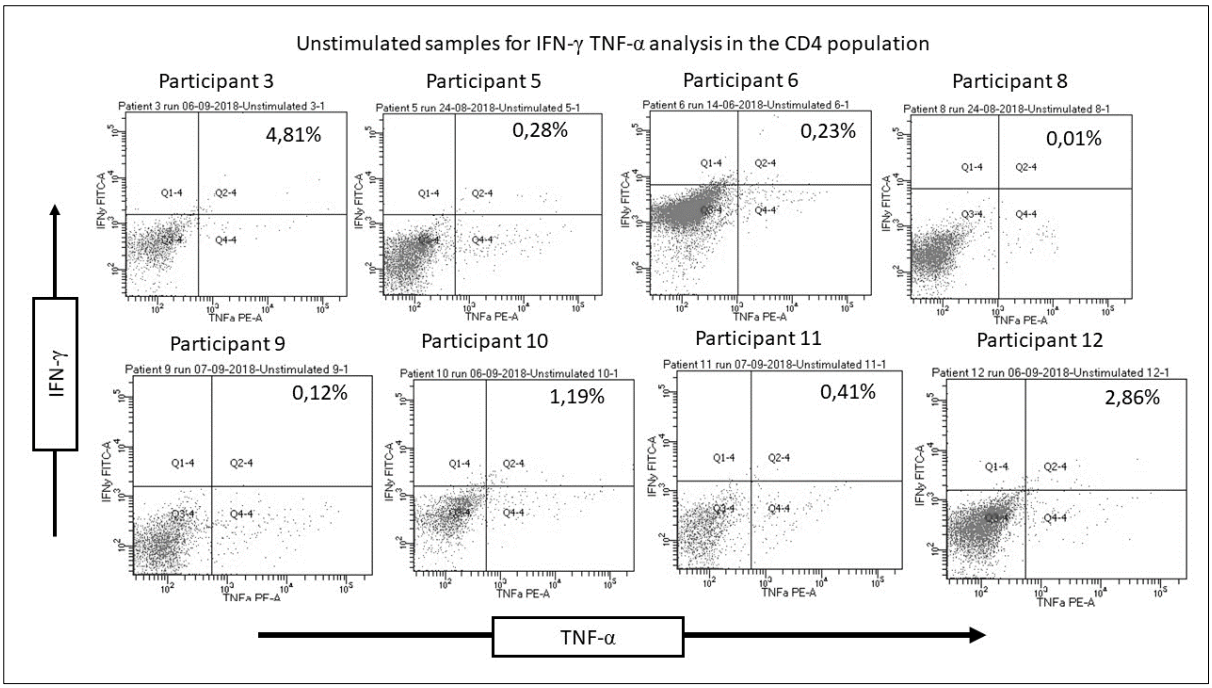


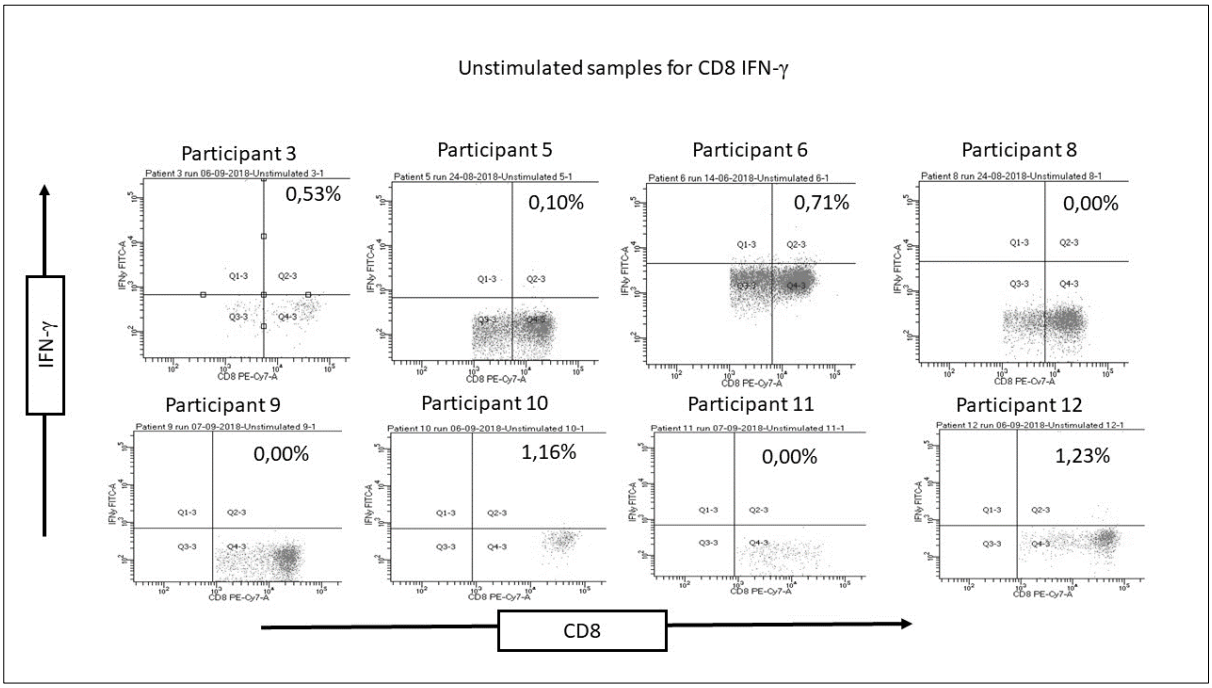
APPENDIX H

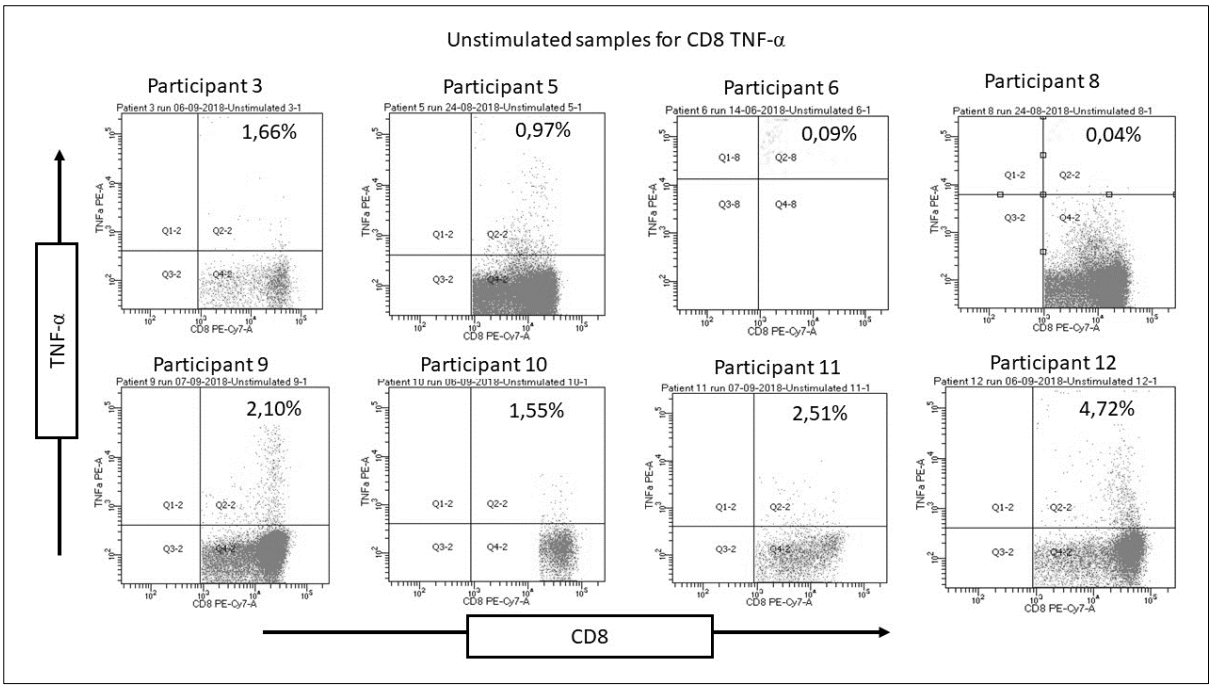
The images below represent the dot plots for the unstimulated samples for each of the participants and the markers analysed. The level of responses for each analysis are indicated as percentage in the appropriate quadrant.

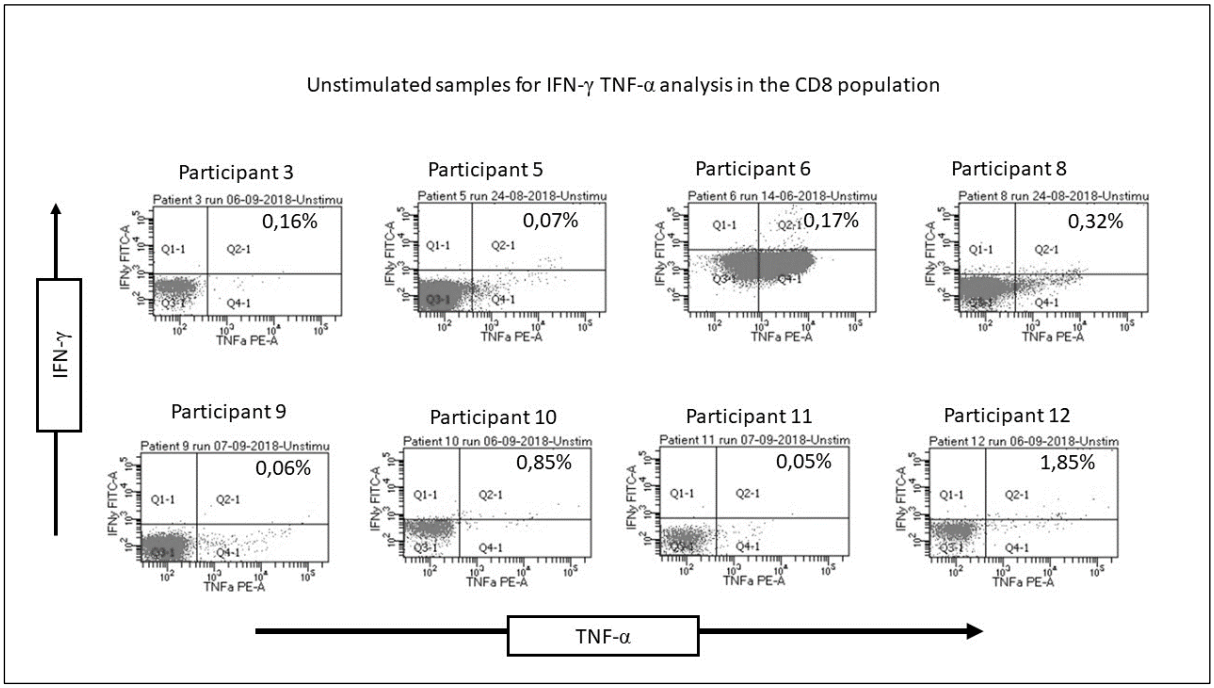


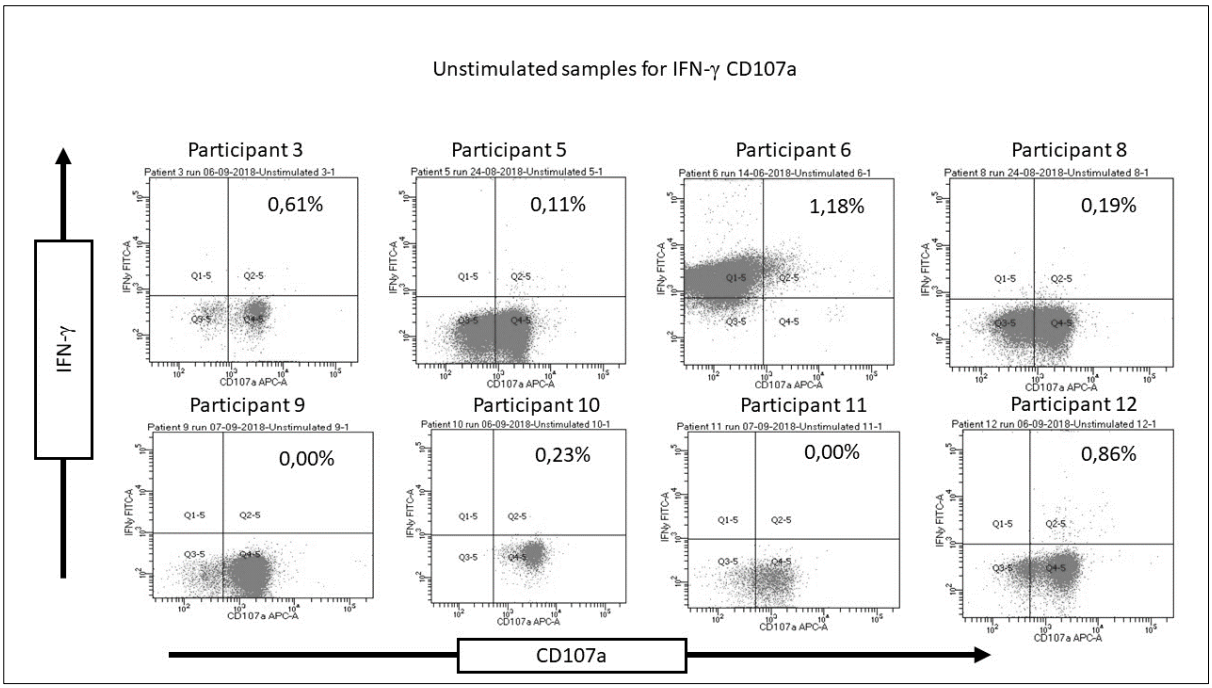



Unstimulated samples for IFN-γ TNF-α analysis in the CD4 population
Participant 3
Participant 5
Participant 6
Participant 8
Participant 9
Participant 10
Participant 11
Participant 12
4,81%
0,28%
0,23%
0,01%
0,12%
1,19%
0,41%
2,86%
IFN-γ
TNF-α


Unstimulated samples for CD8 IFN-γ
Participant 3
Participant 5
Participant 6
Participant 8
Participant 9
Participant 10
Participant 11
Participant 12
0,53%
0,10%
0,71%
0,00%
0,00%
1,16%
0,00%
1,23%
IFN-γ
CD8


Unstimulated samples for CD8 TNF-α
Participant 3
1,66%
Participant 5
0,97%
Participant 6
0,09%
Participant 8
0,04%
Participant 9
2,10%
Participant 10
1,55%
Participant 11
2,51%
Participant 12
4,72%
TNF-α
CD8


Unstimulated samples for IFN-γ TNF-α analysis in the CD8 population
Participant 3
0,16%
Participant 5
0,07%
Participant 6
0,17%
Participant 8
0,32%
Participant 9
0,06%
Participant 10
0,85%
Participant 11
0,05%
Participant 12
1,85%
IFN-γ
TNF-α


Unstimulated samples for IFN-γ CD107a
IFN-γ
CD107a
Participant 3
Patient 3 run 06-09-2018-Unstimulated 3-1
0,61%
Participant 5
Patient 5 run 24-08-2018-Unstimulated 5-1
0,11%
Participant 6
Patient 6 run 14-06-2018-Unstimulated 6-1
1,18%
Participant 8
Patient 8 run 24-08-2018-Unstimulated 8-1
0,19%
Participant 9
Patient 9 run 07-09-2018-Unstimulated 9-1
0,00%
Participant 10
Patient 10 run 06-09-2018-Unstimulated 10-1
0,23%
Participant 11
Patient 11 run 07-09-2018-Unstimulated 11-1
0,00%
Participant 12
Patient 12 run 06-09-2018-Unstimulated 12-1
0,86%
IFNγ FITC-A
CD107a APC-A
Q1-5
Q2-5
Q3-5
Q4-5