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INSTITUTE OF HEALTH SCIENCES

INVESTIGATION OF DELAYED TYPE HYPERSENSITIVITY RESPONSE AGAINST CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS NUCLEOPROTEIN

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Department of Biotechnology

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KIRIM KONGO KANAMALI ATEŞİ VİRÜSÜ NÜKLEOPROTEİNİNE KARŞI OLUŞAN GECİKMİŞ TİP HİPERSENSİTİVİTE CEVABININ ARAŞTIRILMASI

Nesibe Selma ÇETİN

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ÖZET

Kırım Kongo Kanamalı Ateşi Virüsü (KKKAV) bir arbovirüstür ve ixodid kenelerinde bulaşmaktadır. Kırım Kongo Kanamalı Ateşi Virüsünün yol açtığı Kırım Kongo Kanamalı Ateşi (KKKA) ise. Afrika, Asya, Güneydoğu Avrupa ve Ortadoğu'yu kapsayan çok geniş bir coğrafyada, %5 ile %50 arasında değişen fatalite oranına sahip bir hastalıktır. KKKAV *Bunyaviridae* ailesinin bir üyesi olup, *Nairovirus* cinsi altında sınıflandırılmaktadır. Virüs yaklaşık 90-100 nm çapında sferik viriona sahiptir. KKKAV genomu large (L), medium (M), and small (S) olarak adlandırılan üç tane negative sarmallı RNA segmentlerinden oluşmaktadır. RNA'ya bağlı viral RNA ploymerazı L segment tarafından üretilmektedir. S segment nükleoproteinleri, M segment ise Gn ve Gc proteinlerini kodlamaktadır. Viral replikasyonun ve transkripsiyonun konakçı hücrede başlayabilmesi için tüm segmentlerin nücleoproteinler (NP) tarafından sarmalanması gerekmektedir. KKKAV S segmenti yaklaşık 1.7 kb uzunluğunda olup 53 kDa ağırlığındaki nükleoproteini kodlamaktadır. KKKA genellikle hafif ve spesifik olmayan ateşli bir hastalık olarak rapor edilmiştir ancak bazı vakalarda çok şiddetli hemorajik hastalık gelişebilmektedir. Hastalığın 5 ile 14. günleri arasında devam eden hemoraj, çoklu organ bozukluğu ve şok ile sonuçlanabilmektedir. Hücre aracılı immünite kronik, kalıcı ve latent virüs enfeksiyonların seyrinde hayati önem taşımaktadır. Bu yüzden KKKAV enfeksiyonlarının hücresel immünite üzerindeki rolünü aydınlatmak oldukça önemlidir. Bu amaçla, çalışmamızda hayvan modellerinde KKKAV'ye karşı oluşacak DTH reaksiyonunun varlığının araştırılması hedeflenmiştir. Böylelikle KKKAV NP (1449 bç), NP'nin N-terminal parçası, NPNT (387 bç) ve NP'nin C-terminal parçası, NPCT (513 bç) rekombinant olarak prokaryotik ekspresyon sistemlerinde üretilerek DTH üzerindeki etkileri araştırılmıştır. Fare immunizasyon çalışmalarının ardından üç farklı dozda (50 µg/µl, 100 µg/µl, 200 µg/µl) antijen fare ayak tabanlarına enjekte edilmiş ve ayak tabanındaki şişme elektronik kumpas aleti ile ölçülmüştür. 24. saatte oluşan DTH cevabı her bir protein için en yüksek değeri göstermiş ve 72. saate kadar azalmıştır. Antijen enjekte edilen sağ ayak tabanındaki şişlik, kontrol grubuna kıyasla istatistiksel olarak anlamlı bulunmuştur ($p<0,05$). DTH reaksiyonu oluşturmak için 100 µg/µl ideal doz olarak saptanmıştır ve oluşan reaksiyonlar kıyaslandığında NP nin sırasıyla NPNT ve NPCT'ye göre daha antijenik olduğu gösterilmiştir.

ABSTRACT

Crimean–Congo hemorrhagic fever virus (CCHFV) is an arbovirus and transmitted both vertically and horizontally by ixodid ticks. Crimean–Congo hemorrhagic fever (CCHF) caused by CCHFV is reported in a wide geographic range including Africa, Asia, Southeast Europe and Middle East with a fatality rate from 5 to 50 %. CCHFV is a member of the family *Bunyaviridae* and classified in *Nairovirus* genus. Virus has spherical virions and a diameter of approximately 90-100 nm. CCHFV genome is composed of tripartite single-stranded negative RNA segments, called large (L), medium (M), and small (S) segment. RNA dependent viral RNA ploymerase is produced by L segment, S segment encodes nucleoproteins and M segment expresses Gn and Gc. To initiate the viral replication and transcription in the host cell, the segments are needed to be encapsidated by nucleoproteins (NP) .The S segment of CCHFV is approximately 1.7 kb long and encodes 53 kDa nucleoprotein (NP) made up of globular domain with a prominent arm. CCHFV infection is generally reported as a mild, nonspecific febrile illness but in some cases, severe hemorrhagic disease is also developed. Persisting hemorrhage, multi-organ failure and shock result in fatality on day 5-14 of illness. Cell mediated immunity (CMI) is of the essence resulting in chronic, persistent and latent virus infections. Therefore it is essential to clarify the role of cellular immunity in CCHFV infection. In this study, we aimed to investigate the presence of DTH reactions against CCHFV in animal model. Therefore, CCHFV NP (1449 bp), N-terminal part of NP, NPNT (387 bp) and C-terminal part of NP, NPCT (513 bp) were recombinantly produce in procaryotic expression systems and investigated their effect on DTH. Following immunization steps, antigens were injected in three different dosage (50 µg/µl, 100 µg/µl, 200 µg/µl) and footpad swelling was measured by SPI External Electronic Caliper Gages. For each protein DTH response has peaked at 24 hr and decreased by 72 hr. Footpad swelling was statistically significant on right hind pad injected with antigen for each groups, compared to negative control ($p<0,05$). 100 µg/µl was found to be optimum dose for DTH response and considering to DTH response NP was more antigenic than NPNT and NPCT, respectively.

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LIST OF ABBREVATIONS AND SYMBOLS

aa	: Amino acids
APC	: Antigen presenting cells
BCG	: Bacille Calmette–Guérin
BSL-4	: Biosafety level 4
CCHF	: Crimean–Congo hemorrhagic fever
CCHFV	: Crimean–Congo hemorrhagic fever virus
CFA	: Complete Freund Adjuvant
CHF	: Crimean hemorrhagic fever
CMI	: Cell mediated immunity
DHF	: Dengue hemorrhagic fever
DIC	: Disseminated intravascular coagulation
DTH	: Delayed type hypersensitivitiy
DUGV	: Dugbe virus
HAZV	: Hazara virus
HFVs	: Hemorrhagic fever viruses
hpi	: Hours post infection
hRSV	: Human respiratory syncytial virus
HSV-1	: Herpes simplex virus type-1
ICAM1	: Leukocyte adhesion molecule 1
IFA	: Incomplete Freund Adjuvant
IFN-γ	: Interferon gamma
IFNs	:Interferons
ISG15	: Interferon stimulated gene 15
KO	: Knock out
LASV	: Lassa virus
LB	: Luria Bertani
MAbs	: Monoclonal antibodies
MAVS	: Mitochondrial antiviral-signaling protein
MHC	: Major histocompatibility complex
moDC	: Monocyte-derived dendritic cells
MPs	: Macrophages

MV	: Measles virus
NP	: Nucleoprotein
NS	: Non-structural proteins
NSD	: Nairo sheep disease
NSVs	: Negative strand RNA viruses
ORF	: Open reading frame
OTU	: Ovarian tumor
RdRp	:RNA- dependent RNA polymerase
RIG-I	:Retinoic acid-inducible gene I
RNP	: Ribonucleoprotein complexes
RVFV	: Rift Valley Fever Virus
s.c.	: Subcutaneous
SKI-1/S1P	: Serine protease subtilisin-kexin isoenzyme-1/site-1-protease
STAT-1	: Signal transducer and activator of transcription 1
SUMO	: Small ubiquitin-related modifier
TCR	: T-cell receptor
Th1	: Type 1 T-helper
Treg	: Regulatory T cells
Ub	: Ubiquitin
VCAM1	:Vascular cell adhesion molecule 1
VHF	: Viral hemorrhagic fever

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1. INTRODUCTION

1.1. Viral Hemorrhagic Fever

The term of viral hemorrhagic fever (VHF) describes a virus-induced acute febrile, hemorrhagic disease reported from wide areas of the world. This terminology is first used by Russian and Japanese scientists in the 1930's to define typical clinical symptoms including fever and varying degrees of hemorrhage occurring in the Manchurian-Russian-Korean triangle of East Asia [1]. Hemorrhagic fever viruses (HFVs) are enveloped, single-stranded RNA viruses consisting of 14 viruses belong to four viral families: *Arenaviridae, Bunyaviridae, Filoviridae* and *Flaviviridae* (Table 1-1) [2]. Crimean Congo Hemorrhagic Fever Virus (CCHFV) belonging to *Bunyaviridae* family has become more of an issue for Turkey since when the first case of CCHF was announced in 2002 and until now over 6300 cases have been identified [3, 4].

Family, virus	Disease	Distribution	Reservoir	Means of transmission
Arenaviridae				
Lassa	Lassa fever	West Africa	Rodent	Aerosolization of rodent excreta/body fluids and direct contact
Junin	Argentine HF	Argentina	Rodent	
Machupo	Bolivian HF	Bolivia	Rodent	
Guanarito	Venezuelan HF	Venezuela	Rodent	
Sabia	Brazilian HF	Brazil	Rodent?	
Bunyaviridae				
Rift Valley fever	Rift Valley fever	Sub-Saharan Africa, Nile Delta	Wild and domestic mammals	Mosquito; contact with animal carcasses
Crimean–Congo HF	Crimean–Congo HF	Africa, Asia, Crimea, Middle East	Hares, birds, ticks, domestic animals	Tick; contact with infected animals; nosocomial
Hantaviruses*	HF w/renal syndrome	Asia, West and Central Europe	Rodent	Aerosolization of rodent excreta/body fluids and direct contact
	Hantavirus pulmonary syndrome	North and South Americas		
Filoviridae				
Marburg	Marburg HF	Sub-Saharan Africa	Unknown	Person to person nosocomial
Ebola	Ebola HF	Sub-Saharan Africa	Unknown	
Flaviviridae				
Yellow fever	Yellow fever	Tropical Americas, Sub-Saharan Africa	Monkeys	Mosquito
Dengue	Dengue fever, dengue HF, dengue SS	Asia, Africa, Pacific Islands, Americas	Monkeys, humans	Mosquito
Kyasanur Forest disease	Kyasanur Forest disease	India	Monkeys, rodents, shrews	Tick
Omsk	Omsk HF	Russia	Rodents, muskrats	Tick

Table 1-1. The hemorrhagic fever (HF) viruses, their distribution, and principal mode of transmission

1.2. Crimean-Congo Hemorrhagic Fever Virus

Crimean–Congo hemorrhagic fever virus (CCHFV) is an arbovirus and transmitted both vertically and horizontally by ixodid ticks, especially *Hyalomma* spp. ticks and some vertebrates like hares, hedgehogs, rodents, and birds serve as a reservoir for viral replication without displaying any symptoms. Crimean–Congo hemorrhagic fever (CCHF) is caused by CCHFV and occurs sporadically throughout much of Africa, Asia, Southeast Europe and Middle East with up to 50 % fatality rate (approximately %30) [5]. CCHFV is the most geographically disseminated tick-born virus among others and since the last decades the incidence (Fig.1-1) and geographic distribution (Fig. 1-2) of CCHF cases have remarkably increased [5, 6].

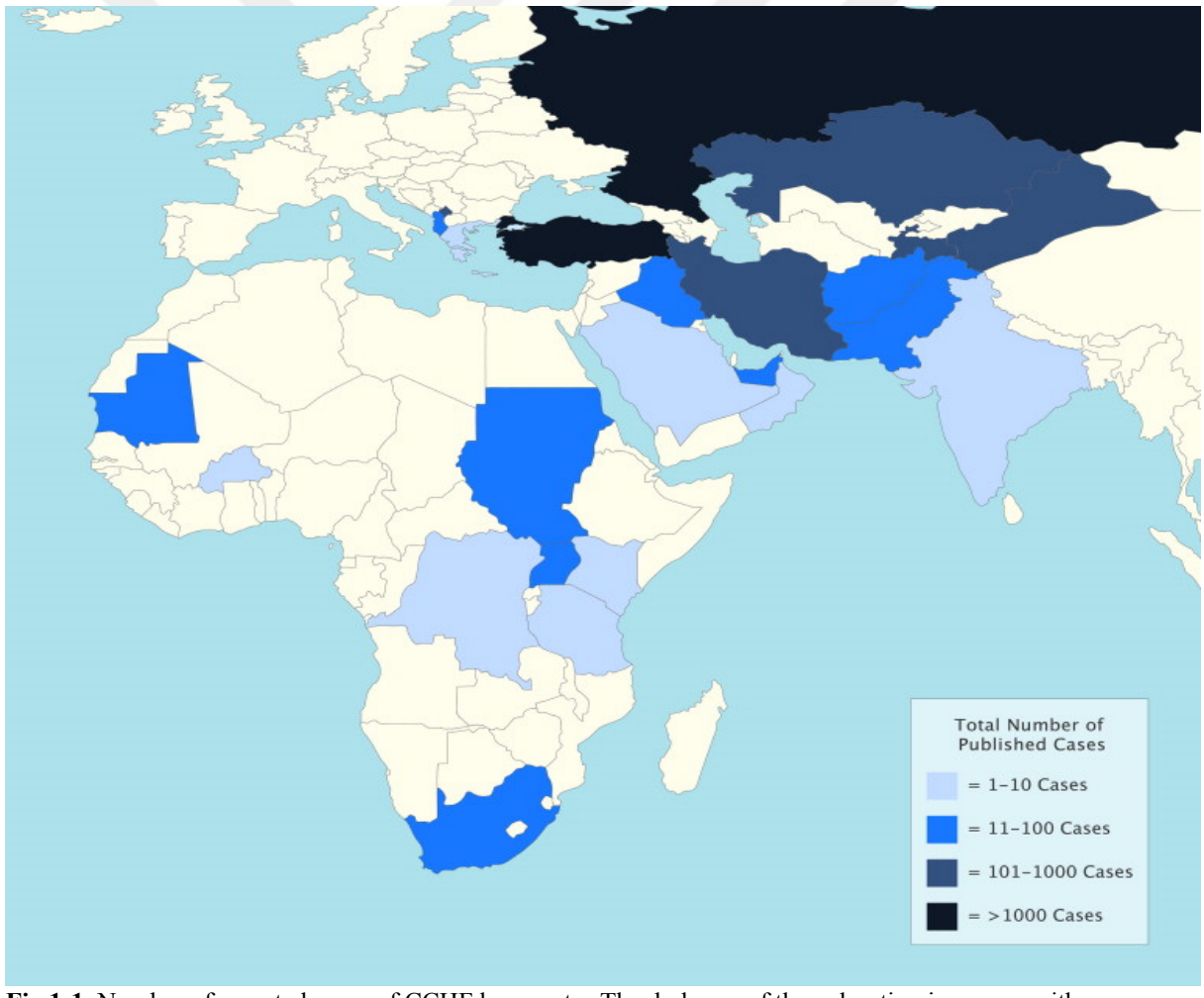


Fig 1-1. Number of reported cases of CCHF by country.The darkness of the coloration increases with case numer as defined by the key (5)

Even though CCHFV has very wide range of genetic variability with 20% sequence differences for the S segment and 31% for the M segment among virus isolates, viruses showing close genotypes can be present in far distinct regions and also distinct subtype of viruses can be found in the same region. The widespread dispersion of CCHFV could be explained by ticks carried on migratory birds. Indeed, birds migrating between Russia and Africa were counted as one of the reasons behind the Turkish epidemics and the other being the international livestock trade [7]. Moreover, broad host range of virus might cause the genetic variability by reassortment of genetic segments during co-infection of ticks or vertebrates, which enables to appearance of novel viruses [5].

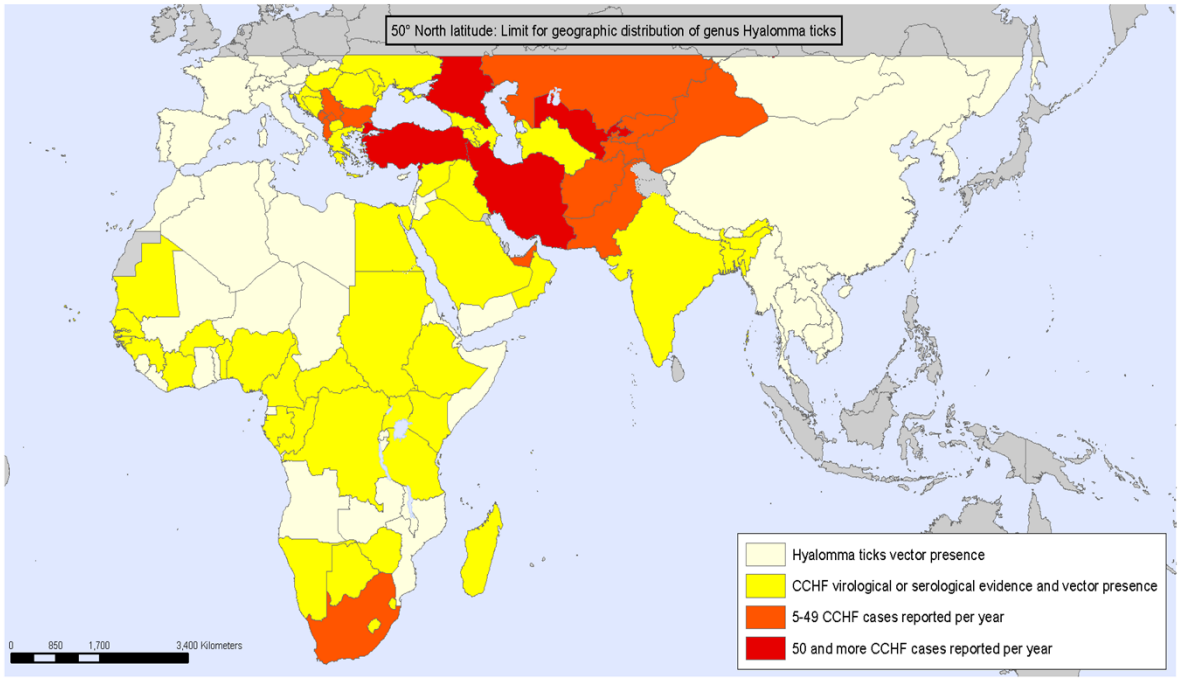


Fig.1-2. Geographic distribution of CCHF according to WHO. Countries in red report more than 50 human cases, and those in orange report fewer than 50 cases. Countries in yellow have not reported human cases, but CCHFV has been isolated, or its presence has been inferred from serologic studies, and a transmission-competent tick vector is also present [5]

1.2.1. History

In 12th century, a persian physician Ismail Jorjani described a hemorrhagic disease caused by a louse or tick parasitizing a blackbird and in his book, Zakhireye Khwarazmshahi he called the disease as “kara khalak“ [8]. *Hyalomma* ticks are common vector for CCHF and frequently found on blackbirds. Currently, this disease considered to be CCHF according to his clinical and epidemiological descriptions [9]. In Uzbekistan, CCHF has also been denominated as khungribta (blood taking), khunymuny (nose bleeding), or karakhalak (black

death) for ages [10, 11] since 16th and 17th centuries the term 'black dead' entered European literature to refer to plague [12].

In modern terminology, CCHF has two historical background; firstly Crimean hemorrhagic fever (CHF) described in 1944-1945 when about 200 Soviet soldiers were infected in Crimea [13, 14]. In 1967, Chumakov and his colleagues in Moscow first isolated CHFV from newborn white mice [15, 16]. Then, isolation of virus by this method from a patient (Drosdov) in Astrakhan Drosdov led to use as a prototype strain for much experimental work all around the world. The researches working on viruses causing hemorrhagic fever discovered that CCHFV was antigenically indistinguishable from the Congo virus (Casals, 1969; Chumakov et al. 1969), originally isolated in 1956 from Congo [17, 18]. Afterwards, this virus began to be called CHF–Congo virus and later it was replaced with Crimean–Congo hemorrhagic fever virus [11].

1.2.2. Classification

CCHFV is a member of the family *Bunyaviridae* and classified in *Nairovirus* genus. *Bunyaviridae* also contains other 4 genus comprising over 350 arthropod-borne viruses; *Orthobunyavirus, Phlebovirus, Hantavirus* and *Tospovirus* [19]. The nairoviruses consist of 34 predominantly tick-borne viruses and are distinguished from other bunyaviruses due to size of their L segments [5]. They are divided into seven serogroups (Table 1-2) and predominantly cause disease in human [20, 21]. CCHFV and Hazara virus (HAZV) are listed in the CCHF serogroup. HAZV was firstly isolated from ticks on wild rodents in Pakistan and until now, there is no report about HAZV to cause disease in humans [22]. Because CCHFV is highly pathogen and require biosafety level 4 (BSL-4) laboratories for experimental works, HAZV can pave the way of research with antiviral agents against CCHFV [23].

1.3. Structure and genome

CCHFV has spherical virions and a diameter of approximately 90-100 nm [24]. Viral envelope has two glycoproteins Gn and Gc in the lipid bilayer and they are recognized by cellular receptors to attach to the host cell surface (Fig 1-3.) [5]. CCHFV genome is composed of tripartite single-stranded negative RNA segments, called large (L), medium (M), and small (S) segment. These segments encode structural proteins: RNA dependent viral RNA ploymerase is produced by L segment, S segment encodes nucleoproteins and M

segment expresses Gn and Gc (Fig. 1-4). Each of the 3 segments contains single open reading frame (ORF) and terminal complementary sequences at 5'-UCUCAAAGA and at 3'AGAGUUUCU. These terminal sequences are conserved in all nairoviruses [25, 26]. This highly conserved ends might

Table 1-2. Nairovirus serogroups and their host tick relationships (81)

Serogroup	Virus	Strain	Isolation	Geographic Location	Disease	Tick Vector
CCHF group (3)[a]				Africa, Asia, Europe		Predom. *Hyalomma*; also *Dermacentor*, *Rhipicephalus*, *Ixodes*, *Haemaphysalis*
	CCHF			Africa, Asia, Europe	Human	Predom. *Hyalomma*; also *Dermacentor*, *Rhipicephalus*
		IbAr10200	1966	Sokoto, Nigeria		*Hyalomma excavatum*
	Hazara			Asia		*Ixodes redikorzevi*
		JC 280	1964	Kaghan Valley, Gitidas, Hazara District, Pakistan		*Ixodes redikorzevi*
Dera Ghazi Khan group (6)				Africa, Asia, Australia		Predom. *Argas*
	Abu Hammad			Africa, Asia		*Argas hermanni*
		Eg Art 1194	1971	Abu Hammad Shargiya, Egypt		*Argas hermanni*
	Abu Mina			Africa		*Argas streptopelia*
		Eg An 4996	1963	Bahig, Egypt		*Argas streptopelia*
Hughes group (10)				Americas, Europe, Africa, Asia		Predom. *Carios*; also *Argas*
	Farallon			North America		*Carios capensis*
		USA Ar 846	1964 or 65	Farallon Islands, California		*Carios capensis*
	Punta Salinas			Peru, Tanzania	Human	*Carios amblus*, *Argas arboreas*
		Cal. Ar 888	1967	Punta Salinas, Huacho, Peru		*Carios amblus*
	Raza			North America		*Carios denmarki*
		829	1962	Raza Island, Gulf of Mexico, Mexico		*Carios denmarki*
NSD group (2)				Africa, Asia		*Amblyomma*, *Boophilus*, *Hyalomma*, *Rhipicephalus*, *Haemophysalis*
	Dugbe			Africa	Human	Predom. *Amblyomma*; also *Boophilus*, *Hyalomma*, *Rhipicephalus*
		ArD44313[b]	1985	Bouroufaye, Senegal		*Amblyomma variegatum*
	NSD			Africa, Asia	Human, livestock	*Rhipicephalus appendiculatus*, *Haemophysalis intermedia*
		RV082	?	Kenya		*Rhipicephalus appendiculatus*
		Ganjam IG 619	1954	India		*Haemophysalis intermedia*
Qalyub group (4)				Africa		*Ornithodoros*
	Bandia			Senegal		*Ornithodoros sonrai*
		IPD/A 611	1965	Bandia Forest, Thies Region, Senegal		*Ornithodoros* sp.
	Qalyub			Egypt		*Ornithodoros erraticus*
		Eg Ar 370	1952	Qalyub, Qalyubiya Province, Egypt		*Ornithodoros erraticus*
Sakhalin group (7)				Europe, N. America, Asia, Australia		*Ixodes*
	Tillamook			North America		*Ixodes uriae*
		RML 86	1970	Oregon, USA		*Ixodes uriae*
Thiafora group (2)				Europe, Africa		unknown
	Erve			Europe	Human	unknown
		An 221	1982	France		unknown

CCHF—Crimean Congo hemorrhagic fever virus; NSD—Nairobi sheep disease.

[a] Number of viruses within the group is indicated in parentheses.

[b] Dugbe virus L sequence was obtained from GenBank entry NC_004159.

form predicted secondary structures (panhandle or corkscrew model) due to intrastrand

basebairs which provides functional promoter region for the binding of viral polymerase, and form of non-covalent closed circular structure of segments [5, 25, 27]. To initiate the viral replication and transcription in the host cell, the segments are needed to be encapsidated by nucleoproteins (NP) and by the RNA- dependent RNA polymerase (RdRp) [5].

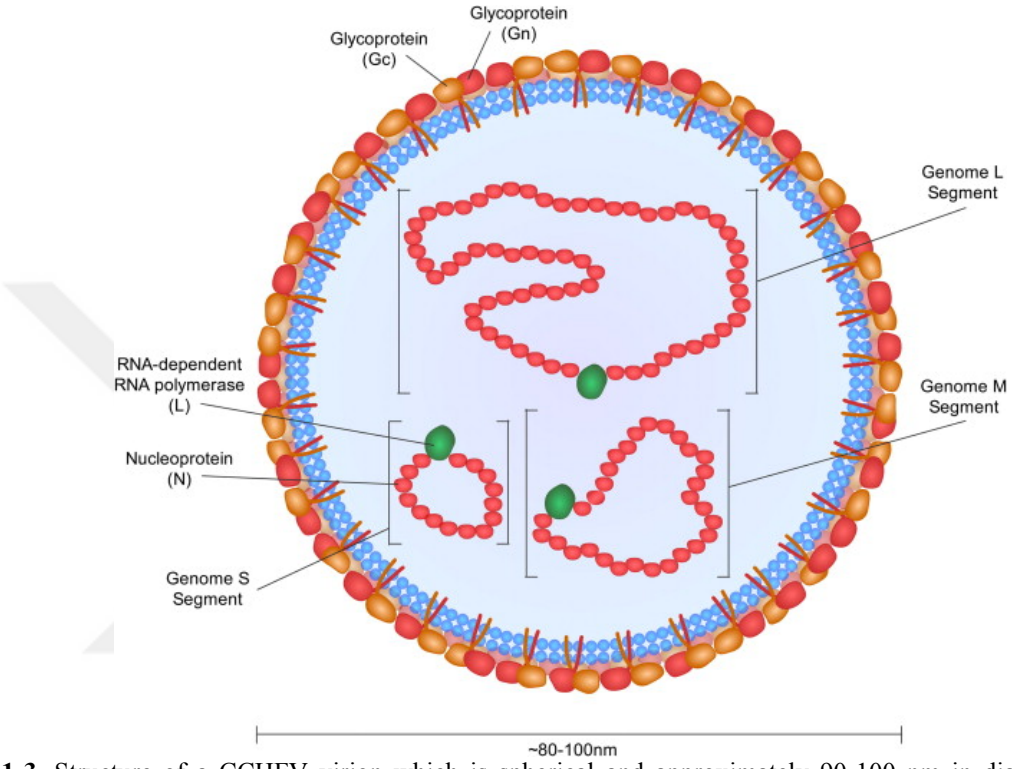


Fig.1-3. Structure of a CCHFV virion which is spherical and approximately 90-100 nm in diameter. Virion consists of three single stranded negative RNA genome segment surrounded by nucleoproteins (NPs) complexed with the RNA dependent RNA polymerase (RdRp) and it is encapsidated by glycoproteins (Gn and Gc) in the lipid bilayer [5]

Also one or two non-structural proteins (NS) are expressed from some viruses in *Bunyaviridae* which called NSs and NSm encoded by S and M segment, respectively. They can act as an interferon antagonist, a regulator of replication and determinant of host range [28]. Previously it was thought that, unlike other bunyaviruses CCHFV does not express any NSs or NSm but recent studies reports that NSm is cleaved during maturing process of glycoproteins encoded by M segment of CCHFV [29-32]. However, the function of this non-structural protein NSm in CCHFV is unkown [33]. Besides that S segment of CCHFV encodes also a non-structural protein NSs in positive sense [34]. A recent study of Barnwal et al. indicates that NSs induces apoptosis as activated by caspases due to mitochondrial membrane permeabilization [35].

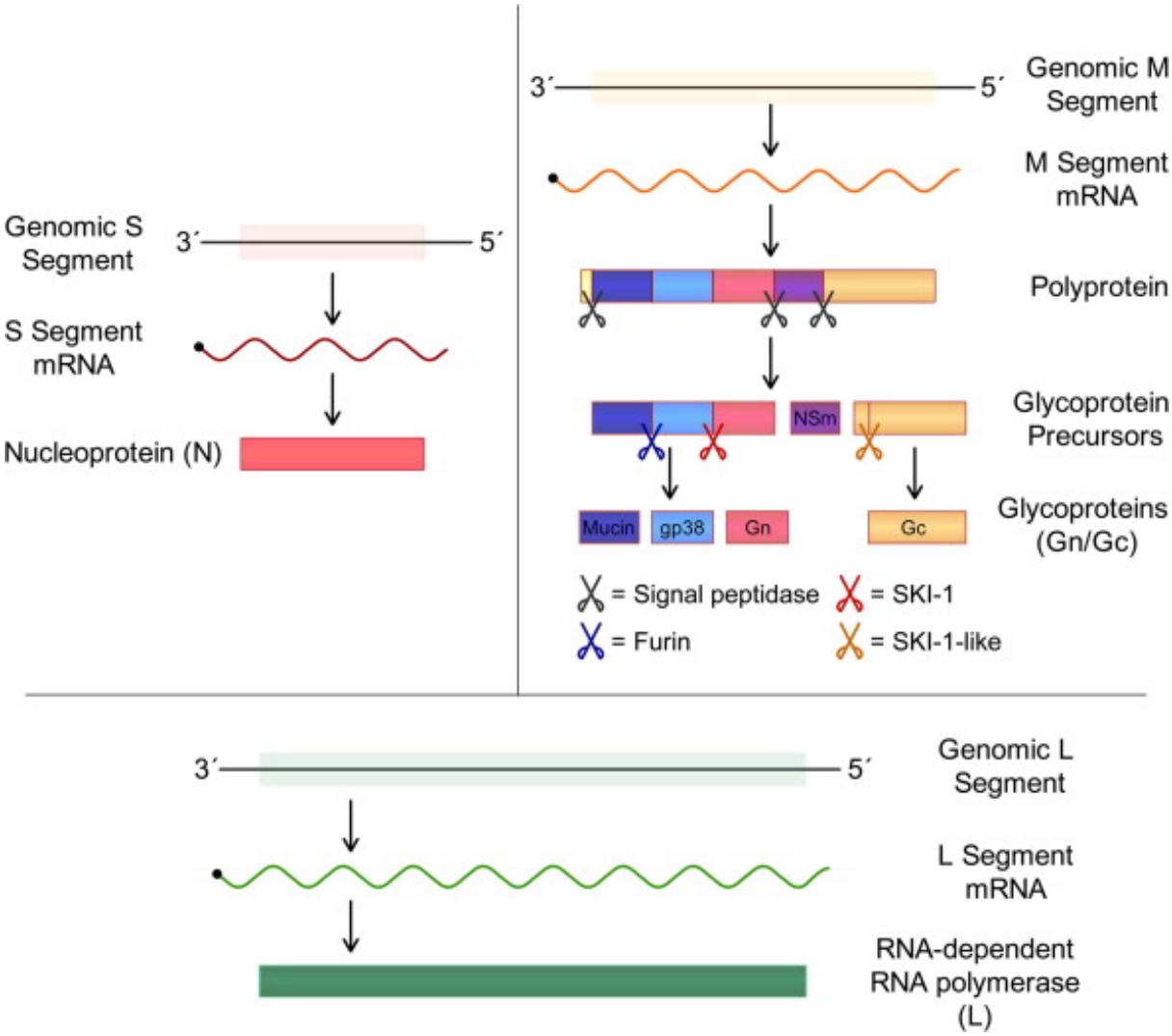


Fig.1-4. CCHFV segment and their encoded proeins [5]

1.3.1. S segment

The S segment of CCHFV is approximately 1.7 kb long and encodes 53 kDa nucleoprotein (NP) made up of globular domain with a prominent arm comprising two long alpha helices containing a conserved caspase-3 cleavage site (Fig. 1-5) [35, 36]. The structural function of NP is the encapsidation of viral RNA due to homo-oligomerization to form ribonucleoprotein complexes (RNP). In addition to this, globular region of NP with viral polymerase (L protein) is critical for viral replication [5, 31]. Besides, the CCHFV polymerase activity is enhanced during viral vRNA transcription by the disruption of the caspase-3 cleavage site [37]. NP interacts also with actin which provides perinuclear localization of NP [38]. However, it is known that the interaction of the viral proteins with is actin filaments enhance replication of

the virus as is the case in paramyxoviruses such as Newcastle disease virus and Sendai virus [31, 39]

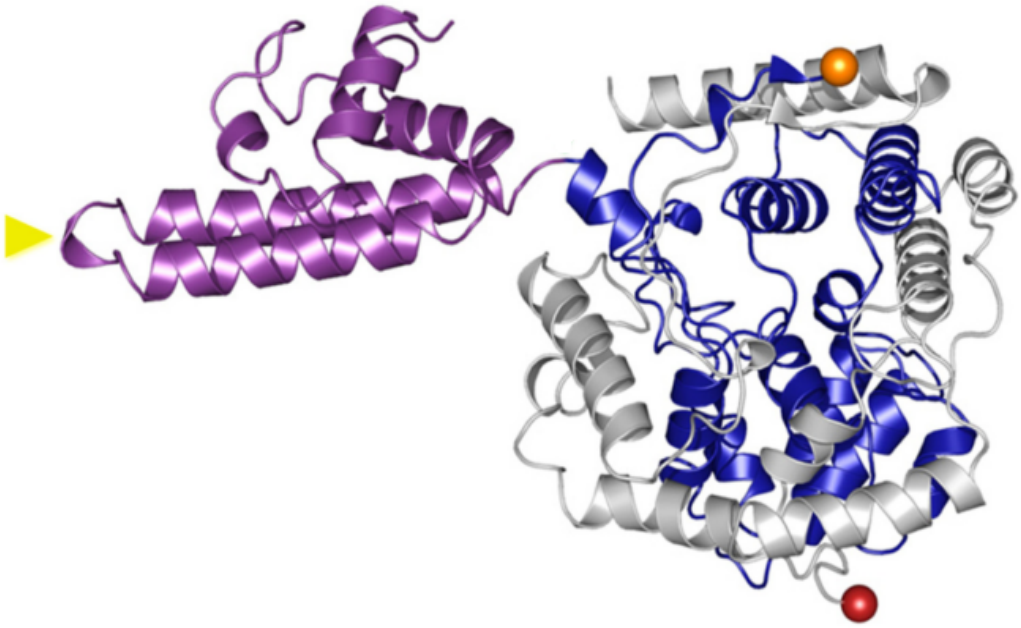


Fig.1-5. Ribbon model of the CCHFV NP protein. Helices are coloured as gray for N-terminal portion, dark blue for C-terminal portion of globular domain and purple for protruding arm. The red sphere indicates the N terminus, and the gold sphere indicates the C terminus. Yellow arrow shows the caspase-3 DEVD cleavage motif [35]

Interestingly, after structural analysis for CCHFV NP, it is concluded that CCHFV NP resembles Lassa virus (LASV) NP more than other bunyaviruses [35]. Furthermore, it is exhibited by phylogenetics analysis of L and NP sequences among segmented negative-strand RNA viruses (sNSVs) that nairoviruses are more closely related to arenaviruses than to any other bunyaviruses and as a result revision of current sNSV taxonomy might be essential [35].

1.3.2. M segment

As with other *Bunyaviridae*, CCHFV M segment is approximately 5.4 kb long [40] and translated into a single polypeptide of 1689 amino acids (aa) in length which is cotranslationally cleaved by the signal peptidase and post-transcriptionally modified in Golgi to form the type I transmembrane glycoproteins Gn (37 kDa) and Gc (75kDa), named for their relative proximity to the respective ends of the polyprotein, and a nonstructural protein NSm [29, 33]. Generation of the mature glycoproteins occur with a series of very complex

endoproteolytic events. Maturing process of 225 kDa M polyprotein begins with its cotranslational cleavage into the glycoprotein precursors PreGn (140 kDa) and PreGc (85 kDa) presumably by signal peptidase [29, 33]. N terminal of both Gn and Gc is generated by the serine protease subtilisin-kexin isoenzyme-1/site-1-protease (SKI-1/S1P) cleavage in the ER/cis-Golgi [41, 42]. After that, mucin-GP38 domain, named as GP85 when it is in monomer form and as GP160 when alternatively secreted as dimer, is cleaved by furin into GP38 and a mucin like domain [29, 41, 42] (Fig. 1-6). Mucin like domains are very unique for genera *Nairovirus* and notably hypervariable and rich in serine, threonine, and proline amino acids; heavily O glycosylated, which is similar to the mucin-like domain of the Ebola virus glycoprotein [43-45].

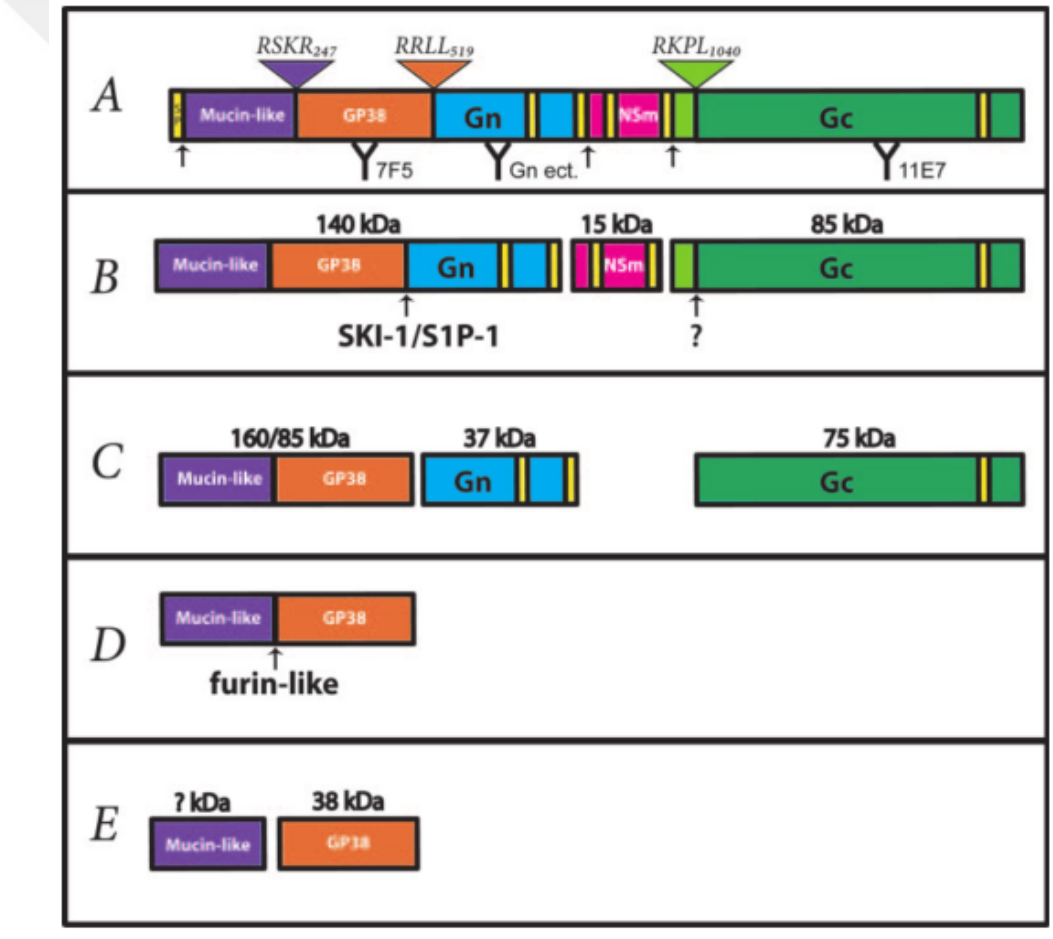


Fig. 1-6. Proteolytic process of CCHFV M segment. A) M polyprotein domains are coloured yellow for potential transmembrane domains; purple for mucin-like domain; orange for GP38;blue for Gn; pink for NSm and green for Gc. Signal peptidase cleavage sites are indicated by black arrows and furin-like (RSKR247), SKI-1/S1P (RRLL519), and SKI-1/S1P-like (RKPL1040) cleavage sites are illustrated by inverted triangles. B) PreGn and PreGc is cleaved by SKI-1/S1P and PreGc convertase (indicated arrows) the early secretory pathway. C) Generation of a nonstructural mucin-like GP38 protein of either 160 or 85 kDa, and the structural glycoproteins Gn (37 kDa) and Gc (75 kDa). D) The mucin-like GP38 domain. E) Furin-like enzyme cleavage of GP85/GP160 into GP38 glycoprotein (38 kDa) and a mucin-like protein of unknown mass (? kDa) [29]

Secretion of Gn and Gc in trans-golgi is an interdependent process. Gn is indispensible for transportation of Gc from ER to golgi due to presumably having golgi localization signal on it [46-48] and also has a chaperone-like function for correctly folding of Gc [49]. However, Bertolotti-Ciarlet et al. has showed that only monoclonal antibodies (MAbs) against Gc, but not against Gn, were able to neutralize virus *in vitro*, suggested to be more important for infection [46]. Interestingly, a recent research has indicated that CCHFV Gn contains dual CCHC-type zinc finger motif on its C- terminal cytoplasmic tail, which can bind viral RNA [5, 50]

1.3.3. L segment

Like all nairoviruses, CCHFV L segment is about 12.2 kb in length, aproximately twice the size of those of other bunyaviruses. CCHFV L segment has single open reading frame comprising of 12164 nucleotides and encodes 3944 amino acid polyprotein, viral RNA-dependent RNA polymerase (RdRp), which has 62% nucleotide and amino acid identity with nairovirus Dugbe virus (DUGV) [40, 51]. L segment contains a high conservative ovarian tumor (OTU)-like cysteine protease domain domain (residues 35 to 152), a zinc finger type C2H2 domain (residues 606 to 632) and a leucine zipper motif (residues 1386–1407), suggests that CCHFV L polyproteins may have other functions than being a polymerase. In addition to this, a sequence in the CCHFV L is indicated as highly similar to a eukaryotic DNA topoisomerase I active site motif, like in DUGV L at amino acid position 76-94 (Fig. 1-8) [51].

Viral OTU domains are present in eukaryotic, viral and bacterial proteins and functions as a core element of a cystein protease which can hydrolyze ubiquitin (Ub) and interferon stimulated gene 15 (ISG15) conjugates form target protein [52, 53]. Viruses capable to use this activity can evade two different cytokine pathways, IFNαβ and TNFα, in innate immunity as well as adaptive cellular immunity during MHC I and II antigen presentation [54, 55], TLR/IL1 signaling [56] and induction of type I IFN by the cellular viral sensor retinoic acid-inducible gene I (RIG-I) [53, 57]. Predictably, a study of Frias-Staheli et al. is reported that CCHFV L protein can deconjugate Ub and ISG-15 from cellular target proteins as a viral immune evasion mechanism (Fig. 1-7) [53]. Nevertheless, a minigenom replication assay is indicated that OTU domain containing CCHFV L protein does not

provide any evidence of autoproteolysis to generate additional protein products and OTU protease activity is dispensible for virus RNA replication [52].

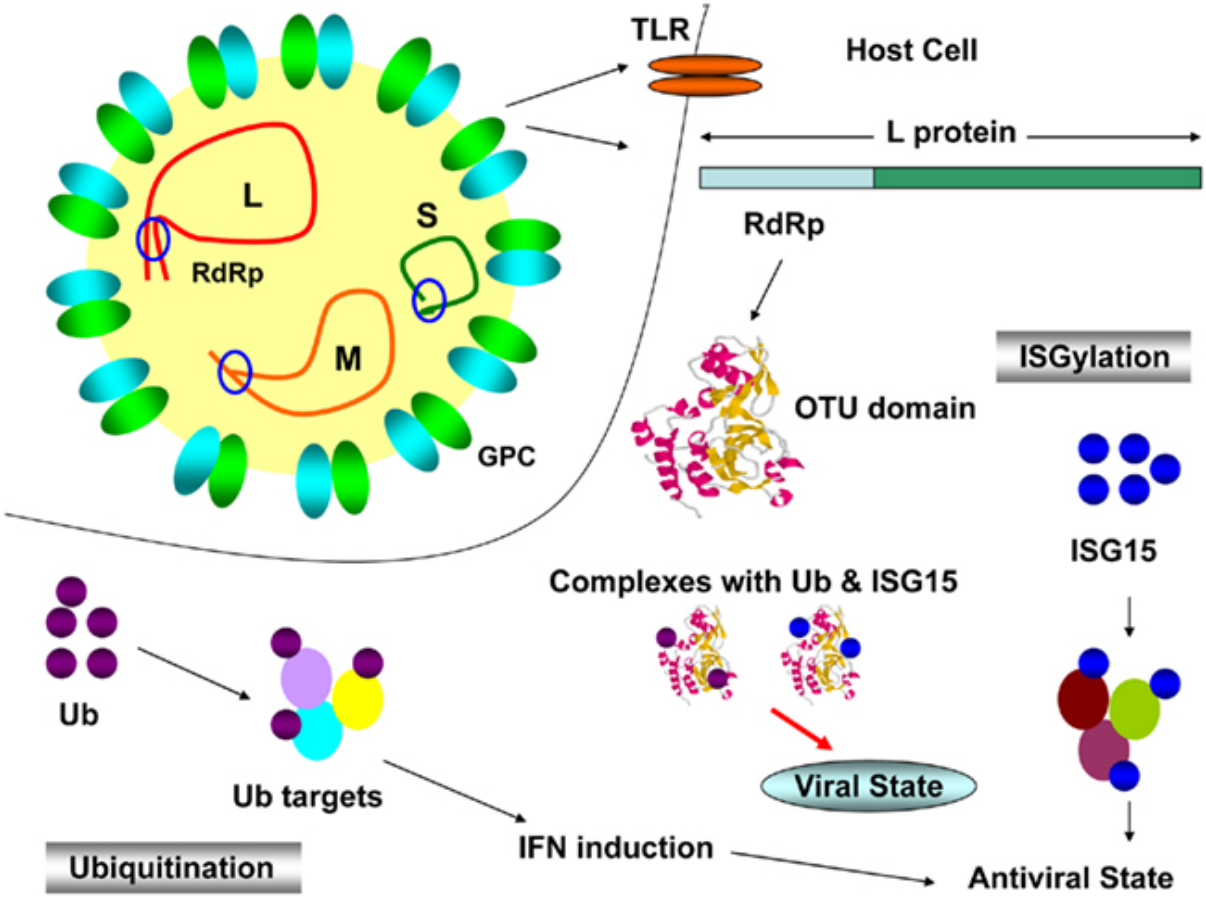


Fig. 1-7. OTU domain of CCHFV L protein deconjugates Ub and ISG-15 from cellular target proteins as a viral immune evasion mechanism [58]

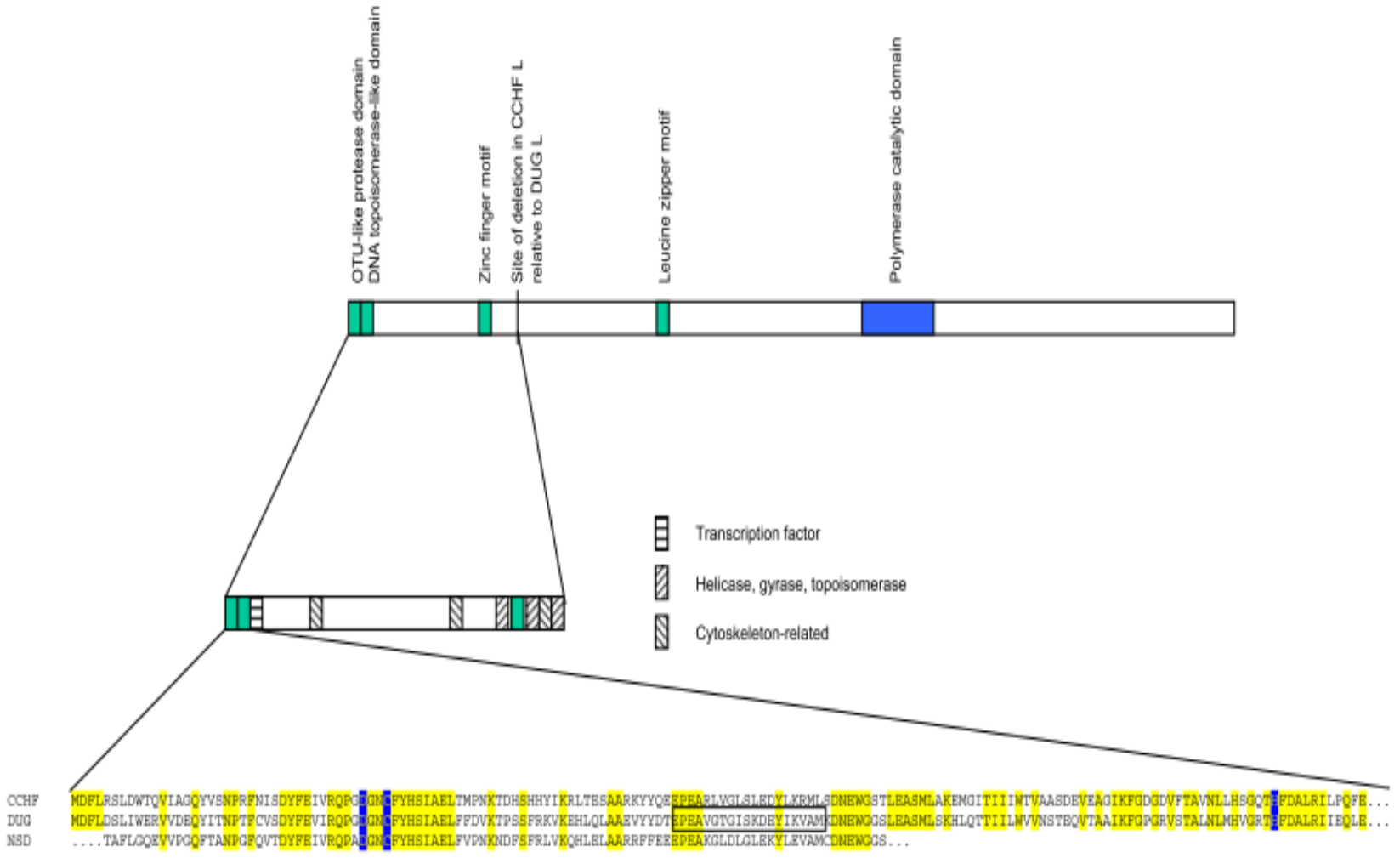


Fig. 1-8. Sequence of CCHF L RNA segment-encoded proteins. The upper panel represents the predicted location of various sequence motifs within the entire CCHF L segment. In the lower panel, the alignment of the amino terminus of the L encoded protein of CCHF, Dugbe, and NSD (partial sequence) viruses are compared. Conserved positions are coloured in yellow. The highly conserved D, C, and H residues (blue) believed to constitute the OTU-like protease core amino acid

motif. The boxed region within the DUG virus sequence indicates the position of the predicted topoisomerase I active site motif which is also seen in both CCHF and NSD viruses [51]

1.4. Life cycle

The knowledge about cellular entry mechanism of CCHFV is currently deficient but Xiao and his collegues has identified that 180-300 amino acid residues of Gc has significant receptor binding capacity and the human cell surface nucleolin as a putative CCHFV entry factor (Fig. 1-9a) [59]. Simon and his collegues has indicated that CCHFV utilizes clathrin-dependent endocytosis, requires low pH for productive infection like other Bunyaviruses [60] and cholesterol in events following CCHFV binding and internalization [61]. Internalization of virus within the clathrin-coated vesicles occurs by fusing with endosomes and then lysosomes. Acidic environment in lysozyme induces conformational changes, which allow the virus to fuse with endosomal membranes (Fig. 1-9b) [62]. Moreover, Simon and his collegues has found that virus internalization was dependent on intact microtubules, and depolymerization of microtubules inhibited the expression of CCHFV RNA following progeny virus production. Within the first hour after infection, CCHFV is delivered by microtubules to the cellular sites where the viral transcription and replication will take place [63].

Replication starts with production of mRNA from negative sense viral RNA segment by virion associated viral polymerase in cytoplasm of host cell [64]. According to the study of Simon et al. about the kinetics of CCHFV RNA expression and synthesis in vitro, CCHFV positive sense RNA (mRNA and copy RNA) are principally increased during the first 6 h of infection while negative sense RNA (vRNA) is kept constant till vRNA levels increased on 16 h post infection (hpi) in Vero E6 cells [63].

Bunyaviruses utilize generally same cap snatching mechanism for transcription with those of other arenaviruses and orthomyxoviruses [65] through approximately 11-15 nt capped RNA fragments used as primer to start transcription of nonpolyadenylated mRNA on the three viral RNA segments [37, 66]. CCHFV NP has unique endonuclease activity unlike other members of family. Even so, monomer form of CCHFV NP shows extremely low or no cap-binding affinity *in vitro*, compared to LASV NP which has highly structural smilarity with CCHFV [67]. Although cap-snatching mechanism of CCHFV is still unknown, it is reported that CCHFV NP superhelical polymer presents these RNA primer fragments, which leads to conformational change of subunits following liberation of monomeric NP. Thus, vRNA would be prepared for transcription and replication [37].

It is convinced that NP plays major role in viral replication. In order to start CCHFV viral replication, both interaction of NP with viral polymerase and enough NP should be present to encapsidate viral mRNA and genomic RNA to prevent degredation [68]. During microtubule dependent replication of CCHFV, actin filaments are involved in viral NP internalization to perinuclear region [38].

During the maturation of virions in the family of *Bunyaviridae*, the S, M, and L RNPs are packaged intracellularly into budding virions through cisternae within the Golgi structure [68-70]. In infected cells, N and L are translated on free ribosoms in cytosol and internalized to a non-Golgi perinuclear compartment via actin dependent mechanism [38, 71]. On the other hand, CCHFV NP proteins are found in Golgi at the late stage of viral assembly [68]. Syntesis of CCHFV glycoproteins is a multistep process. Primarily, a polyprotein from whole M segment is produced in ER. After SKI-1/S1P cleavage process, Gn is transported to Golgi complex by its Golgi localization signal in hydrophobic region of cytoplasmic tail while Gc is retained in ER until Gn is co-expressed, suggesting that the proper transport of two virion-forming glycoproteins is important for virus replication [29, 47]. Final assembled proteins in Golgi generate mature virions which are transported to the cell surface and released by secretory pathway toinfect other cells (Fig. 1-9j) [64]. Involvement of microtubules affects CCHFV entry, replication, assembly and egress [63].

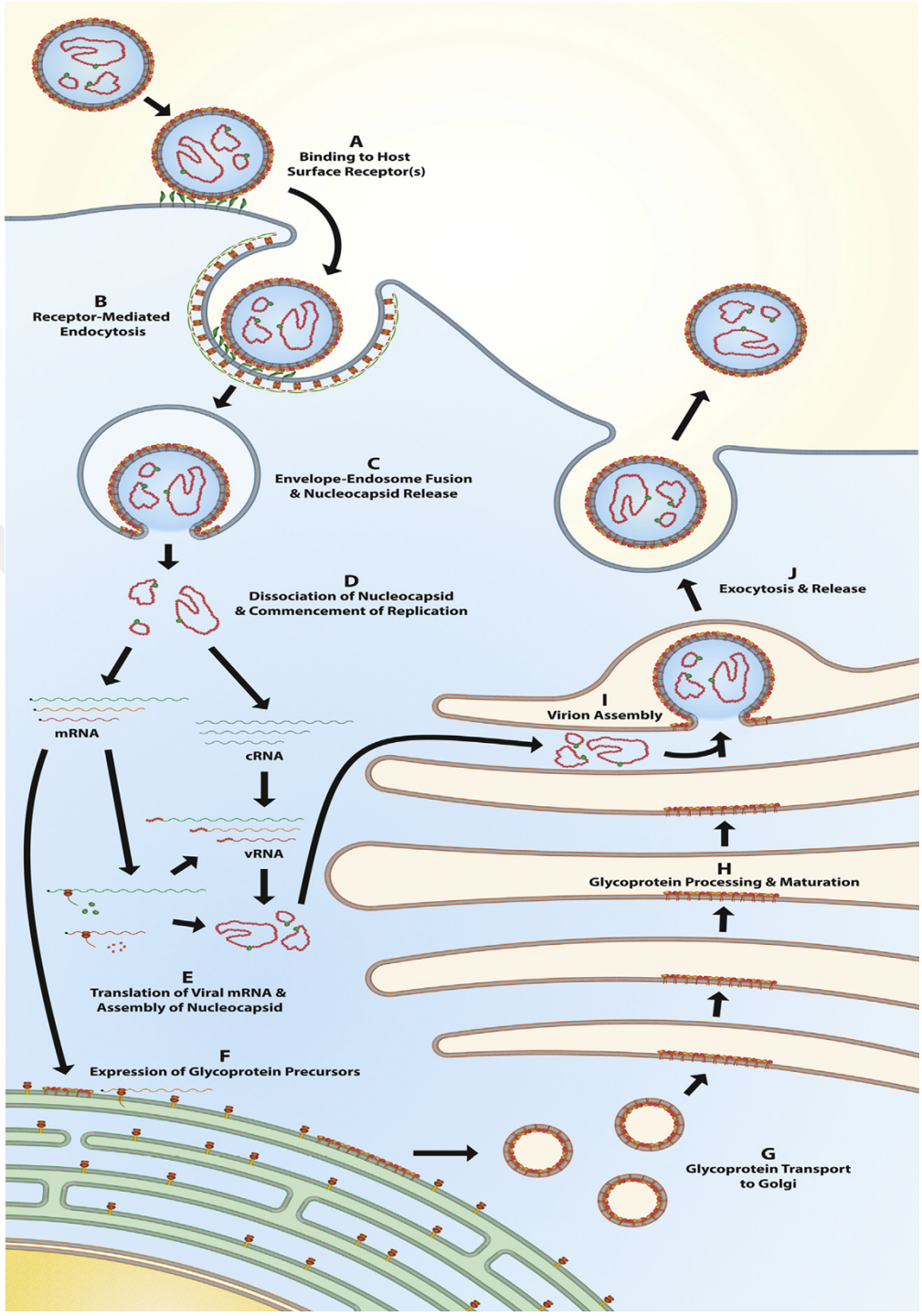


Fig. 1-9. Life cycle of CCHFV. A)Viral attachment to surface receptor (unknown) B) Internalization through clathrin-dependent, receptor-mediated endocytosis. C) Fusion between the envelope and endosomal membranes by reduced pH in endosome D) Dissociation of the nucleocapsids and generation of mRNA and cRNA by RdRp. E) Translation of viral proteins and genomic vRNA production following new nucleocapsids formations. F) M polyprotein production in ER. G) Cleavage of olyprotein into Gn and Gc precursor forms transported to the Golgi complex H) Maturing process of glycoproteins, I) Formation of new virions. J) Virion egress [5]

1.5. Transmisson of CCHFV

CCHFV is an arthropod-borne virus and causes zoonosis despite circulating in an enzootic tick–nonhuman vertebrate–tick cycle and developing only a transient viremia without apparent of illness [5, 11]. At least 31 ticks species and one biting midge (Culicoides spp.) have been identified as CCHFV reservoirs [9, 11, 72]. But in fact, not all of these reservoirs serve as CCHFV competent vectors. Virus can be simply transmitted by a recent blood meal from a viremic host into them [9]. *Hyalomma* as a member of family *Ixodidae* is the predominant vector of CCHFV and posses a rigid shield or scutum unlike *Argasidae* containing soft ticks [40, 73]. Because of its wide geographic distrubition extending over southern Europe, a part of Middle East and Central Asia, *H. marginatum* is the most important vector for CCHFV [74] and commonly collected from humans and animals in endemic regions of Turkey (Fig. 1-10) [75]. *H. marginatum rufipes* (mainly in Africa) and *H. anatolicum anatolicum* are also common vector for CCHFV. The maintance of CCHFV can be achived by transstadially (from larva to nymph to adult) and interseasonally in several tick species and transovarially to the F_1 generation (in some cases to F_2) in *Hyalomma marginatum marginatum, H. marginatum rufipes, Dermacentor marginatus* and *Rhipicephalus rossicus* [11].

Transmission of CCHFV to tick vectors is occured principally during the spring and summer months, and is supported by viral replication in different host tissues reaching the highest titers in the salivary glands and reproductive organs during metamorphosis of competent tick vectors feeding on host blood [76]. CCHFV is vertically transmitted by adult females producing eggs and by adult males to females during copulation [5, 77-80].

All species maintain their life on at least two different hosts: larvae and nypmphs feed on the same individual host while feeding and molting or until dropping off, but adults feed on second host, particularly mammals. As an example, immature *H. m. marginatum and H. m. rufipes* feed on birds, hares and hedgehogs and adults on cattle and other large mammals, while *H. a. anatolicum* feed on domesticated mammals through their entire lifecycle [81]. Furtermore, some species feed on more than two host which means they drop off their host each molt. *Hyalomma* ticks are hunting ticks and their larvae and nymphs quest aggressively to feed on several hosts, including hedgehogs, hares and ground-feeding birds while the adults actively seek out sheep, cattle and other large animals including humans [5, 11].

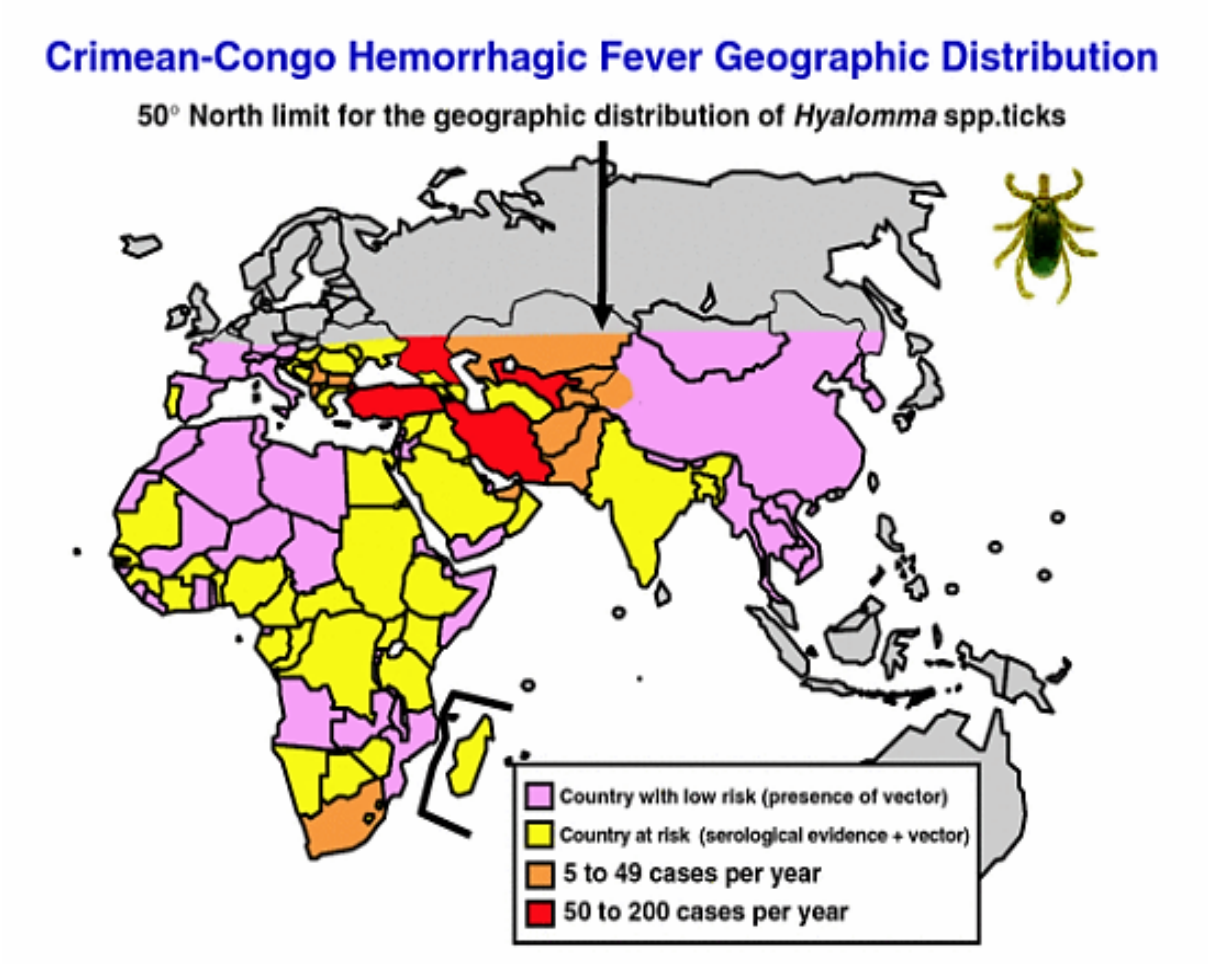


Fig.1-10. Geographic distribution and relatively incidence rate of Hyalomma spp. ticks [82]

Like other naioroviruses, co-evolution of CCHFV with several tick species which have themselves evolved along with the vertebrates as sources of blood meals may contribute to the wide genetic diversity of the virus [5, 83].

Humans are actually not a main component of tick life cycle but actually a dead-end host. They are bitten occasionally by infected ticks when entered in enzootic area, which leads to CCHF in humans while other animals develop only asymptomatic transient viremia. Because of this, farmers, livestock owners and herders, abattoir workers, and veterinarians carry the highest risk of contracting CCHFV [7, 84-87]. CCHF can also caused by direct contact with the blood of infected animals and by contact with the virus-containing body fluids of a patient during the first 7–10 days of illness (Fig. 1-11) [5].

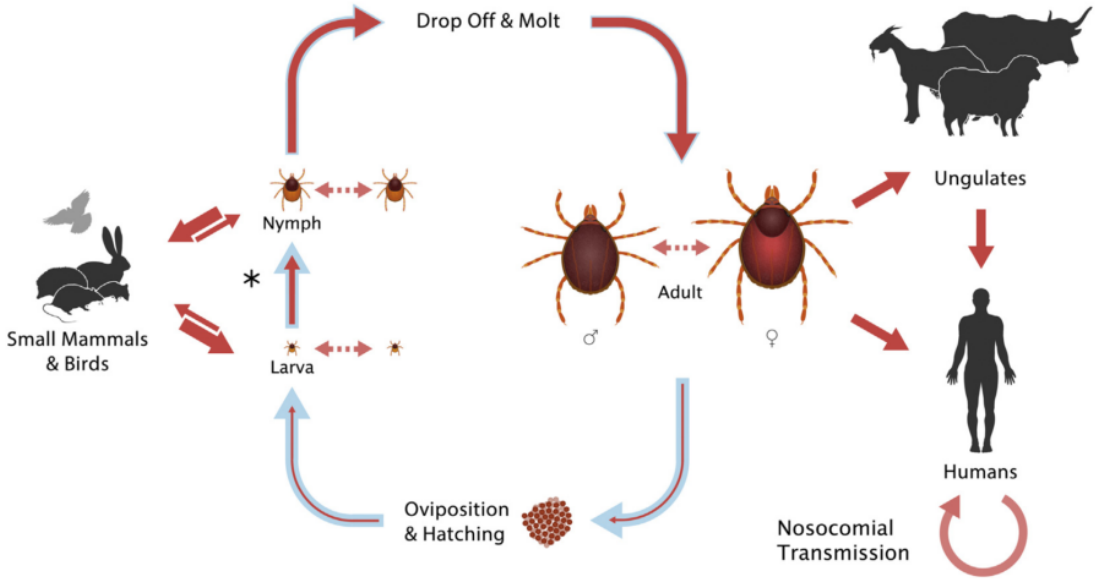


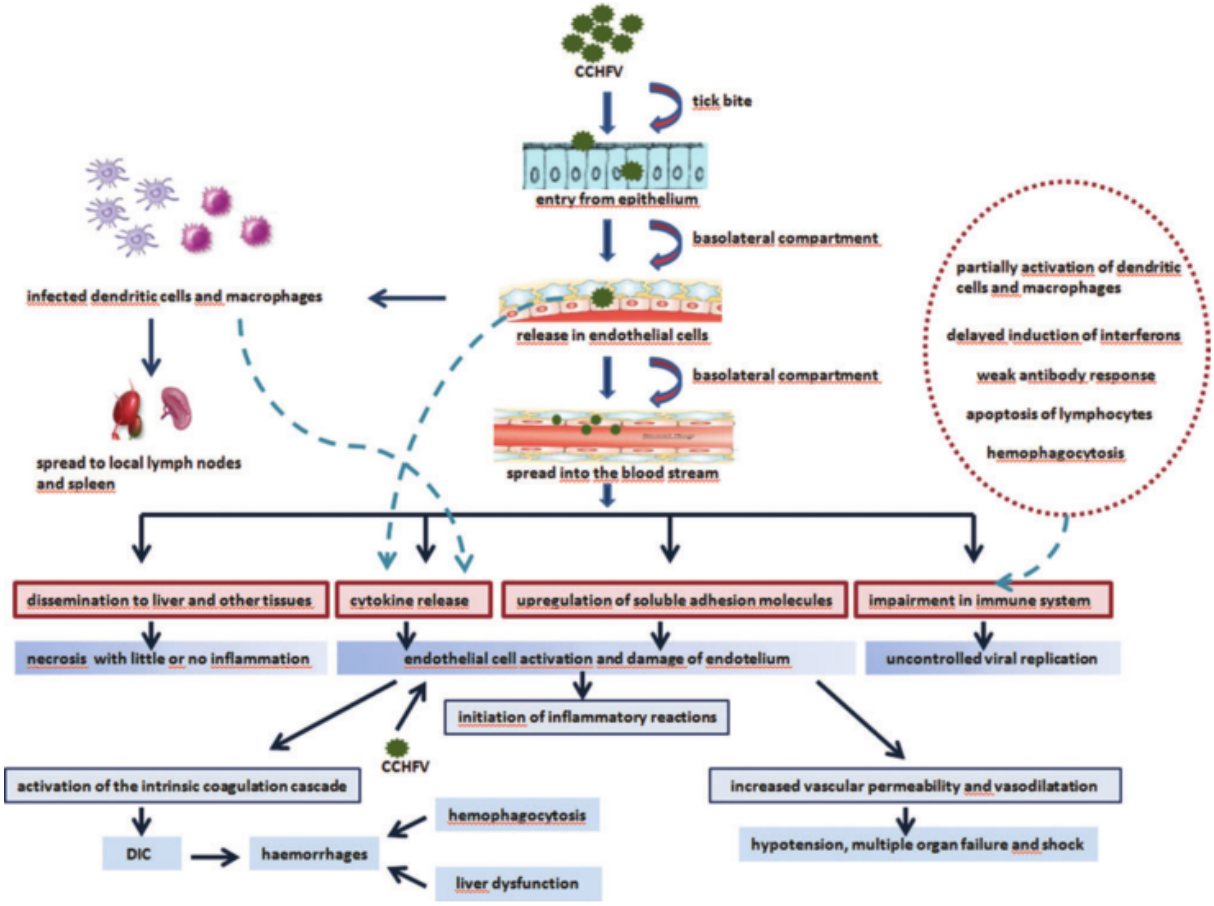
Fig. 1-11. Routes of transmisson for CCHFV during the life cycle of Hyalomma spp. ticks. Blue arrows indicate the course of the tick life cycle. After hatching, larvae feed on first hosts containing small mammals and birds. Some tick larvae and nyhmphs feed on two host while other species dropp off each molt (three-host ticks). This transmission is indicated by asteriks. Beside that, virus can be spread by ticks through co-feeding (dashed arrow). Then adult ticks take blood from large animal and after copulation virus is transmitted to eggs. Viral transmission to human can occur in different ways like infected ticks bites and direct blood contact with infected animals or humans, which is therefore marked as solid red arrows. The thickness of the red arrow indicates the efficiency of transmission [5].

1.6. Pathogenesis

CCHFV pathogenesis is poorly understood because of necessity of BSL-4 laboratory conditions and lack of animal models. Blood analyses, autopsies, and liver biopsies of patients provide limited knowledge about pathogenesis [9].

Virus overcomes epithelium as a first barrier through tick bite. Basolateral membran reserving viral attachment proteins enable viral entry and viral release into the bloodstream, which causes systemic dissemination [88]. Internalization of the virus into host cells is mediated by the clathrin-, pH- and cholesterol-dependent pathway [61] by binding of the envelope glycoprotein Gc to cell-surface-associated receptors suggested human cell-surface nucleolin [59]. Upon activation of endothelial cells both directly and indirectly by cytokines like IL-1β, IL-6, and TNF-α released from dendritic cells, inflammatory responses is initiated and leukocytes are recruited [89, 90]. Upregulation of the leukocyte adhesion molecules ICAM1, vascular cell adhesion molecule 1 (VCAM1), and E-selectin increases vascular permeabilization [91]. Expression level of soluble ICAM1 (sICAM1) is correlated with elevated CCHFV titers and disease severity [92, 93]. After the viral proliferation in tissue

resident macrophages and dendritic cells, virus can be transmitted to local lymph nodes, spleen, and finally to systemic circulation of the host [88]. Excessive release of the cytokines named as cytokine storm have toxic effects on the endothelium causing to increased vascular



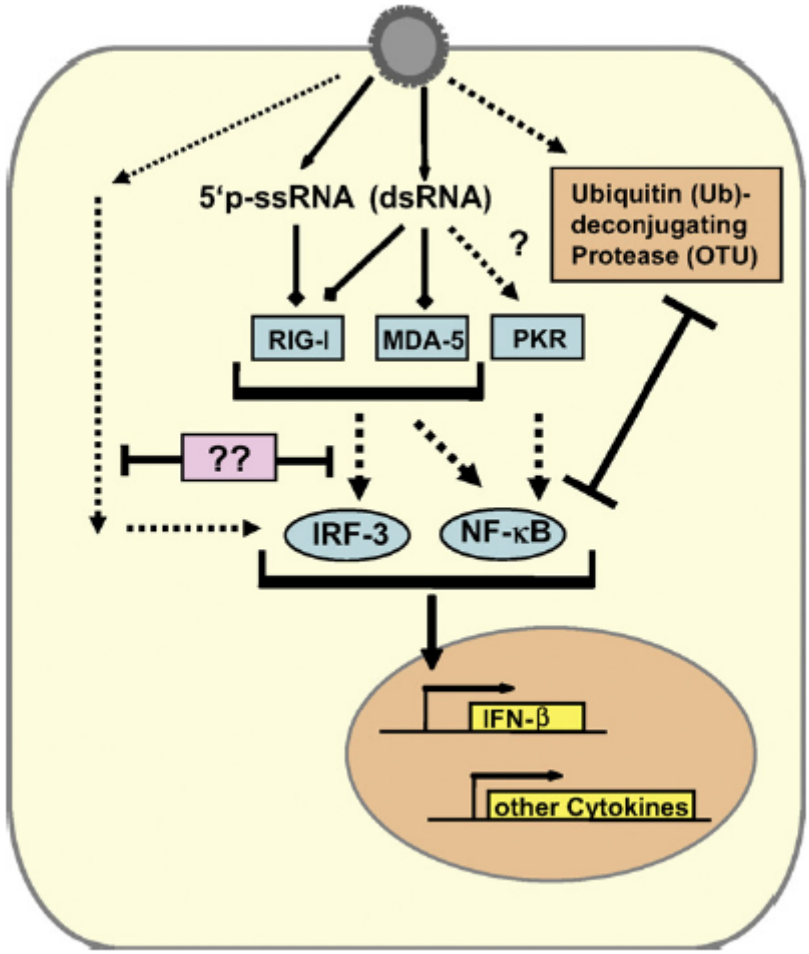
permeability, vasodilatation, multiple organ failure, and shock (Fig. 1-12) [94].

Fig. 1-12. Pathogenesis of CCHF. Endothelial cells are crossed over by CCHFV upon tick bite and are activated either directly by the virus or indirectly by virus-induced soluble mediators. Endothelial cell activation leads to the inflammatory reactions,increase of vascular permeability, and activation of the intrinsic coagulation cascade which then causes haemorrhages, hypotansion, multiple ogan failure and shock. CCHFV can be replicated uncontrollably by blocking several immune mechanism. DIC, disseminated intravascular coagulation [94]

According to animal studies, CCHFV replicates in blood on the first day of infection, in liver and spleen on the following day and then spreads systemically to lung, kidney, and finally to brain as a result of increased vascular permeability causing disruption of the blood–CSF barrier as seen on other viral hemorrhagic fevers [4, 95-98].

Connolly-Andersen and her colleagues reported that monocyte-derived dendritic cells (moDCs) and macrophages (MPs) were permissive for CCHFV replication following release of IL-6, Il-10 and TNF-α [99, 100]. TNF-α and IL-6 is highly correlated with disease

severity [101, 102]. Supernatants of infected DCs activated endothelial cells by up-regulating ICAM-1 expression [100]. Additionally, CCHFV-infected DCs also partially matured to an antigen-presenting cell as a result of inadequate upregulation of major histocompatibility



complex II (MHC II) priming of naive T cells [94, 99]. Hyperactivation of monocytes and macrophages leads to cytopenias, an excessive phagocytosis of blood cells [103, 104].

Fig. 1-13. Escape mechanism of CCHFV from type I IFN response. The monophosphorylate 5' ends of the CCHFV ssRNA is recognized byRIG-I unlike other bunyaviruses. PKR is presumably activated,

either by trace amounts of dsRNA or by cell stress resulting from infection. IRF-3 is triggered by an unknown mechanism. CCHFV L protein containin OTU protease antagonizes the ntiviral effects of ISG15 and inhibits NF-kB-dependent signaling [105].

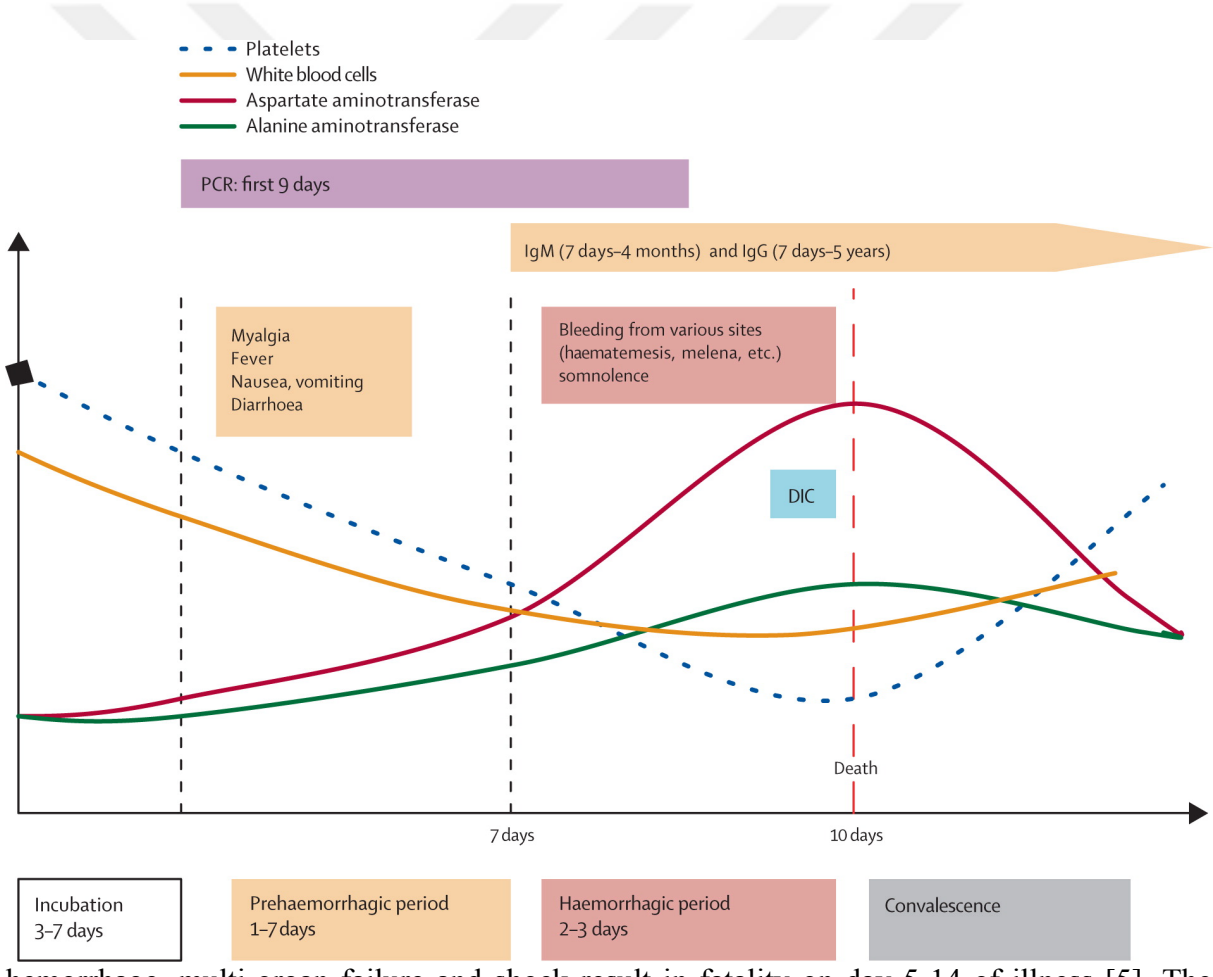
As a member of innate immune system, interferons (IFNs) are very important for a rapid and efficient antiviral response by limiting the spread of the infection. Upon viral replication, secretion of type I IFNs (IFN-α and β) and subsequently upregulation of interferon-stimulated genes (ISGs) are induced [106, 107]. Therefore, they can regulate

inhibiton of viral protein expression, cell proliferation and apoptosis. However, IFN treatment at 1 hr after infection has no significant impact against CCHFV replication but it is effective to apply before infection [105]. MxA, a component of IFN-pathway is responsible for the inhibition of primer virus replication, which co-localize and interact with CCHFV nucleocapsid protein in the perinuclear region of infected cells [108, 109]. It is of interest to investigate, wheter CCHFV counteracts IFN signalling by several independent strategies. For example, CCHFV introduce viral ssRNA carrying 5' monophosphate, not 5' triphosphate triggering RIG-I signaling pathway in other bunyaviruses. This evasion mechanism can be explained by a possible phosphate cleavage during genome replication or by an anti-IFN factor like NSs of Rift Valley Fever Virus (RVFV) and La Crosse virus [110, 111]. Although triphospate on 5' end is a characteristic motif for recognition by RIG-I, a recent study has reported that RIG-I recognizes CCHFV RNA, resulting in downstream type I IFN antiviral signaling and subsequent ISG stimulation [112]. On the other hand, the CCHFV OTU would block signaling by RIG-I and mitochondrial antiviral-signaling protein (MAVS) due to its deubiquitinase and deISGylase activities, which is almost seen in other nairoviruses targeting activated RIG-I to control innate immune signaling [113]. The OTU domain is a superfamily of ubiquitin (Ub)-deconjugating proteases and cleaves Ub and interferon stimulated gene product 15 (ISG15) from covalently conjugated proteins mediating innate antiviral responses. Frias-Staheli and her collegues showed that the CCHFV OTU protease deconjugate Ub and ISG15 from cellular target proteins and antagonizes the antiviral effects of ISG15 thereby inhibits NF-kB-dependent signaling (Fig. 1-13) [53]. Beside its effect on NFkB signaling, protein ubiquitination plays also a critical role in the induction of adaptive cellular immune system such as MHC class I and II antigen presentation [54, 55, 114]. Deubiquitination might also affect other cellular processes subverted by viruses for their own advantage, such as the proteasome-mediated protein degradation system, multiple signal transduction events, or cell cycle progression[53].

1.7. Clinical features and treatment

CCHFV infection is generally reported as a mild, nonspecific febrile illness but in some cases, severe hemorrhagic disease is also developed. Clinical course of infection could be explained by four phases: incubation, prehaemorrhagic, haemorrhagic, and convalescence periods (Fig. 1-14) [11]. Although incubation period take 3-7 days following tick bite, period

of time can be decreased or increased depending on the route of exposure and viral load. The prehaemorrhagic period range from 1 to 7 days and reveals itself in fever (39–41°C) persisting for 4-5 days, headache, myalgia, and dizziness [7, 9, 11, 85, 115-117]. Diarrhoea, nausea, and vomiting are also reported in some cases [116, 117]. Hyperaemia of the face, neck, and chest, congested sclera, and conjunctivitis are also commonly observed in this period of disease. In case of asymptomatic course, RT-PCR would be performed for diagnosis [5, 6]. The haemorrhagic phase begins usually on day 3-5 and takes 2-3 days [6]. Common haemorrhagic manifestations are petechiae, conjuntivitis and large cutaneous ecchymoses as well as gastrointestinal, gingival, cerebral, vaginal, abdominal and urinary tracts bleeding [117]. About 30% of patients are subject to hepato- and splenomegaly [11]. Persisting



hemorrhage, multi-organ failure and shock result in fatality on day 5-14 of illness [5]. The convalescence period begins about 10–20 days if patients could survive CCHF but disease can take its effect up to a year [6]. However, such health problems after convalescent period does not reported by recently published articles from Turkey and Iran [85, 118].

Fig. 1-14. Clinical and laboratory course of CCHF DIC=disseminated intravascular coagulation [6]

1.8. Treatment

A number of studies have been reported on treatment of CCHF. Accordingly, a fully effective treatment is not attenable at the moment [119]. Because its initial nonspecific symptoms resemble myriad of other infections, CCHF patients can not be treated properly [120, 121]. Patients developing hypotension and hemorrhage are usually hospitalized for monitoring and supportive therapy including the administration of thrombocytes, fresh frozen plasma, and erythrocyte preparations. These supportive therapies are accompanied by laboratory tests such as complete while blood cells count, serum electrolytes and transaminases, renal function tests, and coagulation parameters [120, 122]. Initially, it is reported that a combination of high-dose methylprednisolone, intravenous immunoglobulin and fresh-frozen plasma was effective to cure CCHF [5, 123].

In recent publications, ribavirin is debated as an effective antiviral agent and widely administered [5]. Furthermore, its inhibitor effect on CCHFV replication has been noted in a minigenome system [52], in virus-infected cells [124-126], in newborn mice [127] and in signal transducer and activator of transcription 1 (STAT-1) KO mice [4]. These effects were noted in a concentration-dependent manner both *in vivo* and *in vitro* [5]. Detailed reviews about ribavirin therapy concluded that ribavirin is beneficial in CCHFV infections despite the lack of inscrupulous evidence [120, 128]. For example, a meta analysis has found no significant correlation between survival rate, length of hospitalization or need for blood perfusion and ribavirin treatment [129].

Passive immunotherapy has been carried out by injecting of anti-CCHFV antibodies prepared from convalesent patients, intramuscularly or intravenous for prophylactic and therapeutic purposes, particularly for high-risk patients [130-132].

1.9. Delayed Type Hypersensitivity Response

During the initial course of viral haemorrhagic fever infections, immune system mount a significant inflammatory response leading to disseminated intravascular coagulation following haemorrhage, multiple organ failure and septic shock [133].

The delayed type hypersensitivitiy (DTH) response is known to occur during the repeated exposure to the antigens. Consequently, the role of DTH response played during the

initial encounter with CCHFV is currently unknown. However, an essential arms of protective immunity against a microbial agent usually contain an effective DTH response. Furthermore, DTH reactions is usually helpful in gauging the breadth of T cell responsiveness [134-136].

Delayed type hypersensitivity response is primarily a function Th1 type response where CD4+ T lymphocytes are activated. This response sometimes could be to the detriment of the host and there are many example of viral, bacterial and parasitic infections where the DTH response against microbial antigens trigger tissue destruction causing harm to the host [137]. For this intial response to role played during granulomas, edemas, and exudates are formed after induction of DTH mediators and in case of intensive response not only tissues and small vessels but also larger vessels are damaged, which causes hemorrhages [137].

In this study, we aimed to investigate the presence of DTH reactions against CCHFV in animal model.

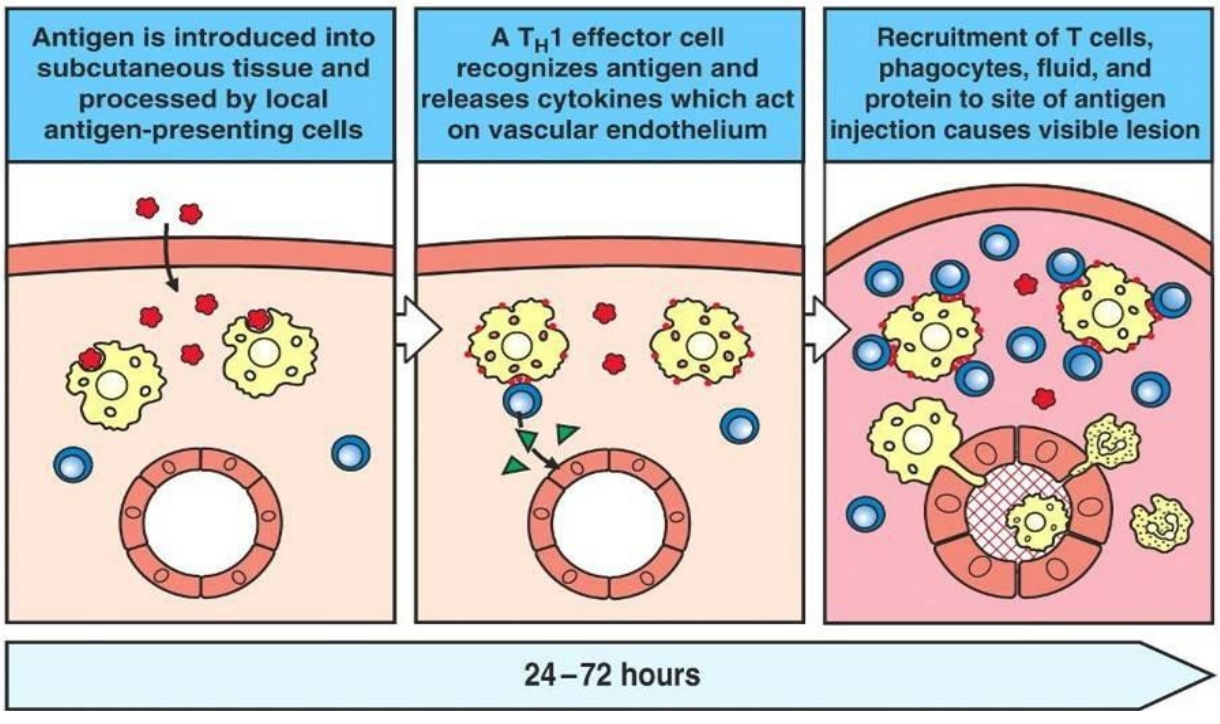


Fig. 1-15. Development of DTH response on site of infection. Following secondary exposure to antigen, TH1 cells recognizes processed antigen by APCs and release cytokines acting on vascular endothelium. Recruiting an inflammatory cell infiltrate causes the accumulation of fluid and protein which forms visible lesion [138]

Delayed type hypersensitivity (DTH) is initially described by Koch as tuberculin test used to determine whether an individual has previously been infected with *Mycobacterium tuberculosis* [138]. Following exposure, mycobacterial antigen is processed by antigen presenting cells (APC) and introduced by major histocompatibility complex (MHC) class II molecules to $CD4^+$ lymphocytes and subsequently circulating sensitized memory T cells are generated [139]. After intradermal injection of small amounts of tuberculin derived from *M. tuberculosis*, a local T cell-mediated inflammatory reaction will be develop over 24–72 hours for individuals exposed to the bacterium, either by infection with the pathogen or by immunization with BCG, an attenuated form of *M. tuberculosis* [138]. This response is mediated by T_H1 cells and several inflammatory cytokines are secreted to recruit and activate macrophages and other non-specific inflammatory cells (Fig. 1-16) [140]. Stimulated expression of adhesion molecules on endothelium leads to increase local blood vessel permeability, allowing plasma and accessory cells to enter the site; this causes a visible swelling (Fig. 1-15) [138]. Each of these phases takes several hours and so the fully developed response appears only 24–48 hours after challenge.

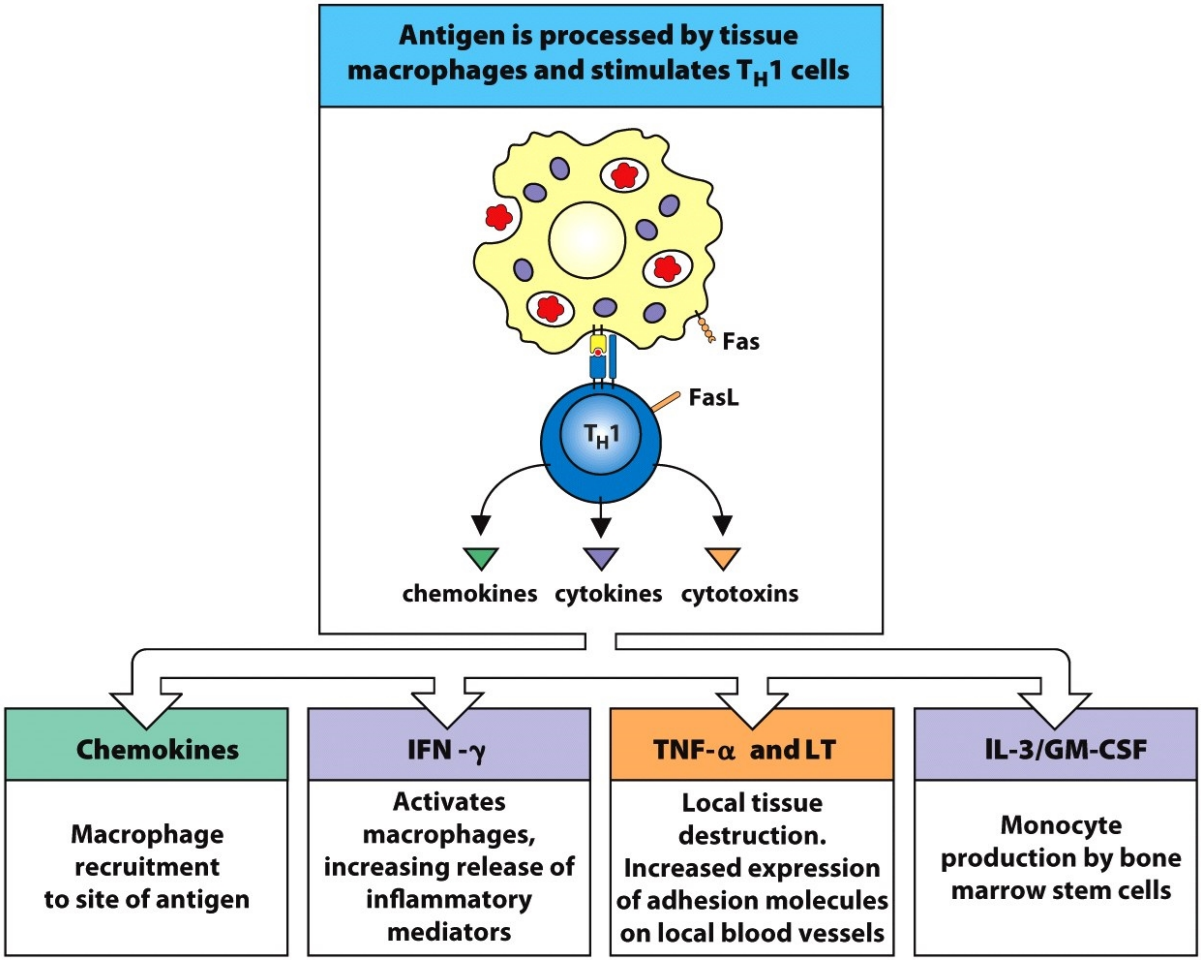


Fig.1-16. DTH is directed by chemokines, cytokines and cytotoxins released by sensitized TH1 cells [138]

2. MATERIALS AND METHODS

2.1. Plasmid Constructions and Bacterial Hosts

2.1.1. Cloning vector for CCHFV NP

CCHFV Kelkit'06 NP (1449 bp) retrieved from GenBank (Accession no. GQ337053) and their estimated antigenic sequences by Kolaskar Tongaonkar (Kolaskar ve Tongaonkar, 1990; Immune Epitope Database and Analysis Program- http://tools.immuneepitope. org/tools/bcell/ iedb_ input0), $NP^{124-507}$ (390 bp) and $NP^{886-1392}$ (513 bp) were optimized for bacterial expression, synthesized and cloned into pUC19 by GenScript®. They were transformed into chemically competent DH5α *E.coli* cells.

Optimized gene sequences to be cloned:

1) NP (1449 bp) sequence

5’ atg gaa aac aaa att gaa gtg aat agc aaa gat gaa atg
aac aaa tgg ttc gaa gaa ttt aaa aaa ggc aac ggt ctg atg
gat acg ttt acc aac agc tat tct ttc tgc gaa aac gtt ccg
aat ctg gat aaa ttt gtc ttc cag atg gca agt gct acg gat
gac gcc caa aaa gac agc att tat gcg tct gcc ctg gtg gaa
gcc acc aaa ttt tgc gca ccg att tac gaa tgt gcg tgg gtg
agc agc acc ggc atc gtt aaa aaa ggt ctg gaa tgg ttc gaa
aaa gat agt ggc acc att aaa tcc tgg gac gaa aac tat gct
gaa ctg aaa gtg gat gtt ccg aaa atc gaa cag ctg gca aat
tac cag caa gcg gcc ctg aaa tgg cgc aaa gat att ggc ttt
cgt gtt aac gcc aat acc gca gct ctg agc aac aaa gtc ctg
gca gaa tat aaa gtg ccg ggt gaa atc gtc atg agc gtg aaa
gaa atg ctg tct gat atg att cgt cgc cgt aac ctg atc ctg aat
cgc ggc ggt gac gaa aat ccg cgc ggt ccg gtt tca cgt gaa
cat gtc gaa tgg tgt cgt gaa ttt gtg aaa ggc aaa tac att atg
gcc ttc aac ccg ccg tgg ggt gat att aat aaa agt ggc cgc tcc
ggt atc gca ctg gtt gct acc ggc ctg gcg aaa ctg gcc gaa
acg gaa ggc aaa ggt gtc ttt gat gaa gcg aag aaa acc
gtg gaa gcg ctg aat ggt tat ctg gat aaa cat cgc gat gaa
gtg gac aaa gca tca gct gac tcg atg att acg aac ctg ccg aaa
cac atc gcg aaa gcc cag gaa ctg tac aaa aat agt tcc gca
ctg cgt gct cag ggc gcg caa atc gat acc ccg ttt tca tcg ttc
tat tgg ctg tac aaa gcg ggt gtt acg ccg gaa acc ttt ccg acg att
tct cag ttt ctg ttc gaa ctg ggc gaa caa ccg cgt ggc acc aag
aaa atg aaa aaa gcg ctg ctg tca acg ccg atg aaa tgg ggc aaa
aaa ctg tat gaa ctg ttt gcc gat gac tcg ttc cag caa aac cgc atc
tac atg cat ccg gct gtg ctg acc gcg ggt cgt att agt gaa atg
ggc gtg tgc ttc ggt acg atc ccg gtt gca aac ccg gat gac gca
gca cag ggc tca ggt cac acc aaa tcg att ctg aat ctg cgt acc
agc acc gaa acc aac aat ccg tgt gcg aaa acc att gtc aaa ctg
ttt gaa atc cag gaa acg ggc ttc aat att caa gat atg gac atc

gtt gca agc gaa cat ctg ctg cac cag agt att gtc ggc aaa cag
tcc ccg ttt caa aac gcg tat aat gtt aag ggt aac gcc acc tct
gca aat att atc taa 3’

2) $NP^{124\text{-}507}$ (390 bp) sequence

5’ atg aac ctg gat aaa ttc gtg ttc caa atg gcc tcc gca
acc gat gac gct caa aaa gac tca atc tac gcc tca gcc
ctg gtc gaa gcg acc aaa ttt tgc gcc ccg att tat gaa
tgt gcg tgg gtt agc tct acg ggc atc gtc aaa aaa ggt
ctg gaa tgg ttc gaa aaa gat agt ggc acc att aaa tcc
tgg gac gaa aac tat gcg gaa ctg aaa gtg gat gtt ccg
aaa atc gaa cag ctg gcc aat tac cag caa gcg gcc ctg
aaa tgg cgt aaa gac att ggc ttt cgc gtc aac gca aat
acg gca gct ctg agc aac aaa gtg ctg gct gaa tac aaa
gtt ccg ggt gaa atc gtc atg agc gtg aaa gaa atg taa 3’

3) $NP^{886\text{-}1392}$ (513 bp) sequence

5’ atg gcc ctg cgt gct caa ggc gct caa atc gac acc ccg
ttc tca tca ttc tat tgg ctg tat aaa gct ggc gtg acc ccg
gaa acc ttt ccg acg att tcc cag ttt ctg ttc gaa ctg ggc
gaa caa ccg cgt ggc acc aag aaa atg aaa aaa gcg ctg
ctg tca acc ccg atg aaa tgg ggc aaa aaa ctg tat gaa
ctg ttt gcc gat gac tcg ttc cag caa aac cgt att tac atg
cat ccg gca gtt ctg acg gct ggt cgc att agc gaa atg ggc
gtg tgc ttc ggc acc atc ccg gtt gca aac ccg gat gac gca
gca cag ggc agc ggt cac acc aaa tct atc ctg aat ctg cgc
acc agc acg gaa acc aac aat ccg tgt gca aaa acg att gtc
aaa ctg ttt gaa atc cag gaa acc ggc ttc aat att caa gat atg
gac atc gtc gct tct gaa cat ctg ctg cac cag agt atc gtg ggt
aaa caa tcc taa 3’

2.1.2. Preparation of competent cell

All the cells used for transformation for this assay were plated on Luria Bertani (LB) agar (1.0% Tryptone, 0.5% yeast Extract, 0.5% sodium chloride, 1.5% agar) and a single colony from overnight grown culture were inoculated into 25 ml LB (1.0% tryptone, 0.5% yeast Extract, 0.5% sodium chloride) in a 250 mL bottle and incubated at 37°C for 4-6 hours. Cells were chilled for 10 minutes and harvested at 4100 rpm for 10 minutes at 4°C. Pellets were gently resuspended in 10 ml cold 0.1M CaCl, incubated on ice for 20 minutes and centrifuged at 4100 rpm for 10 minutes at 4°C. After resuspension in 5mL cold 0.1MCaCl/15% glycerol, cells were dispensed in microtubes (300µL/tube) and frozen in -80°C for storage.

2.2. Transformation

One µl isolated plasmid DNA (100 pg) or 5 µl ligation mixture were gently added into competent cells and incubated on ice for 10 minutes. Cells were heat shocked at 42°C for 2 minutes, immediately transfered on ice and incubated for 10 minutes. 250 µl S.O.C medium 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate and 20 mM glucose) was added to the mixture and the cells were incubated at 37°C with shaking 220 rpm for 1 hour. Following incubation, different amount of culture was spread on a prewarmed LB agar plate supplemented with relative antibiotics and incubated overnight at 37°C.

2.2.1. Analyzing positive transformants

Antibiotic selection

Transformed cells were plated on LB agar with appropriate antibiotic. For the cells containing pUC19 vectors, ampicilin were used to a final concentration of 100µg/ml while 50µg/ml kanamycin were added to LB agar to select the cells containing pETSUMO and pET28b expression vectors.

Polymerase Chain Reaction

Isolated plasmids (see Subheading 2.4) were used as template for CCHFV NP specific PCR. The reaction was conducted in Veriti® 96-Well Thermal Cycler (Applied

Biosystem) using cycling conditions as outline in Table 2-1. Two and half µl isolated plasmid was mixed with 2.5 µl 1 µM primers (Table 2-2) and 50 µM dNTPs in 1 µl (D7295, SIGMA), 5 µl 10X PCR Buffer (P2192, SIGMA), 0.5 µl 1u Taq polymerase (D6677, SIGMA) and 1.5 µl 25mM $MgCl_2$ (M8787, SIGMA) were added into 50 µl PCR mixture. Tm was calculated using OligoAnalyzer3.1 (Integrated DNA Technologies, Inc, Coralville, IA, USA).

Table 2-1. Time and temperature set up for PCR

Step	Number of cycles	Phase	Duration	Temperature
1	1	Initial melt	5 min	95 °C
2	25	Melt	30 s	95 °C
3	25	Annealing	30 s	52 °C
4	25	Extension	30 s	72 °C
5	1	Final extension	10 min	72 °C

Table 2-2. Primers used for TA cloning

Name	Sequence	Target gene
F1	5'atg gaa aac aaa att gaa gtg 3'	NP
R1	5' tta gat aat att tgc aga ggt g 3'	
F2	5' atg aac ctg gat aaa ttc gtg ttc c 3	NPNT
R2	5' tta cat ttc ttt cac gct cat gac 3'	
F3	5' atg gcc ctg cgt gc 3'	NPCT
R3	5' tta gga ttg ttt acc cac g 3'	

Restriction analysis

Analysis of inserts directionally cloned into pET28b were performed by using restriction enzymes. To cut 1µg plasmid DNA, 0.2 µl *XhoI* (10 u/µl), 0.2 µl *NcoI* (10 u/µl) and 10X Buffer (CutSmart® Buffer, New England Biolabs, Inc.) were mixed in a total volume of 20 µl. The mixture was incubated at 37°C for 1 hour and enzymes were inactivated at 80°C for 20 minutes.

Sequencing

Before starting to expression all positive transformants were analyzed by sequencing performed by Prof. Dr. Ali Osman Kilic at Karadeniz Technical University.

2.3. Construction Of Plasmids Expresssing CCHFV NP

To investigate CCHFV NP induced DTH response, 3 complete NP: 1449 bp, N-terminal part of NP, NPNT: 387 bp and C-terminal part of NP, NPCT: 513 bp) were separately amplified by PCR and cloned into Champion™ pET SUMO expression vector (Thermo Fisher Scientific). The cloning was TA based cloning system, which allows to express gene of interest as a fusion to N-terminal polyhistidine (6xHis) tag and SUMO protein for increased expression and solubility of recombinant fusion proteins and generation of native protein following cleavage by SUMO Protease. CCHFV NP and its both N- and C-terminal parts were also cloned into pET28b (kindly provided by Asst. Prof. Esen Bakhautdin, Fatih University, Istanbul) by using restricticton enzymes in a directional cloning strategy.

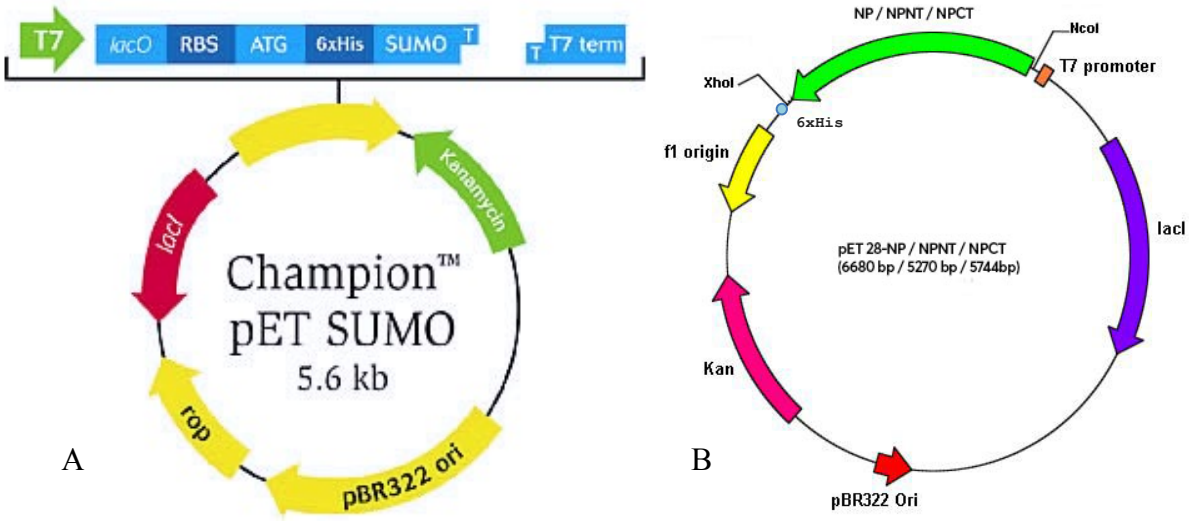


Fig. 3-1. Plasmid constructs expressing CCHFV NPs. Gene of interests were inserted A) downstream of SUMO protein following N-terminal 6XHis tag into pETSUMO expression vector via TA cloning B) by using restriction enzymes XhoI and NcoI before C-terminal 6XHis tag into pET28b vector via directional cloning.

2.3.1. TA cloning

To add single deoxyadenosine (A) to the 3´ends of PCR products for properly TA cloning, HOT FIREPol® DNA polymerase (Solis Biodyne) with proofreading activity was used primarily among the extension period during repeated cycles, then a Taq polymerase lacking 3'→ 5' exonuclease activity (D1806, SIGMA) and100 µM adenine were added and incubated at 72°C for 10 minutes in final extension. Primers used in PCR are listed in Table 4. PCR products were directly ligated with pET SUMO vector to avoid degradation of the single 3 A-overhangs over time. For ligation, 1:3 molar ratio of vector:insert was mixed with 10X Ligation Buffer and 4 Weiss units T4 DNA Ligase (both included with kit, K300-01, Thermo Fisher Scientific) and incubated at 15°C for overnight. Afterwards, all ligation reaction was transformed as described in subheading 2.2 into One Shot® Mach1^{TM}-T1^{R} Chemically Competent *E. coli* cells (C8620-03, Thermo Fisher Scientific) used only for plasmid propagation. After cloning, plasmids are named as pBVU1 for complete NP sequence, pBVU2 for N-terminal part of NP and pBVU3 for C-terminal part of NP. Plasmids acquired from One Shot® Mach1^{TM}-T1^{R} *E. coli* were transformed as described in subheading 2.2 into BL21(DE3) One Shot® Chemically Competent *E. coli* (C6000-03, Thermo Fisher Scientific).

2.3.2. Directional cloning

Cloning was achieved by using *XhoI* and *NcoI* restriction enzymes. Restriction sites were introduced into PCR products by primers (Table 2-3). PCR was accomplished by mixing 2.5 µl template DNA with 1µM primers and 10µl 5x HOT FIREPol® Blend Master Mix (04-25-00120, Solis Biodyne) in 50 µl reaction volume. The reaction was conducted in Veriti® 96-Well Thermal Cycler using cycling conditions as outline in Table 2-4.

Table 2-3. Primers used in directional cloning

Name	Sequence	Target gene
Forward_NcoI_12	5' ccg gcc atg gaa aac aaa att g 3'	NP
Reverse_XhoI_13	5' cgg cgc tcg agg ata ata ttt gc 3'	
Forward_NcoI_14	5' ccg gcc atg gac ctg gat aaa ttc 3'	NPNT
Reverse_XhoI_15	5' cgg cgc tcg agc att tct ttc 3'	
Forward_NcoI_16	5' ata tcc atg gcc ctg cgt g 3'	NPCT
Reverse_XhoI_17	5' cgt gac tcg agg gat tgt tta cc 3'	

Table 2-4. Reaction conditions set up for PCR

Step	Number of cycles	Phase	Duration	Temperature
1	1	Initial melt	15 min	95 °C
2	2	Melt	15 sec	95 °C
3	2	Annealing	30 sec	42 °C
4	2	Extension	1 min	72 °C
5	30	Melt	15 sec	95 °C
6	30	Annealing	30 sec	55 °C
7	30	Extension	1 min	72 °C
8	1	Final extension	10 min	72 °C

Restriction digestion

Sticky ends on 5' ends of PCR products and pET28b required for cloning were generated with *XhoI* (R6379, SIGMA) and *NcoI* (R0193S, New England Biolabs) enzymes. Briefly, 1µg plasmid DNA or PCR products eluted from gel was digested with 5 units of each enzyme (*XhoI* and *NcoI*) and 10X Buffer (CutSmart® Buffer, New England Biolabs, Inc.) at 37°C for 1 hour and enzymes were inactivated at 80°C for 20 minutes.

Gel elution

Following polymerase chain reaction (PCR) or restriction digestion samples were electrophoresed on a 1% agarose gel at 100V for 45 minutes. The correct size band was excised and DNA was purified using GenElute™ Gel Extraction Kit (NA1111, SIGMA). Gel slices were solubilized in 3 gel volume of the Gel Solubilization Solution by incubating at 50°C and vortexing briefly every 2-3 minutes until the gel was completely dissolved; the DNA was precipitated with 1 volume of 100% isopropanol (Merck); the solution loaded onto a prepared binding column (56500, SIGMA) and centrifuged at 16000xg for 60s; the column was washed with 700µL Wash Solution and dried by centrifugation at 16000xg for 60s and eluted in 50µL of Elution Buffer. All buffer used in gel elution procedure was included with kit.

Ligation

Compatible cohesive ends of DNA fragments generated by restriction enzymes, *XhoI* and *NcoI* were ligated using Rapid DNA Ligation Kit (K1422, Thermo). Briefly, 200 ng linearized vector was mixed with 180 ng insert, 5X Rapid Ligation Buffer and 5 units of T4 DNA Ligase. The reaction mixture was incubated at 22°C for 5 min. Then, 5 µl ligation reaction was used for transformation into One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli* (C6010-03, Thermo Fisher Scientific) as described in subheading 2.2.

2.4. Plasmid Isolation

Positive transformants were inoculated in 2 ml LB and incubated overnight at 37°C with vigorous shaking. Plasmid isolation was accomplished using QIAprep Spin Miniprep kit (27104-Qiagen). Overnight culture was pelleted by centrifugation at 8000 rpm for 23 minutes; the bacterial pellet was resuspended in 250µL P1 buffer (50 mM Tris hydrochloride,

pH 8.0; 10 mM EDTA; 100µg/mL RNase A), lysed by addition of 250µL of P2 buffer (200 mM sodium hydroxide; 1% sodium dodecyl sulfate w/v) and neutralized with 350µL of N3 (4.2 M guanidine-HCl; 0.9 M potassium acetate; pH 4.8) buffer; majority of cellular protein and membranes were removed from the DNA containing supernatant by centrifugation at 13300 rpm for 10 minutes. The supernatant was transferred to a DNA binding column and centrifuged at 13300 rpm for 60s; the DNA washed with 500µL of buffer PB and 700µL of buffer PE; the column was dried by centrifugation at 13300 rpm for 60s and DNA was eluted in 50µL of buffer EB.

2.5. Expression

Positive transformants were analyzed by sequencing (see Subheading 2.2). To optimize protein expression, a pilot expression was performed with each protein. For this purpose, 10 ml of LB containing 50 µg/ml kanamycin and 1% glucose was incubated with 500 µl of the overnight culture at 37°C with vigorous shaking until OD_{600} has reached 0.4-0.6. The culture was splitted into two 5 ml cultures and induced with 1 mM IPTG to one of the cultures. 500 µl from each culture was collected for each time point during 6 hr expression, centrifuged at 13000 rpm for 30 seconds,dissociated from supernatants and analyzed by SDS-PAGE to determine both optimized expression time and protein solubility.

2.5.1. SDS-PAGE

Supernatant samples were diluted 1:2 with SDS Sample Buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approx. 6.8) while pellets were resuspended directly in 80 µl SDS Sample Buffer. All samples were boiled at 95°C for 5 minutes and centrifuged briefly. 10 µl each of each sample was loaded on 8-12% Tris Glycine Polyacrylamide Gel. Proteins were run at 120 V for 20 minutes and then at 150 V for 1 hour. Gel was stained with enough Coomassie Stain (0.1% Coomassie R250, 10% acetic acid, 40% methanol), microwaved on high power for 1 minute, incubated for 15 minutes on a rocking table, rinsed with water and then incubated in destaning solution (10% acetic acid, 40% methanol) on a rocking table until gel background was sufficiently clear.

2.5.2. Scaling-up expression for purification

An overnight culture with LB containing 50 µg/ml kanamycin and 1% glucose was prepared for each positive BL21(DE3) transformation strains, named as 1.8 for NP, 2.2 for NPNT and 3.5 for NPCT after sequencing. Next day, when OD_{600} reached 1-2, overnight culture was diluted 1:50 in LB containing 50 µg/ml kanamycin and 1% glucose and incubated at 37°C with 220 rpm to an OD_{600} 0.4-0.6. Expression was induced with 1 mM IPTG and performed at 37°C with 220 rpm for 3 hours. The cells were harvested by centrifugation 4100 rpm for 10 minutes at +4°C and washed 3 times with PBS.

2.6. His-tagged Protein Purification

All proteins expressed using pETSUMO and pET28b vector, were purified under denaturing contiditions. Before purification, cells were incubated on ice for 30 minutes; resuspended in Lysis Buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, 10 mM imidazole pH 8.0); sonicated with 3 short burst of 10 sec followed by intervals of 10 sec for cooling (SONOPULS HD 2070); freeze-thawed 3 times; incubated at room temperature with agitation for 1 hour; centrifuged at 13000 rpm for 30 minutes and clear lysates were collected while pellets were stored at -20°C only for SDS-PAGE analysis.

Purification was carried out with HisPur™ Ni-NTA Spin Columns (88226, Thermo). After equilibration of column with lysis buffer, clear lysates were loaded on column and incubated on orbital shaker at room temperature for 1 hr. Columns were centrifuged at 700xg for 2 min; washed three times with two resin-bed volumes of Wash Buffer (8 M urea, 100 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 8.5); centrifuged at 700xg for 2 min and proteins were eluted from the resin three times by adding one resin-bed volume of Elution Buffer (8 M urea, 100 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 8.5).
Denatured proteins were then refolded in 5 eluates volume of PBS and concentrated using Centricon® Plus-70 Centrifugal Filter Units (UFC701008 used for NPNT and NPCT; UFC703008 used for NP, Merck Millipore).

2.6.1. Protein concentration determination

Purified protein amounts were determined using Coomassie (Bradford) Protein Assay Kit (23200, Thermo). 5µl concentrated protein was mixed with 250 µl of the Coomassie Reagent in a microplate well; incubated for 10 minutes at room temperature and

absorbance was measured at 595 nm with a iMark™ Microplate Absorbance Reader (Bio-Rad). In the same way, a standart curve was done with serial dilution of BSA starting from 2 mg/ml, included with kit. Concentration of unkown samples were calculated due to linear reggression equation formed with standart BSA values (concentration plotted on the x-axis and absorbance plotted on the y-axis).

2.7. Western Blot

Cells were lysed in lysis buffer and resolved by standard SDS-PAGE. Proteins were transferred to an Amersham Hybond ECL Nitrocellulose Membrane (RPN303D, GE healthcare) and membranes were blocked in PBST (5% skim milk, PBS, 0.05%Tween-20) at 4 C° for 1 hr. The membranes were incubated with anti-HisG antibodies (1:5000) followed by anti-HisG HRP conjugated antibodies (1:5000) to confirm the expression of CCHFV proteins tagged with polyhistidine. Additionally, two more western blot were performed to elicit whether expressed proteins were recognizable by antibodies of immunized mice or CCHFV patients. For this purpose, membranes were incubated with mice sera (1:2000) followed by goat anti-mouse IgG-HRP (in 1:4000 dilution, Santa Cruz Biotechnology) and with human sera (1:100) inactivated at 60°C for 1 hr followed by goat anti-human IgG-HRP (in 1:2000 dilution, Santa Cruz Biotechnology) Chemiluminescent detection was developed using WesternBright Sirius Chemiluminescent Detection Kit (K-12043-D10, Advansta) according to the manufacturer's protocol.

2.8. Delayed Type Hypersensitivity

First immunization of 6-8 week old male Balb/C mice (5 mice per group) was implemented by subcutaneous injection of 50 µg/ml, 100 µg/ml and 200 µg/ml CCHFV NP/ NPNT/ NPCT proteins fused with 6His-SUMO tag emulsified in Complete Freund Adjuvant (CFA). For the control, 5 more mice were immunizied only with PBS. With 2 weeks intervals, mice were boosted two more times subcutaneously (s.c.) with the same dose of antigen used in prime immunization but this time they were emulsified in Incomplete Freund Adjuvant (IFA). Skin test was performed 1 week after last immunization by injection of 50 µl of 100 µg/ml CCHFV NP/ NPNT/ NPCT without SUMO fusion proteins in right hind footpad and same volume of PBS in the left. Footpad thickness was measured daily for 3 days after challenge by SPI External Electronic Caliper Gages (155069).

3. RESULTS

3.1. Production Of Recombinant NPs of CCHFV

To assess cell mediated delayed type hypersensitivitiy of CCHFV, NP and two predicted antigenic regions of NP were generated recombinantly in prokaryotic expression systems. Two different expression vectors were used for production of proteins: pET SUMO based on TA cloning and pET28b on directional cloning.

3.1.1. pET SUMO expression system

This expression system based on TA cloning utilizes SUMO protein to allow expression, purification, and generation of native proteins in *E. coli* and offers an increase in the expression of recombinant proteins and enhance the solubility of partially insoluble proteins. Gene of interest will be cloned in frame with the N-terminal peptide containing the polyhistidine (6xHis) tag and SUMO, both cleaved specifically by SUMO Protease resulting in the production of native protein with no extra amino acids added between the cleavage site and the start of your protein.

SUMO is firstly discovered in the *Saccharomyces cerevisiae* as Smt3 protein and then its 11 kDa homolog of the mammalian SUMO-1 protein was described [141]. SUMO, is a member of a ubiquitin-like protein family which attaches to lysine side chains on cellular target proteins and regulates several cellular processes including apoptosis, nuclear transport, and cell cycle progression [142]. But in contrast to ubiquitin modification, SUMO causes change in protein function and activity rather than protein degradation.

pET SUMO expression plasmid was constructed for this study in this way: inserts were amplified by PCR using native Taq DNA polymerase which enables required 3' end deoxyadenine (A) on gene sequences to be cloned; ligated efficiently with linearized pET SUMO vector supplied with single 3 deoxythymidine (T) and transformed to One Shot® Mach1™-T1^{R} competent *E. coli* cells. Positive transformants were analyzed both using PCR (Fig. 3-1). Plasmids were isolated for transformation into One Shot® BL21(DE3) competent *E. coli*. After cloning proteins were called as HSNP, HSNPNT, HSNPCT (H, 6XHis; S, SUMO protein; NP, nucleoprotein of CCHFV; NT; N-terminal part of NP; CT, C-terminal part of NP).

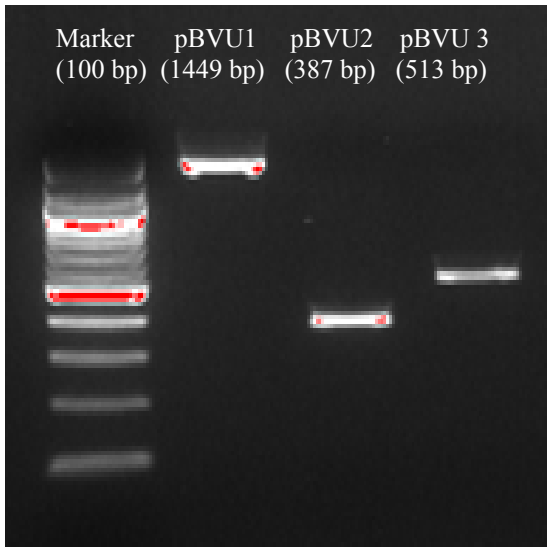


Fig. 3-1. Gel electrophoresis after PCR following transformation of plasmids into One Shot® Mach1™-T1R competent *E. coli*

Towards optimization, proteins were expressed in a variety of conditions like time (1 hr, 2 hr, 3 hr, 4 hr, 5 hr 6 hr), temperature (16°C, 25°C, 30°C, 37°C) and IPTG concentration (5uM, 20 uM, 50uM ve 200 uM, 1mM). No significant effect of temperature has found on protein expression level while highest expression was seen in induction with 1mM IPTG. Therefore, expression of proteins were performed by induction with 1mM IPTG at 37°C for 3 hr (Fig3-2). Due to SDS-PAGE analysis it is exhibited that proteins were insoluble and accumulated in commonly reffered as inclusion bodies.

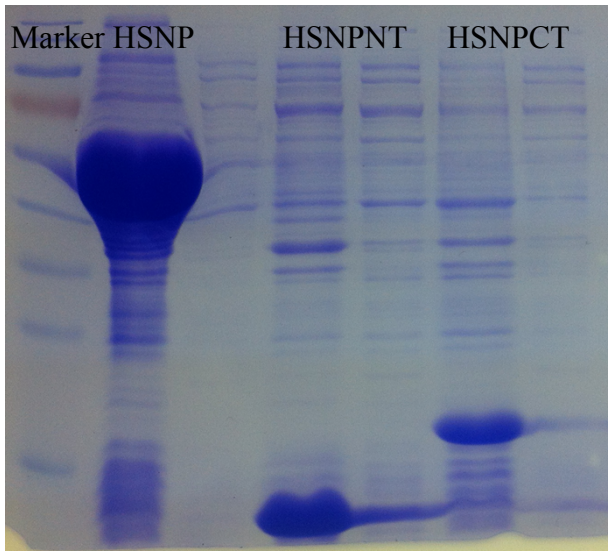


Fig.3-2. SDS-Page analysis after expression. Samples were loaded as pellet and supernatant respectively. H, 6XHis; S, SUMO protein; NP, nucleoprotein of CCHFV; NT; N-terminal part of NP; CT, C-terminal part of NP).

3.1.2. Purification of proteins tagged with 6xHis-SUMO

Following expression, cells were lysed under denaturing condition using urea (8M). Proteins were purified using immobilized metal affinity chromatography via Ni-NTA columns on which histidine residues are captured by nickel ions immobilized on resin and eluted in the presence of high concentration of imidazole.

For the optimum condition, ionic strength of buffers used in purification was increased (500 mM NaCl) to prevent non-specific binding proteins in eluate and different pH values of buffers (6.5, 7.0, 7.4, 8.0, 8.5) were tested for stability increasing the yield of protein. Proteins were efficiently purified under denaturing conditions and refolded in PBS and concentrated by centrifugal filter removing urea completely from protein suspension. After purification, about 45 mg/ml for each protein was derived as measured by Bradford Assay. Purification efficiency was demonstrated by SDS-PAGE analysis (Fig. 3-3)

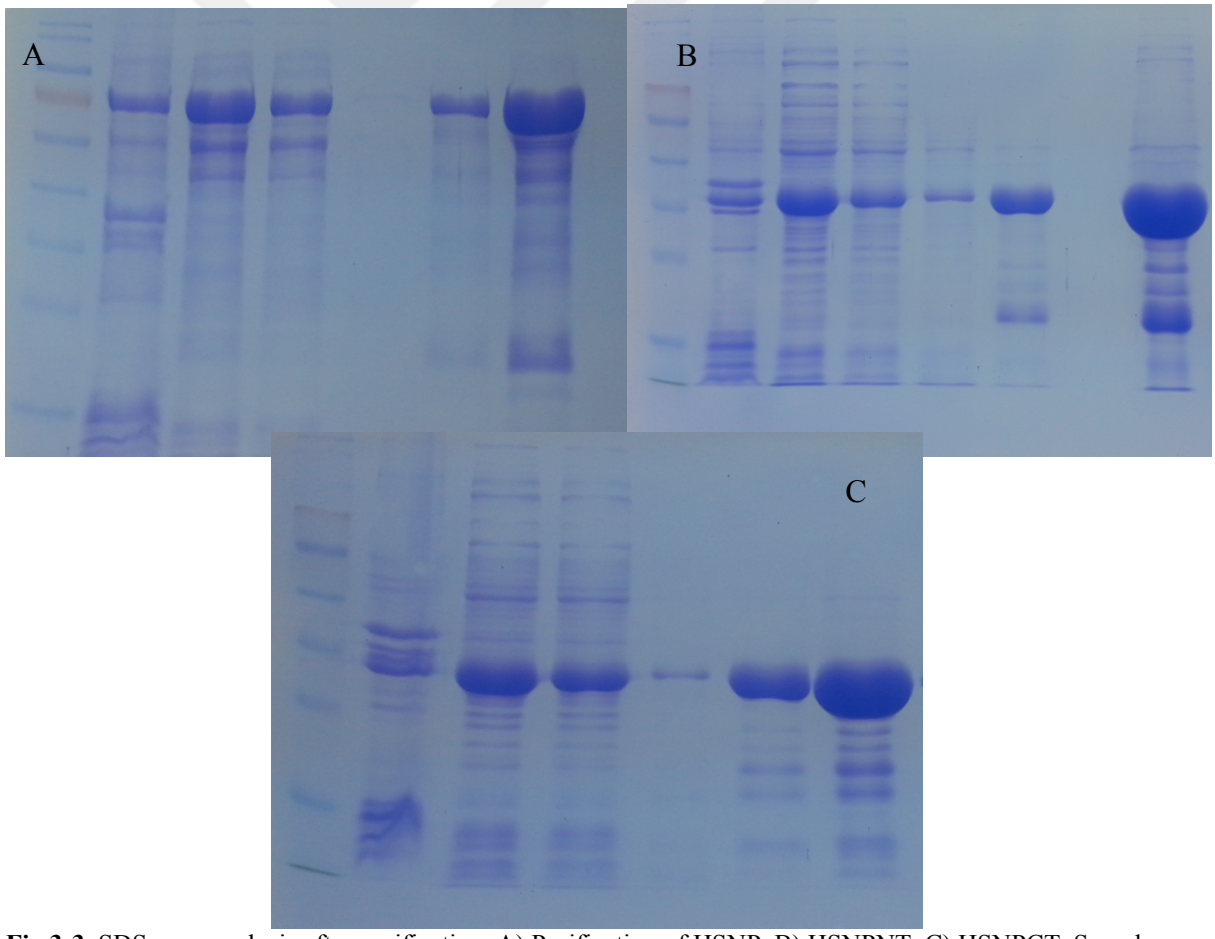


Fig 3-3. SDS page analysis after purification. A) Purification of HSNP. B) HSNPNT. C) HSNPCT. Sample were loaded on gel as pellet, lysis, flowthrough, wash, elution and consentration, respectively.

3.1.3. SUMO cleavage

According to the manufacturer's instructions, one unit of SUMO Protease (12588-018, Invitrogen Life Technologies) cleaves ≥85% of 2 µg protein in 1X SUMO Protease Buffer – Salt (50 mM Tris-HCl, pH 8.0, 0.2% Igepal, 1 mM DTT) at 30°C in 1hr. However, even 4 units enzyme was insufficient to cleave 1 µg fusion protein in spite of different optimizations including incubation temperature, time and salt concentration of buffer. (Fig. 3-4).

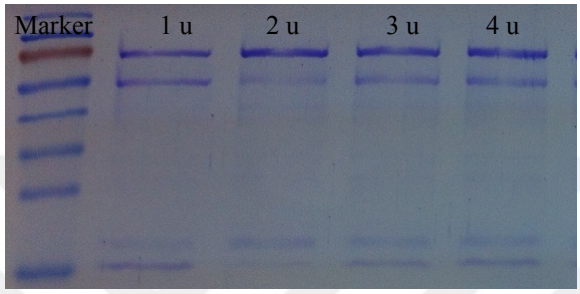


Fig. 3-4. Unefficient SUMO cleavage of proteins with up to 4 units of SUMO protease

3.1.4. Expression of CCHFV NP proteins using pET28b vector

Because of high cost and time consuming effect during the generation of native recombinant protein cleaved by SUMO protease, a different cloning strategy was developed for CCHFV NP proteins fused with only 6XHis tag commonly used in recombinant protein for antibody production. Because of its small size and low immunogenicity, polyhistidine tags does not need to be removed after purification [143]. Therefore CCHFV NP genes were cloned into pET28b and expressed with C-terminal polyhistidine (6x) tag. After that, proteins were called as NPH, NPNTH, NPCTH.

Directional cloning of genes were designed to be inserted into pET28b vector due to restriction enzymes, *XhoI* and *NcoI*. Gene sequences were amplified by restriction sites added primers used in PCR (Table 2-2). Following restriction digestion of both PCR products and pET28b vectors, ligated plasmids were transformed primarly into One Shot® Mach1™-T1^{R} competent *E. coli*. Plasmids were analyzed with both PCR (Fig. 3-5) and restriction digestion (Fig. 3-6). After sequence confirmation of 5 positive colonies for each proteins, positive strains (1.2.3 for NPH, 2.3.4 for NPNTH and 3.1.4 for NPCTH) were selected randomly and used for expression in One Shot® BL21(DE3) Star competent *E. coli* cells following transformation (Table 3-1).

Table 3-1. Sequenced bacterial strains containing pET28b plasmids encoding relative genes . Denomination rules: x: (NP:1, NPCT:2 NPNT:3), y: number for selected colony after positive transformation into One Shot® Mach1™-T1^R, z: number for selected colony after positive transformation into One Shot® BL21(DE3) Star

Host	Name	Protein	Backbone
BL21 Star	1.2.1	NPH (53 kDA)	pET28b
	1.2.2		
	1.2.3		
	1.2.4		
	1.2.5		
	2.3.1	NPNTH (14 kDa)	
	2.3.2		
	2.3.4		
	2.3.5		
	2.3.5		
	3.1.1	NPCTH (19 kDa)	
	3.1.2		
	3.1.3		
	3.1.4		
	3.1.5		

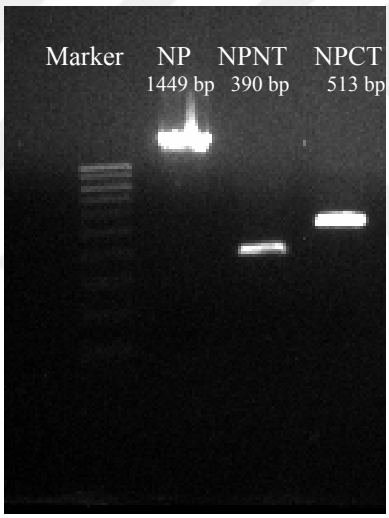


Fig. 3-5. Analysis of inserted CCHFV NP genes into pET28b plasmids by PCR. NP. Nucleoprotein, NT: N-terminal, CT: C-terminal

Each cells were induced with 1 mM IPTG and expressed at 37°C for 3 hour. SDS-PAGE analysis demonstrated that these pET28 based proteins also precipitated as inclusion bodies.

.

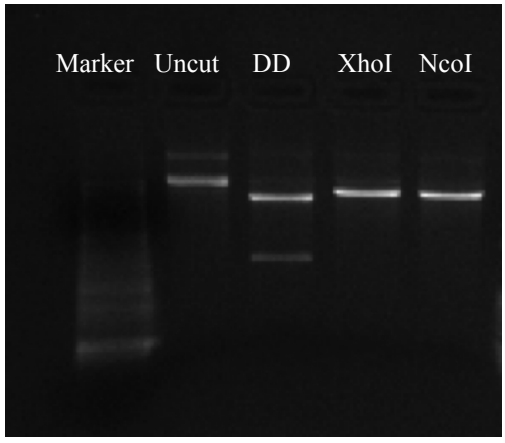


Fig. 3-6. Restriction analysis of isolated plasmids expressing NP. DD: double digest, XhoI: restriction only with XhoI, NcoI: restriction with NcoI.

3.1.5. Purification of proteins tagged with 6xHis

Proteins were purified succesfully under denaturing conditions using buffer without imidazole during binding on column and washing steps to increase the yield of eluted protein. Also binding of non-specific proteins on columns were substantially eliminated by adding 1% Tween-20 and 500 mM NaCl in all buffers. Proteins were eluted against 250 mM imidazole competing agent. Up to 4 mg/ml protein was obtainedd after desalting and refolding through PBS using Centricon® Plus-70 Centrifugal Filter Units.

3.2. Detection of Specific Antibodies Against Recombinantly Produced NPs

Whether recombinant NPs of CCHFV was able induce an antibody response, immunized mice sera, and human sera infected by CCHFV were tested in western blot analysis using recombinant NPs as antigens (Fig. 3-7). The western blot analysis has shown that all recombinant NP proteins had epitotopes recognised by both mouse antibodies generated after immunization with recombinant antigens and human antibodies obtained form CCHFV infected patient sera. Recognition by human antibodies also demonstrates recombinant proteins produced in this study carry native structures.

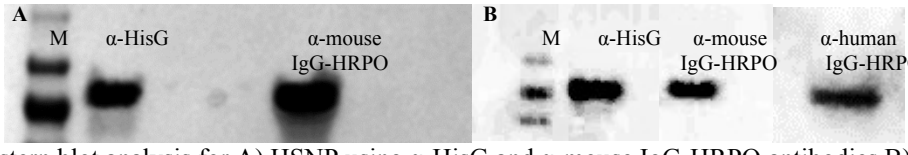


Fig. 3-7. Western blot analysis for A) HSNP using α-HisG and α-mouse IgG-HRPO antibodies B) NPH using α-HisG , α-mouse IgG-HRPO and α-human IgG-HRPO antibodies.

3.3. Recombinant CCHFV NPs Displays Cell Mediated Delayed Type Hypersensitivity In Mice

Mice were immunized s.c. with HSNP, HSNPNT, HSNPCT in a concentration of 50 µg/ml, 100 µg/ml and 200 µg/ml and with PBS for control.After prime immunization with antigen in a suspension with CFA; 3 more boost was applied s.c. with IFA. For a specific DTH response against CCHFV NPs, a potential effect of SUMO protein must have been eliminated. Therefore following 2 weeks after last immunization 100 µg/ml NPH, NPNTH, NPCTH was injected into right hind footpad while PBS into left. Footpad thickness was measured between 24-72 hours after injection. Swelling reaction was conspicuously visible, peaked at 24 hr and decreased by 72 hr for all groups. In behalf of their immunogenicty, N-terminal part of CCHFV NP developed higher DTH response than C-terminal part.

According to statistical analysis of DTH measurement, antigen injected footpads swelling was statistically significant for each groups, compared to negative control ($p<0,05$). However, DTH response has peaked at 24 hr for each protein. Between 3 different dose of injection to each proteins, 100 µg/µl was found to be optimum dose for DTH response although 200 µg/µl for NPCT and 50 µg/µl for NPNT were appeared to cause more swelling but results were not significant. Most potent DTH response was observed against NP, followed by NPNT. NPCT has showed weakest DTH (Fig. 3-8).

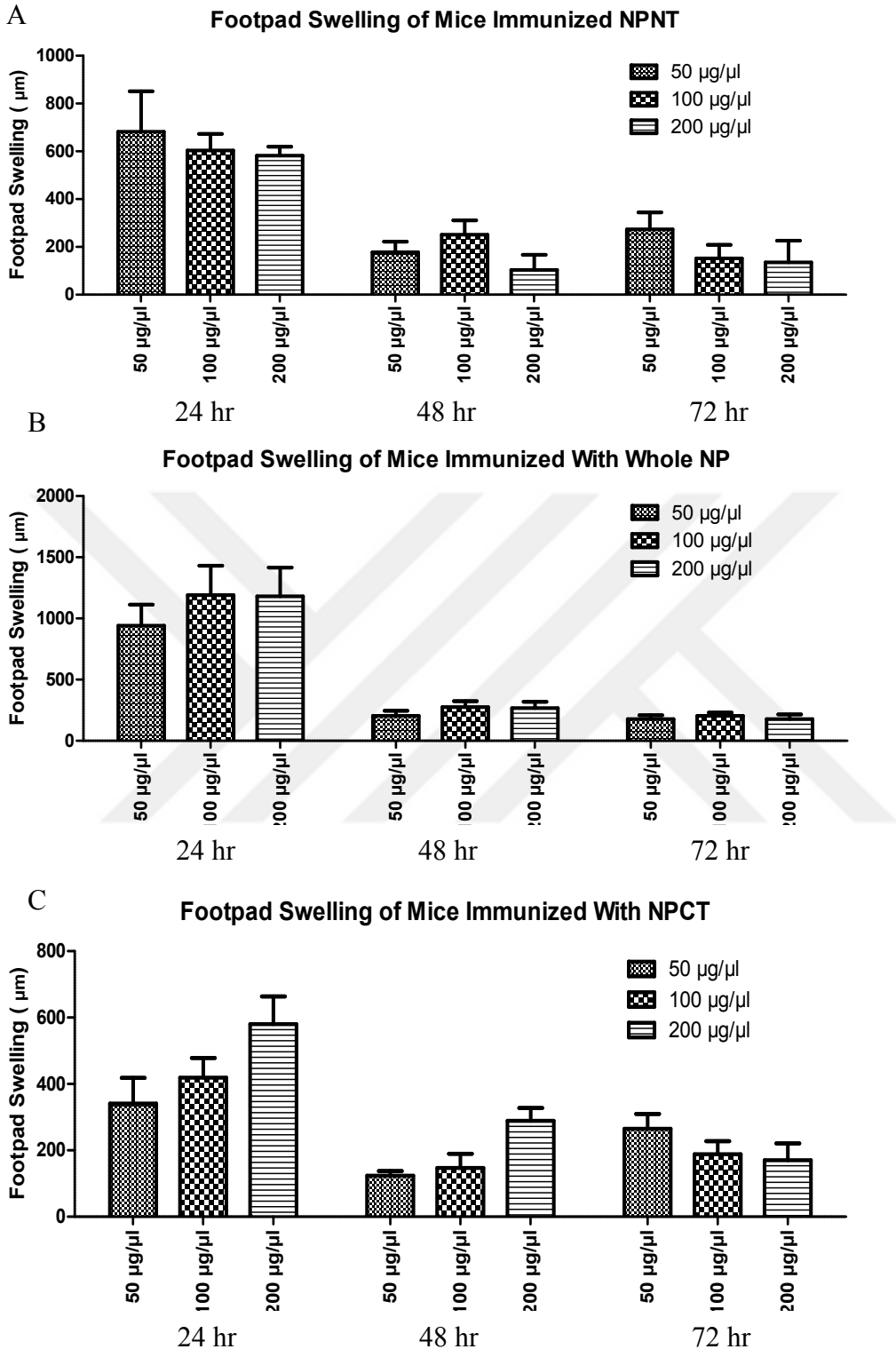


Fig.3-8. DTH measurement of antigen injected footpad swelling. A) Injection of NPH, B) of NPNTH, C) NPCTN in a concentration of 50 μg/μl, 100 μg/μl, 200 μg/μl. Swelling thickness was measured by SPI External Electronic Caliper Gages for 72 hr.

4. DISCUSSION

In this study, delayed type hypersensitivity response against CCHFV NP protein was analyzed in mice and N-terminal part of CCHFV NP developed stronger antigenicity than C-terminal part. Studies on cellular immune response to CCHFV and particularly against individual proteins are rather scarce. The exact role played by each protein in developing a cellular immunity against the whole virus have not been studied in detail. Thus any information on the individual proteins and on the cellular immune response will be a wellcomed addition to the data on CCHFV. These information would be used in developing startegies in designing vaccines as well as on antiviral strategies. In this study we chose to look at the delayed type immune response against a relativelly well known CCHFV protein NP. We have investigated the role of recombinantly produced CCHFV NP proteins on cell mediated DTH, and also scrutinized the parts of CCHFV NP leading to $CD4^+$ T cell activation.

The high risk of contagiousness, requirement of BSL-4 facilities and the lack of a suitable animal models limit researches on CCHFV. Therefore, different biotechnological methods were carried out *in vitro* with recombinantly produced viral proteins interacting with their host to understand its function on biology and immunity. *Escherichia coli* is the widely used organisms for the production of recombinant proteins because of its well-established cellular function. Therefore, many molecular tools and protocols are developed for the high-level production of heterologous proteins, such as a vast catalog of expression plasmids, a great number of engineered strains and many cultivation strategies [144]. With this aim, the Champion™ pET SUMO Expression System, produces high levels of soluble protein in *E. coli* was used in this study. Proteins are fused with small ubiquitin-related modifier (SUMO), belonging to the growing family of ubiquitin-related proteins, to enhance the solubility and stability which is then cleaved by the highly specific and active SUMO (ULP-1) protease at the carboxyl terminal, producing a native protein. This expression system is in use since 2009 and their success at high yield of recombinant protein production was reported by many articles as well as in this study [145] [146, 147] [148].

The negative-stranded RNA viruses are causative agents of several important human pathogens, including influenza, measles, mumps, rabies, respiratory syncytial, Ebola,

hantaviruses and also CCHFV [149]. The development of new biotechnological manipulation mechanism on the genomes of negative-stranded RNA viruses enables to structure-function studies and contributes to further information about pathogenicity of these viruses. As an example a biologically active influenza virus RNP complex was reconstituted by transfection of plasmids using reverse genetic system[150].

The genomes of all negative strand RNA viruses (NSVs) are encapsidated by the viral nucleoprotein forming ribonucleoprotein (RNP) complexes, responsible for viral transcription and replication [151]. However, the function of NP has not been completely clarified yet and little is known about the mechanism of NP assembly into RNPs or how the polymerase is able to read through NP-bound RNA templates. However studies on NP seem to be accumulated in recent years trying to understand interaction of NP both with viral and host proteins [152-154] [155, 156] [67, 157, 158].

Besides of cellular immunity, innate immune mechanism against NPs is also intrigiuing issue for researches. For example, human respiratory syncytial virus (hRSV) nucleoprotein antagonize the innate immune response mediated by MDA5 and MAVS [159]. Interferon-Inducible Protein Mx1 inhibits viral replication due to distruption of influenza ribonucleoprotein complexes [160]. In addition. antiviral role of CCHFV NP was reported by Andersson et.al for CCHFV [161]. They have showed that MxA colocalizes with the NP of CCHFV in the perinuclear regions of infected cells and inhibits the NP expression resulting in decreased level of new infectious viral particles [161]. Also suppression of type I interferon response can be mediated by NP of CCHFV strain Hoti [162].

A delayed type hypersensitivity reaction in the skin is based on antigen presentation by APCs (ie, Langerhans cells to sensitized memory T cells) and subsequent T-cell activation via CD3 and T-cell receptor (TCR)(CD3/TCR) complex, eliciting an influx of macrophages, monocyte, and lymphocytes at the site of antigen exposure. Following production of inflammatory cytokines including tumor necrosis factor-α (TNF-α), IL-17A, and IFN-γ by recruiting cells, vasopermeability is increased and adhesion molecules are up-regulated in the vascular endothelium, so that additional cellular components migrate into the local site of antigen presentation [163].

Tumor Necrosis Factor-α is a determinant cytokine for DTH responses and secreted by variety of cells including macrophages and Th1 cells but also by many cells from other lineages. Upon activation of endothelial cells, TNF-α up-regulates expression of adhesion

molecules on vascular endothelial cells, causing cytokine storm due to massive influx of inflammatory cells. Tumor Necrosis Factor-α also induces inflammatory cytokines including IL-1 which is crucial for IFN-γ induced DTH reactions in rodents [164]. Interleukin-12, another key cytokine associated with DTH is mainly produced by APC and Th1 cells. Iinterleukin-12 enhances production of IFN-γ, cytolytic actions of NK and CD8+ T cells and Th1 cell differentiation [163].

In general, cell-mediated immunity (CMI) is a T-cell–mediated defense mechanism and administers antimicrobial actions against microbes that survive within phagocytes or infect nonphagocytic cells. As a manifestation of CMI, T-cell–mediated delayad type hypersensitivity depends on interferon gamma (IFN-γ), a major cytokine released by type 1 T-helper (Th1) cells [163]. However, the contribution of IFN- γ to the induction of DTH differs among antigens. Studies with IFN-γ or interferon gamma receptor (IFN-γR) knock out (KO) mice, reported attenuated DTH responses during herpes simplex virus type-1 (HSV-1) infection [165, 166] but exhibition of normal DTH responses against *Mycobacterium tuberculosis* [167]. Additionally, in IFN-γ KO mice, DTH against KLH was suppressed [168, 169] while were exacerbated against OVA- and mBSA [170, 171]. Therefore, DTH cannot be solely explained by IFN-γ mediated Th1 responses. The identification of Th17 cells, secreting IL-17A, IL-17F, IL-21, IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF), and many other factors shed light on previously observed CMI in the absence of IFN-γ [163]. Studies with IL17a KO mice have showed attenuation in mBSA-induced DTH but increases in *Mycobacterium bovis bacille* Calmette–Guérin (BCG)-induced DTH and impaired mBSA-specific T-cell proliferation and mBSA-specific antibody production, suggesting that IL-17A plays significant role in T-cell sensitization and in antibody production [172, 173]. The role of Th17 and Th1 cells in CMI may vary depending on stimulants [174] although the contribution of Th17 cells in IFN- γ -dependent DTH has not yet been clarified.

Cell-mediated immunity (CMI) has both protective and pathogenic effects in a wide variety of viral infections [175, 176]. The expression of DTH is a manifestation of CMI *in vivo* [177, 178]. T cells takes an immunopathologic role in infections with lymphocytic choriomeningitis virus [179] and coxsackie B3 virus [176] and T cells mediating DTH to influenza virus may contribute to the pathology of influenza pneumonia in infected mice [180]. Delayed type hypersensitivity against Saint Louis encephalitis virus [181], Semliki

Forest virus [178], and Dengue viruses were developed both *in vitro* [182] and *in vivo* assays [183]. Immunosupressive therapy with antithymocyte serum effectively increased the survival time of mice infected with Langat virus [184] and survival rate of nude, athymic mice was significantly higher than normal mice infected with Dengue virus [178]. Although DTH reactions plays a vital role in the primary immune defense mechanism against many viral infections, DTH response following antibody activation could be harmful to host after reinfection with a virus. Coexistence of both protective and pathological features of CMI in most virus infections also theorised by Doherty [185].

Viral infections have both stimulatory and suppressive effects on CMI responses in different cases. For example upon infection with a variety of viruses, depression of CMI to heterologous antigens has been reported in humans as well as in experimental animals [186]. In Dengue virus infection model, DTH to sheep red blood cells was significantly depressed [187], and approximately one-third of T cells and macrophages were killed or destroyed [178]. Depression of CMI responses is explained by a variety of mechanisms [159], but a disturbance or modulation of immune regulatory mechanisms (e.g., T-helper or T- suppressor cells) triggered during viral infections is usuaaly accounted as a culprit [186]. Interferon gamma also exerts regulatory actions on $CD4^+$ T cells such as induced T cell death and contribution to lymphocyte homeostasis [188]. Interferon gamma stimulates also converting of $CD4^+$ $CD25^-$ Th cells into inducible $CD4^+$ $CD25^+$ regulatory T (Treg) cells by inducing Foxp3 expression. Treg cells induced by IFN-γ produce IL-10 and TGF-ß in the late stage of DTH–induced inflammation to self-limit DTH reactions [188]. Therefore, deficiency of IFN-γ is eventually associated with excessive DTH reactions in patients [163]. It should be considered that effector Th cells can change lineage-specific functions according to their microenvironment. Th17 cells have flexibility to secrete Th1 cytokines and also switch to Treg phenotype, which assists for maintaining immune homeostasis, minimizing tissue damage caused by DTH responses and for effective microbial clearance [189, 190]. T reg cells produce TGF-ß and IL-10, a counter-regulatory cytokine, and IL-10 down-regulates production of Th1/Th17 cytokines or counteracts to Th1/Th17 cytokines [163].

Cell mediated immunity is considered to be significant in chronic, persistent and latent virus infections [191]. Therefore it is essential to clarify the role of cellular immunity in CCHFV infection. Because of its uncomplicated and practical usage, skin test is widely applied in determining the level of cellular immunity of some virus infections [192, 193]. As

an example, during Measles virus (MV) infection leads to a strong but temporary depression of cell-mediated immunity (CMI) which distrubts the viral elimination process [194]. Immunosuppresion during measles infection was observed by skin test with tuberculin or other antigens for several weeks after recovery from infection [195, 196]. In Dengue haemorrhagic fever, significant inflammatory and hypersensitivity damage to endothelial cells is induced by antigen specific T-cells and specific antibodies, which leads to haemorrhage. CD4 helper T cells, macrophages and polymorphonuclear leukocytes are main cell types inducing DTH in many viral infections caused by enveleoped viruses [140].

Since initial clinical features of Dengue hemorrhagic fever (DHF) and CCHFV are smilar, it is useful to look through pathogenesis of DHF [197]. Cell mediated immunity, expressed by a systemic manifestation of a delayed-type hypersensitivity (DTH) reaction, is the main event that results in the shock and hemorrhage observed in cases of DHF [183]. Humoral and CMI (T cells mediating DTH and cytotoxic T cells) are believed to be crucial for the recovery from primary infection of Dengue virus [183]. However, a transient immunosuppression is induced at the same time, resulting in immunoregulatory disturbances (e.g., imbalances in T-helper and T-suppressor cells) and the formation of enhancing antibodies. In the case of a second infection with heterologous Dengue virus, enhancing antibodies promote virus replication in monocytes (Fig. 5-1). Thereby virus can spread to bone marrow, liver, spleen, gut, lymphoid tissues, and histiocytes in the skin. These Dengue-infected monocytes may become the target cells for an immune elimination response as proposed by Halstead [1-3] and T cells mediating DTH or cytotoxic T cells release a variety of factors (e.g., vascular permeability factor, complement-activating factors, and thromboplastin), causing shock and hemorrhage [178]. Endogenous pyrogens could be also released by activated macrophages, which may lead to fever [178]. The possible role played by DTH response in the pathogenesis of Dengue infection has also been addressed by others [178, 198].

In this study, the presence of DTH reactions against CCHFV NP was investigated in Mouse model. The results of this study demonstrate that, CCHFV NP contains $CD4^+$ T cell epitopes and especially $NP^{124-507}$ includes highly antigenic regions of NP supporting the results of others [199-202]. Lymphoproliferation assay performed with the same panel of recombinantly produced NPs also support DTH response date obtaine here (unpublished data). Further studies on the exact nature of $CD4^+$ T cell epitopes on NP and other viral

proteins as well as other arms of CMI and humoral immunity will contribute to the delineation of the full picture on immunology and pathogenesis of CCHFV infection.

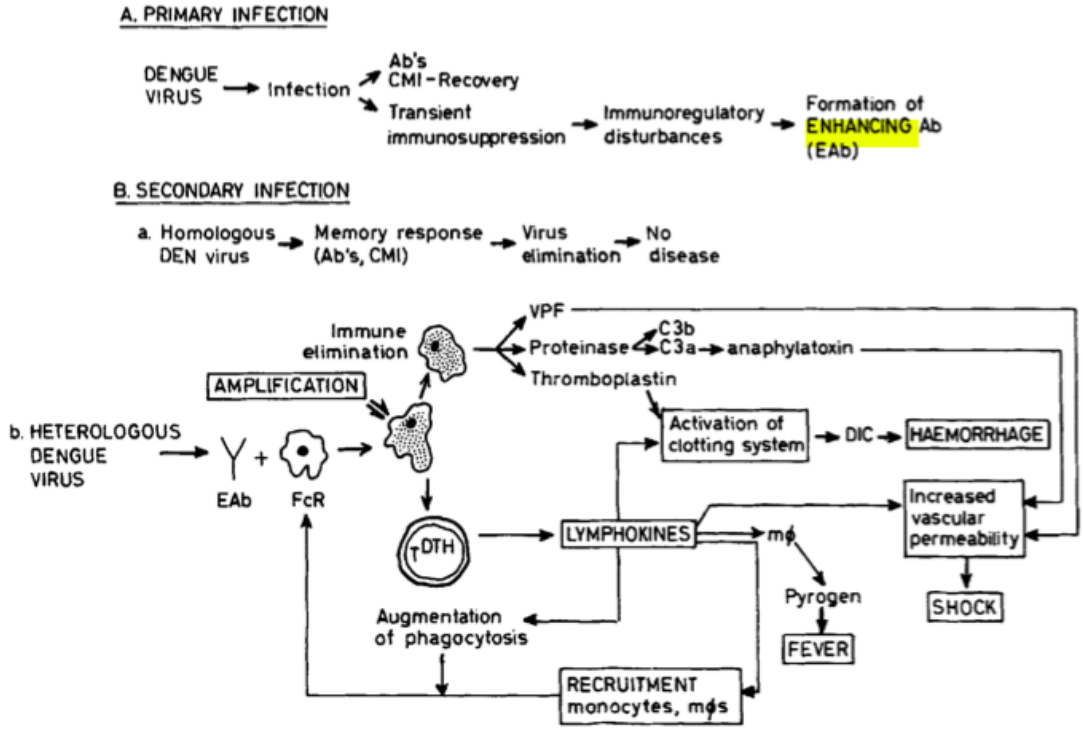


Figure 4-1. DTH during the pathogenesis of dengue infections. Ab's = antibodies; EAb = enhancing antibody; CMI = cell-mediated immunity; VPF = vascular permeability factor; DIC = disseminated intravascular coagulation; FcR = receptor for Fe portion of immunoglobulin; me = macrophage; TDTH = T cells mediating DTH.

6. Uncategorized References

1. Mayer, C.F., *Epidemic hemorrhagic fever of the Far East, or endemic hemorrhagic nephroso-nephritis; a short outline of the disease, with supplemental data on the*

results of experimental inoculation of human volunteers. Mil Surg, 1952. **110**(4): p. 276-84.
2. Chen, J.P.C., T. M., *Hemorrhagic fever virus-induced changes in hemostasis and vascular biology.* Blood Coagulation & Fibrinolysis, 2000. **11**(5): p. 461-483.
3. Chinikar, S., et al., *Crimean-Congo hemorrhagic fever in Iran and neighboring countries.* J Clin Virol, 2010. **47**(2): p. 110-4.
4. Bente, D.A., et al., *Pathogenesis and immune response of Crimean-Congo hemorrhagic fever virus in a STAT-1 knockout mouse model.* J Virol, 2010. **84**(21): p. 11089-100.
5. Bente, D.A., et al., *Crimean-Congo hemorrhagic fever: history, epidemiology, pathogenesis, clinical syndrome and genetic diversity.* Antiviral Res, 2013. **100**(1): p. 159-89.
6. Ergönül, Ö., *Crimean-Congo haemorrhagic fever.* The Lancet Infectious Diseases, 2006. **6**(4): p. 203-214.
7. Karti, S.S., Odabasi, Z., Korten, V., Yilmaz, M., Sonmez, M., E.A. Rahmet Caylan, Necmi Eren, Iftihar Koksal, Ercument Ovali, Bobbie R. Erickson,, and S.T.N. Martin J. Vincent, James A. Comer, Pierre E. Rollin, and Thomas G. Ksiazek, *Crimean-Congo Hemorrhagic Fever in Turkey.* Emerging Infectious Diseases, 2004. **10**(8): p. 1379–1384.
8. Keshtkar-Jahromi, M., et al., *Crimean-Congo hemorrhagic fever in Iran.* Antiviral Res, 2013. **100**(1): p. 20-8.
9. Whitehouse, C.A., *Crimean-Congo hemorrhagic fever.* Antiviral Res, 2004. **64**(3): p. 145-60.
10. Chumakov, M.P., *On 30 years of investigation of Crimean hemorrhagic fever* Tr. Inst. Polio Virusn. Entsefalitov Akad. Med. Nauk SSSR
1974. (22): p. 5-18.
11. Hoogstraal, H., *The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa.* J Med Entomol, 1979. **15**(4): p. 307-417.
12. Dols, M.W., *The Black Death in the Middle East : by Michael W. Dols*. 1977: Princeton University Press. 390 pp.
13. Chumakov, M.P., *A new tick-borne virus disease - Crimean hemorrhagic fever.* 1945 p. pp. 13–45.
14. Chumakov, M.P., *A new virus disease—Crimean hemorrhagic fever.* Nov. Med. , 1947(4): p. 9-11.
15. Chumakov, M.P., et al., *[New data on the viral agent of Crimean hemorrhagic fever].* Vopr Virusol, 1968. **13**(3): p. 377.
16. Butenko, A.M., Chumakov, M.P., Bashkirtsev, V.N., Zavodova, T.I., and E.A. Tkachenko, Rubin, S.G., Stolbov, D.N., *Isolation and investigation of Astrakhan strain ("Drozdov") of Crimean hemorrhagic fever virus and data on serodiagnosis of this infection.* Nauchn. Sess. Inst. Polio Virus Ensefalitis, 1968(3): p. 88-90 (in Russian; in English, NAMRU3-T866).
17. Simpson, D.I., et al., *Congo virus: a hitherto undescribed virus occurring in Africa. I. Human isolations--clinical notes.* East Afr Med J, 1967. **44**(2): p. 86-92.
18. Woodall, J.P., Williams, M.C., Simpson, D.I.H., *Congo virus: a hitherto undescribed virus occurring in Africa. II. Identification studies.* East Afr. Med. J. , 1967(44): p. 93-98.

19. Andersson, I., *Crimean Congo Hemorrhagic Fever Virus interferon-induced antiviral mechanisms and immune evasion strategies*, in *Department of Microbiology, Tumor and Cell Biology*. 2008, Karolinska Institutet: Stockholm, Sweden.
20. Nichol, S.T., *Bunyaviruses*, P.M.H. D.M. Knipe, D.E. Griffen, R.A. Lamb, M.A. Martin, B. Roizman, S.E. Strauss, Editor. 2001, Fields Virology: Lippincott Williams & Wilkins, Philadelphia. p. 1603–1634.
21. R.M. Elliott, M.B., C.H. Calisher, R. Goldbach, J.T. Moyer, S.T. Nichol, R. Pettersson, A. Plyusnin, C.S. Schmaljohn, *Family Bunyaviridae*, in *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*, C.M.F. M.H.V. van Regenmortel, D.H.L. Bishop, Editor. 2000, Academic Press: San Diego. p. 599–621.
22. Begum, F., C.L. Wisseman, Jr., and J. Casals, *Tick-borne viruses of West Pakistan. IV. Viruses similar to or identical with, Crimean hemorrhagic fever (Congo-Semunya), Wad Medani and Pak Argas 461 isolated from ticks of the Changa Manga Forest, Lahore District, and of Hunza, Gilgit Agency, W. Pakistan.* Am J Epidemiol, 1970. **92**(3): p. 197-202.
23. Stuart D. Dowall, S.F.-W., Emma Rayner, Geoff Pearson, Janice Pickersgill, Antony Rule, Natasha Merredew, Hazel Smith, John Chamberlain and Roger Hewson, *Hazara virus infection is lethal for adult type I interferon receptor-knockout mice and may act as a surrogate for infection with the human-pathogenic Crimean–Congo hemorrhagic fever virus.* Journal of General Virology, 2012(93): p. 560-564.
24. Flick, R., *Molecular Biology of the Crimean-Congo Hemorrhagic Fever Virus*, in *CRIMEAN-CONGO HEMORRHAGIC FEVER*. 2007, Springer: Dordrecht. p. 35-44
25. Clerex-Van Haaster, C.M., et al., *The 3′ terminal RNA sequences of bunyaviruses and nairoviruses (Bunyaviridae): evidence of end sequence generic differences within the virus family.* The Journal of general virology, 1982. **61 (Pt 2)**: p. 289-292.
26. Morikawa, S., M. Saijo, and I. Kurane, *Recent progress in molecular biology of Crimean-Congo hemorrhagic fever.* Comp Immunol Microbiol Infect Dis, 2007. **30**(5-6): p. 375-89.
27. Marriott, A.C. and P.A. Nuttall, *Large RNA segment of Dugbe nairovirus encodes the putative RNA polymerase.* J Gen Virol, 1996. **77 (Pt 8)**: p. 1775-80.
28. Schmaljohn CS, H.J., *Bunyaviridae: the viruses and their replication*, in *Fields Virology*, D.M.K.a.P.M. Howley, Editor. 2001, Lippincott Williams & Wilkins: Philadelphia. p. 1581-1602.
29. Bergeron, E., M.J. Vincent, and S.T. Nichol, *Crimean-Congo hemorrhagic fever virus glycoprotein processing by the endoprotease SKI-1/S1P is critical for virus infectivity.* J Virol, 2007. **81**(23): p. 13271-6.
30. Erickson, B.R., et al., *N-linked glycosylation of Gn (but not Gc) is important for Crimean Congo hemorrhagic fever virus glycoprotein localization and transport.* Virology, 2007. **361**(2): p. 348-55.
31. Macleod, J.M., et al., *Mapping of the interaction domains of the Crimean-Congo hemorrhagic fever virus nucleocapsid protein.* J Gen Virol, 2015. **96**(Pt 3): p. 524-37.
32. Sanchez, A.J., M.J. Vincent, and S.T. Nichol, *Characterization of the Glycoproteins of Crimean-Congo Hemorrhagic Fever Virus.* Journal of Virology, 2002. **76**(14): p. 7263-7275.
33. Altamura, L.A., et al., *Identification of a novel C-terminal cleavage of Crimean-Congo hemorrhagic fever virus PreGN that leads to generation of an NSM protein.* J Virol, 2007. **81**(12): p. 6632-42.

34. Hewson, R., et al., *Crimean-Congo haemorrhagic fever virus: sequence analysis of the small RNA segments from a collection of viruses world wide.* Virus Res, 2004. **102**(2): p. 185-9.
35. Carter, S.D., et al., *Structure, function, and evolution of the Crimean-Congo hemorrhagic fever virus nucleocapsid protein.* J Virol, 2012. **86**(20): p. 10914-23.
36. Karlberg, H., Y.J. Tan, and A. Mirazimi, *Induction of caspase activation and cleavage of the viral nucleocapsid protein in different cell types during Crimean-Congo hemorrhagic fever virus infection.* J Biol Chem, 2011. **286**(5): p. 3227-34.
37. Wang, Y., et al., *Structure of Crimean-Congo hemorrhagic fever virus nucleoprotein: superhelical homo-oligomers and the role of caspase-3 cleavage.* J Virol, 2012. **86**(22): p. 12294-303.
38. Andersson, I., et al., *Role of actin filaments in targeting of Crimean Congo hemorrhagic fever virus nucleocapsid protein to perinuclear regions of mammalian cells.* J Med Virol, 2004. **72**(1): p. 83-93.
39. Taylor, M.P., O.O. Koyuncu, and L.W. Enquist, *Subversion of the actin cytoskeleton during viral infection.* Nat Rev Microbiol, 2011. **9**(6): p. 427-39.
40. Deyde, V.M., et al., *Crimean-Congo hemorrhagic fever virus genomics and global diversity.* J Virol, 2006. **80**(17): p. 8834-42.
41. Vincent, M.J., et al., *Crimean-Congo Hemorrhagic Fever Virus Glycoprotein Proteolytic Processing by Subtilase SKI-1.* Journal of Virology, 2003. **77**(16): p. 8640-8649.
42. Sanchez, A.J., et al., *Crimean-congo hemorrhagic fever virus glycoprotein precursor is cleaved by Furin-like and SKI-1 proteases to generate a novel 38-kilodalton glycoprotein.* J Virol, 2006. **80**(1): p. 514-25.
43. Jeffers, S.A., D.A. Sanders, and A. Sanchez, *Covalent modifications of the ebola virus glycoprotein.* J Virol, 2002. **76**(24): p. 12463-72.
44. Simmons, G., et al., *Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence.* J Virol, 2002. **76**(5): p. 2518-28.
45. Sullivan, N.J., et al., *Ebola virus glycoprotein toxicity is mediated by a dynamin-dependent protein-trafficking pathway.* J Virol, 2005. **79**(1): p. 547-53.
46. Bertolotti-Ciarlet, A., et al., *Cellular localization and antigenic characterization of crimean-congo hemorrhagic fever virus glycoproteins.* J Virol, 2005. **79**(10): p. 6152-61.
47. Haferkamp, S., et al., *Intracellular localization of Crimean-Congo Hemorrhagic Fever (CCHF) virus glycoproteins.* Virol J, 2005. **2**: p. 42.
48. Andersson, C., *Virus-host interactions: Entry and replication of crimean-congo hemorrhagic fever virus*, in *Department of microbiology, tumor and cell biology*. 2013, Karolinska Institutet: Stockholm.
49. Shi, X., et al., *Visualizing the replication cycle of bunyamwera orthobunyavirus expressing fluorescent protein-tagged Gc glycoprotein.* J Virol, 2010. **84**(17): p. 8460-9.
50. Estrada, D.F. and R.N. De Guzman, *Structural Characterization of the Crimean-Congo Hemorrhagic Fever Virus Gn Tail Provides Insight into Virus Assembly.* The Journal of Biological Chemistry, 2011. **286**(24): p. 21678-21686.
51. Honig, J.E., J.C. Osborne, and S.T. Nichol, *Crimean-Congo hemorrhagic fever virus genome L RNA segment and encoded protein.* Virology, 2004. **321**(1): p. 29-35.

52. Bergeron, E., et al., *Crimean-Congo hemorrhagic fever virus-encoded ovarian tumor protease activity is dispensable for virus RNA polymerase function.* J Virol, 2010. **84**(1): p. 216-26.
53. Frias-Staheli, N., et al., *Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses.* Cell Host Microbe, 2007. **2**(6): p. 404-16.
54. Loureiro, J. and H.L. Ploegh, *Antigen presentation and the ubiquitin-proteasome system in host-pathogen interactions.* Adv Immunol, 2006. **92**: p. 225-305.
55. Shin, J.S., et al., *Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination.* Nature, 2006. **444**(7115): p. 115-8.
56. Chen, Z.J., *Ubiquitin signalling in the NF-kappaB pathway.* Nat Cell Biol, 2005. **7**(8): p. 758-65.
57. Gack, M.U., et al., *TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity.* Nature, 2007. **446**(7138): p. 916-920.
58. Srinivasan, P., et al., *Epitope-based immunoinformatics and molecular docking studies of nucleocapsid protein and ovarian tumor domain of crimean-congo hemorrhagic Fever virus.* Front Genet, 2011. **2**: p. 72.
59. Xiao, X., et al., *Identification of a putative Crimean-Congo hemorrhagic fever virus entry factor.* Biochem Biophys Res Commun, 2011. **411**(2): p. 253-8.
60. Gonzalez-Scarano, F., N. Pobjecky, and N. Nathanson, *La Crosse bunyavirus can mediate pH-dependent fusion from without.* Virology, 1984. **132**(1): p. 222-5.
61. Simon, M., C. Johansson, and A. Mirazimi, *Crimean-Congo hemorrhagic fever virus entry and replication is clathrin-, pH- and cholesterol-dependent.* J Gen Virol, 2009. **90**(Pt 1): p. 210-5.
62. Marsh, M. and A. Helenius, *Virus entry: open sesame.* Cell, 2006. **124**(4): p. 729-40.
63. Simon, M., et al., *Microtubule-dependent and microtubule-independent steps in Crimean-Congo hemorrhagic fever virus replication cycle.* Virology, 2009. **385**(2): p. 313-22.
64. Schmaljohn CS, N.S., *Bunyaviridae*, in *Fields Virology* H.P. Knipe DM, Editor. 2007, Lippincott Williams and Wilkins: USA.
65. Devignot, S., et al., *A Virus-Like Particle System Identifies the Endonuclease Domain of Crimean-Congo Hemorrhagic Fever Virus.* Journal of Virology, 2015. **89**(11): p. 5957-5967.
66. Reguera, J., F. Weber, and S. Cusack, *Bunyaviridae RNA polymerases (L-protein) have an N-terminal, influenza-like endonuclease domain, essential for viral cap-dependent transcription.* PLoS Pathog, 2010. **6**(9): p. e1001101.
67. Guo, Y., et al., *Crimean-Congo hemorrhagic fever virus nucleoprotein reveals endonuclease activity in bunyaviruses.* Proc Natl Acad Sci U S A, 2012. **109**(13): p. 5046-51.
68. Kraus A. A and M. A., *Molecular Biology and Pathogenesis of Crimean–Congo Hemorrhagic Fever Virus.* Future Virology, 2010. **5**(4): p. 469-479.
69. Matsuoka, Y., S.Y. Chen, and R.W. Compans, *Bunyavirus protein transport and assembly.* Curr Top Microbiol Immunol, 1991. **169**: p. 161-79.
70. Walter, C.T. and J.N. Barr, *Recent advances in the molecular and cellular biology of bunyaviruses.* J Gen Virol, 2011. **92**(Pt 11): p. 2467-84.
71. Altamura, L.A., *Crimean-Congo hemorrhagic fever virus: Identification of an NS(M) protein and oligomerization of the viral glycoproteins*. 2008, Faculties of the University of Pennsylvania.

72. Tahmasebi, F., et al., *Molecular epidemiology of Crimean- Congo hemorrhagic fever virus genome isolated from ticks of Hamadan province of Iran.* J Vector Borne Dis, 2010. **47**(4): p. 211-6.
73. Bell-Sakyi, L., et al., *Tick Cell Lines for Study of Crimean-Congo Hemorrhagic Fever Virus and Other Arboviruses.* Vector Borne and Zoonotic Diseases, 2012. **12**(9): p. 769-781.
74. Gargili, A., S. Thangamani, and D. Bente, *Influence of laboratory animal hosts on the life cycle of Hyalomma marginatum and implications for an in vivo transmission model for Crimean-Congo hemorrhagic fever virus.* Front Cell Infect Microbiol, 2013. **3**: p. 39.
75. Ozdarendeli, A., et al., *The complete genome analysis of Crimean-Congo hemorrhagic fever virus isolated in Turkey.* Virus Res, 2010. **147**(2): p. 288-93.
76. Dickson, D.L. and M.J. Turell, *Replication and Tissue Tropisms of Crimean-Congo Hemorrhagic Fever Virus in Experimentally Infected Adult Hyalomma truncatum (Acari: Ixodidae).* Journal of Medical Entomology, 1992. **29**(5): p. 767-773.
77. Dohm, D.J., et al., *Transmission of Crimean-Congo Hemorrhagic Fever Virus by Hyalomma impeltatum (Acari: Ixodidae) after Experimental Infection.* Journal of Medical Entomology, 1996. **33**(5): p. 848-851.
78. Gordon, S.W., K.J. Linthicum, and J.R. Moulton, *Transmission of Crimean-Congo hemorrhagic fever virus in two species of Hyalomma ticks from infected adults to cofeeding immature forms.* American Journal of Tropical Medicine and Hygiene, 1993. **48**(4): p. 576-580.
79. Logan, T.M., et al., *Replication of Crimean-Congo Hemorrhagic Fever Virus in Four Species of Ixodid Ticks (Acari) Infected Experimentally.* Journal of Medical Entomology, 1990. **27**(4): p. 537-542.
80. Gonzalez, J.P., et al., *Sexual and transovarian transmission of Crimean-Congo haemorrhagic fever virus in Hyalomma truncatum ticks.* Research in Virology, 1992. **143**: p. 23-28.
81. Chinikar, S., et al., *Crimean-Congo Hemorrhagic Fever (CCHF)*, in *Zoonosis*, J. Lorenzo-Morales, Editor. 2012, In Tech. p. 193-212.
82. DVM, P.F., et al., *International Surveillance and Control of Crimean-Congo Hemorrhagic Fever Outbreaks*, in *Crimean-Congo Hemorrhagic Fever: A Global Perspective*, O. Ergonul and C.A. Whitehouse, Editors. 2007, Springer Netherlands: Dordrecht. p. 295-303.
83. Honig, J.E., J.C. Osborne, and S.T. Nichol, *The high genetic variation of viruses of the genus Nairovirus reflects the diversity of their predominant tick hosts.* Virology, 2004. **318**(1): p. 10-16.
84. Ahmeti, S. and L. Raka, *Crimean-Congo haemorrhagic fever in Kosova : a fatal case report.* Virol J, 2006. **3**: p. 85.
85. Bakir, M., et al., *Crimean-Congo haemorrhagic fever outbreak in Middle Anatolia: a multicentre study of clinical features and outcome measures.* J Med Microbiol, 2005. **54**(Pt 4): p. 385-9.
86. Ergonul, O., et al., *Characteristics of patients with Crimean-Congo hemorrhagic fever in a recent outbreak in Turkey and impact of oral ribavirin therapy.* Clin Infect Dis, 2004. **39**(2): p. 284-7.
87. Ozkurt, Z., et al., *Crimean-Congo hemorrhagic fever in Eastern Turkey: clinical features, risk factors and efficacy of ribavirin therapy.* J Infect, 2006. **52**(3): p. 207-15.

88. Connolly-Andersen, A.-M., *Pathogenesis of an emerging pathogen CCHF*, in *Department of Microbiology, Tumor and Cell Biology*. 2010, Karolinska Institutet and the Swedish Institute for Infectious Disease Control: Stockholm, Sweden.
89. Roebuck, K.A. and A. Finnegan, *Regulation of intercellular adhesion molecule-1 (CD54) gene expression.* J Leukoc Biol, 1999. **66**(6): p. 876-88.
90. Sumpio, B.E., J.T. Riley, and A. Dardik, *Cells in focus: endothelial cell.* Int J Biochem Cell Biol, 2002. **34**(12): p. 1508-12.
91. Vestweber, D., *Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium.* Immunol Rev, 2007. **218**: p. 178-96.
92. Bodur, H., et al., *Evidence of vascular endothelial damage in Crimean-Congo hemorrhagic fever.* Int J Infect Dis, 2010. **14**(8): p. e704-7.
93. Ozturk, B., et al., *Evaluation of the association of serum levels of hyaluronic acid, sICAM-1, sVCAM-1, and VEGF-A with mortality and prognosis in patients with Crimean-Congo hemorrhagic fever.* J Clin Virol, 2010. **47**(2): p. 115-9.
94. Akinci, E., H. Bodur, and H. Leblebicioglu, *Pathogenesis of crimean-congo hemorrhagic Fever.* Vector Borne Zoonotic Dis, 2013. **13**(7): p. 429-37.
95. Chaturvedi, U.C., et al., *Breakdown of the blood-brain barrier during dengue virus infection of mice.* J Gen Virol, 1991. **72 (Pt 4)**: p. 859-66.
96. Günther, S., et al., *Lassa Fever Encephalopathy: Lassa Virus in Cerebrospinal Fluid but Not in Serum.* Journal of Infectious Diseases, 2001. **184**(3): p. 345-349.
97. Domingues, R.B., et al., *Involvement of the central nervous system in patients with dengue virus infection.* J Neurol Sci, 2008. **267**(1-2): p. 36-40.
98. Kang, S.S. and D.B. McGavern, *Microbial induction of vascular pathology in the CNS.* J Neuroimmune Pharmacol, 2010. **5**(3): p. 370-86.
99. Peyrefitte, C.N., et al., *Differential activation profiles of Crimean-Congo hemorrhagic fever virus- and Dugbe virus-infected antigen-presenting cells.* J Gen Virol, 2010. **91**(Pt 1): p. 189-98.
100. Connolly-Andersen, A.M., et al., *Crimean Congo hemorrhagic fever virus infects human monocyte-derived dendritic cells.* Virology, 2009. **390**(2): p. 157-62.
101. Ergonul, O., et al., *Evaluation of serum levels of interleukin (IL)-6, IL-10, and tumor necrosis factor-alpha in patients with Crimean-Congo hemorrhagic fever.* J Infect Dis, 2006. **193**(7): p. 941-4.
102. Papa, A., et al., *Cytokine levels in Crimean-Congo hemorrhagic fever.* J Clin Virol, 2006. **36**(4): p. 272-6.
103. Favara, B.E., *Hemophagocytic lymphohistiocytosis: a hemophagocytic syndrome.* Semin Diagn Pathol, 1992. **9**(1): p. 63-74.
104. Fisman, D.N., *Hemophagocytic syndromes and infection.* Emerg Infect Dis, 2000. **6**(6): p. 601-8.
105. Weber, F. and A. Mirazimi, *Interferon and cytokine responses to Crimean Congo hemorrhagic fever virus; an emerging and neglected viral zonoosis.* Cytokine Growth Factor Rev, 2008. **19**(5-6): p. 395-404.
106. Samuel, C.E., *Antiviral actions of interferons.* Clin Microbiol Rev, 2001. **14**(4): p. 778-809, table of contents.
107. Andersson, I., et al., *Crimean-Congo hemorrhagic fever virus delays activation of the innate immune response.* J Med Virol, 2008. **80**(8): p. 1397-404.
108. Andersson, I., et al., *Human MxA Protein Inhibits the Replication of Crimean-Congo Hemorrhagic Fever Virus.* Journal of Virology, 2004. **78**(8): p. 4323-4329.

109. Andersson, I., et al., *Type I interferon inhibits Crimean-Congo hemorrhagic fever virus in human target cells.* J Med Virol, 2006. **78**(2): p. 216-22.
110. Billecocq, A., et al., *NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription.* J Virol, 2004. **78**(18): p. 9798-806.
111. Bouloy, M., et al., *Genetic evidence for an interferon-antagonistic function of rift valley fever virus nonstructural protein NSs.* J Virol, 2001. **75**(3): p. 1371-7.
112. Spengler, J.R., et al., *RIG-I Mediates an Antiviral Response to Crimean-Congo Hemorrhagic Fever Virus.* J Virol, 2015. **89**(20): p. 10219-29.
113. van Kasteren, P.B., et al., *Arterivirus and Nairovirus Ovarian Tumor Domain-Containing Deubiquitinases Target Activated RIG-I To Control Innate Immune Signaling.* J Virol, 2012. **86**(2): p. 773-85.
114. Liu, Y.C., J. Penninger, and M. Karin, *Immunity by ubiquitylation: a reversible process of modification.* Nat Rev Immunol, 2005. **5**(12): p. 941-52.
115. Ergonul, O., *Crimean-Congo hemorrhagic fever virus: new outbreaks, new discoveries.* Curr Opin Virol, 2012. **2**(2): p. 215-20.
116. Schwarz, T.F., H. Nsanze, and A.M. Ameen, *Clinical features of Crimean-Congo haemorrhagic fever in the United Arab Emirates.* Infection, 1997. **25**(6): p. 364-7.
117. Swanepoel, R., et al., *The clinical pathology of Crimean-Congo hemorrhagic fever.* Rev Infect Dis, 1989. **11 Suppl 4**: p. S794-800.
118. Mardani, M. and M. Keshtkar-Jahromi, *Crimean-Congo hemorrhagic fever.* Archives of Iranian Medicine, 2007. **10**(2): p. 204-214.
119. Papa, A., et al., *Recent advances in research on Crimean-Congo hemorrhagic fever.* J Clin Virol, 2015. **64**: p. 137-43.
120. Keshtkar-Jahromi, M., et al., *Crimean-Congo hemorrhagic fever: current and future prospects of vaccines and therapies.* Antiviral Res, 2011. **90**(2): p. 85-92.
121. Ergonul, O., *Treatment of Crimean-Congo hemorrhagic fever.* Antiviral Research, 2008. **78**(1): p. 125-131.
122. Leblebicioglu, H., et al., *Case management and supportive treatment for patients with Crimean-Congo hemorrhagic fever.* Vector Borne Zoonotic Dis, 2012. **12**(9): p. 805-11.
123. Dilber, E., et al., *High-dose methylprednisolone in children with Crimean-Congo haemorrhagic fever.* Trop Doct, 2010. **40**(1): p. 27-30.
124. Huggins, J.W., *Prospects for treatment of viral hemorrhagic fevers with ribavirin, a broad-spectrum antiviral drug.* Rev Infect Dis, 1989. **11 Suppl 4**: p. S750-61.
125. Watts, D.M., et al., *Inhibition of Crimean-Congo hemorrhagic fever viral infectivity yields in vitro by ribavirin.* Am J Trop Med Hyg, 1989. **41**(5): p. 581-5.
126. Paragas, J., et al., *A simple assay for determining antiviral activity against Crimean-Congo hemorrhagic fever virus.* Antiviral Res, 2004. **62**(1): p. 21-5.
127. Tignor, G.H. and C.A. Hanham, *Ribavirin efficacy in an in vivo model of Crimean-Congo hemorrhagic fever virus (CCHF) infection.* Antiviral Res, 1993. **22**(4): p. 309-25.
128. Soares-Weiser, K., et al., *Ribavirin for Crimean-Congo hemorrhagic fever: systematic review and meta-analysis.* BMC Infect Dis, 2010. **10**: p. 207.
129. Ascioglu, S., et al., *Ribavirin for patients with Crimean-Congo haemorrhagic fever: a systematic review and meta-analysis.* J Antimicrob Chemother, 2011. **66**(6): p. 1215-22.
130. Vassilev, T., et al., *A reference preparation for human immunoglobulin against Crimean/Congo hemorrhagic fever.* Biologicals, 1991. **19**(1): p. 57.

131. Papa, A., et al., *Crimean-Congo hemorrhagic fever in Bulgaria.* Emerg Infect Dis, 2004. **10**(8): p. 1465-7.
132. Christova, I., et al., *Crimean-Congo hemorrhagic fever, southwestern Bulgaria.* Emerg Infect Dis, 2009. **15**(6): p. 983-5.
133. Barkagan, Z.S., *Hemorrhagic diseases and syndromes*, in *Medizina*. 1988: Moscow. p. 3–31.
134. Patterson, S.B., M.L. Landrum, and J.F. Okulicz, *Delayed-type hypersensitivity and hepatitis B vaccine responses, in vivo markers of cellular and humoral immune function, and the risk of AIDS or death.* Vaccine, 2014. **32**(27): p. 3341-4.
135. Ruiz-Riol, M., et al., *Influenza, but not HIV-specific CTL epitopes, elicits delayed-type hypersensitivity (DTH) reactions in HIV-infected patients.* Eur J Immunol, 2013. **43**(6): p. 1545-54.
136. Kran, A.M., et al., *Delayed-type hypersensitivity responses to HIV Gag p24 relate to clinical outcome after peptide-based therapeutic immunization for chronic HIV infection.* Apmis, 2012. **120**(3): p. 204-9.
137. Black, C.A., *Delayed type hypersensitivity: current theories with an historic perspective.* Dermatol Online J, 1999. **5**(1): p. 7.
138. Janeway, C.A.J., Travers, P., Walport, M., et al. , *The Immune System in Health and Disease*, in *Immunobiology*. 2001, Garland Science: New York.
139. Cruse, J.M., Lewis, R.E., *Type I, II, III, IV Hypersensitivity*, in *Atlas of Immunology*. 2010, CRC Press. p. 428-431.
140. Parija, S.C., *Type IV (Cell Mediated) Hypersensitivity*, in *Textbook of Microbiology and Immunology*. 2012, Elsevier. p. 154-163.
141. Saitoh, H., R.T. Pu, and M. Dasso, *SUMO-1: wrestling with a new ubiquitin-related modifier.* Trends Biochem Sci, 1997. **22**(10): p. 374-6.
142. Muller, S., et al., *SUMO, ubiquitin's mysterious cousin.* Nat Rev Mol Cell Biol, 2001. **2**(3): p. 202-10.
143. Sharma, S.e.a. *Evidence that hexa histidine tags on therapeutic proteins are not immunogenic in patients* 2014 [cited 2016; Available from: http://conference.ncri.org.uk/abstracts/2009/abstracts/C117.htm.
144. Rosano, G.L. and E.A. Ceccarelli, *Recombinant protein expression in Escherichia coli: advances and challenges.* Front Microbiol, 2014. **5**: p. 172.
145. Liu, X., et al., *SUMO fusion system facilitates soluble expression and high production of bioactive human fibroblast growth factor 23 (FGF23).* Appl Microbiol Biotechnol, 2012. **96**(1): p. 103-11.
146. Peciak, K., et al., *Expression of soluble and active interferon consensus in SUMO fusion expression system in E. coli.* Protein Expr Purif, 2014. **99**: p. 18-26.
147. Bis, R.L., et al., *High yield soluble bacterial expression and streamlined purification of recombinant human interferon alpha-2a.* Protein Expr Purif, 2014. **99**: p. 138-46.
148. Truong, L., et al., *High-level expression, purification, and characterization of Staphylococcus aureus dihydroorotase (PyrC) as a cleavable His-SUMO fusion.* Protein Expr Purif, 2013. **88**(1): p. 98-106.
149. Palese, P., et al., *Negative-strand RNA viruses: genetic engineering and applications.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11354-8.
150. Pleschka, S., et al., *A plasmid-based reverse genetics system for influenza A virus.* J Virol, 1996. **70**(6): p. 4188-92.
151. Turrell, L., et al., *The role and assembly mechanism of nucleoprotein in influenza A virus ribonucleoprotein complexes.* Nat Commun, 2013. **4**: p. 1591.

152. Ruigrok, R.W., T. Crepin, and D. Kolakofsky, *Nucleoproteins and nucleocapsids of negative-strand RNA viruses.* Curr Opin Microbiol, 2011. **14**(4): p. 504-10.
153. Khan, S.H., et al., *New opportunities for field research on the pathogenesis and treatment of Lassa fever.* Antiviral Res, 2008. **78**(1): p. 103-15.
154. Martinez-Sobrido, L., et al., *Differential inhibition of type I interferon induction by arenavirus nucleoproteins.* J Virol, 2007. **81**(22): p. 12696-703.
155. Harmon, B., et al., *Identification of Critical Amino Acids within the Nucleoprotein of Tacaribe Virus Important for Anti-interferon Activity.* Journal of Biological Chemistry, 2013. **288**(12): p. 8702-8711.
156. Pinschewer, D.D., M. Perez, and J.C. de la Torre, *Role of the virus nucleoprotein in the regulation of lymphocytic choriomeningitis virus transcription and RNA replication.* J Virol, 2003. **77**(6): p. 3882-7.
157. Green, T.J., et al., *Common mechanism for RNA encapsidation by negative-strand RNA viruses.* J Virol, 2014. **88**(7): p. 3766-75.
158. Tite, J.P., et al., *Anti-viral immunity induced by recombinant nucleoprotein of influenza A virus. II. Protection from influenza infection and mechanism of protection.* Immunology, 1990. **71**(2): p. 202-7.
159. Lifland, A.W., et al., *Human respiratory syncytial virus nucleoprotein and inclusion bodies antagonize the innate immune response mediated by MDA5 and MAVS.* J Virol, 2012. **86**(15): p. 8245-58.
160. Verhelst, J., et al., *Interferon-inducible protein Mx1 inhibits influenza virus by interfering with functional viral ribonucleoprotein complex assembly.* J Virol, 2012. **86**(24): p. 13445-55.
161. Andersson, I., et al., *Human MxA protein inhibits the replication of Crimean-Congo hemorrhagic fever virus.* J Virol, 2004. **78**(8): p. 4323-9.
162. Fajs, L., K. Resman, and T. Avsic-Zupanc, *Crimean-Congo hemorrhagic fever virus nucleoprotein suppresses IFN-beta-promoter-mediated gene expression.* Arch Virol, 2014. **159**(2): p. 345-8.
163. Jyonouchi, H. *Delayed-type Hypersensitivity* 2015 [cited 2016; Available from: http://emedicine.medscape.com/article/886393-overview.
164. Oboki, K., et al., *IL-33 is a crucial amplifier of innate rather than acquired immunity.* Proc Natl Acad Sci U S A, 2010. **107**(43): p. 18581-6.
165. Bouley, D.M., et al., *Characterization of herpes simplex virus type-1 infection and herpetic stromal keratitis development in IFN-gamma knockout mice.* J Immunol, 1995. **155**(8): p. 3964-71.
166. Molesworth-Kenyon, S.J., J.E. Oakes, and R.N. Lausch, *A novel role for neutrophils as a source of T cell-recruiting chemokines IP-10 and Mig during the DTH response to HSV-1 antigen.* J Leukoc Biol, 2005. **77**(4): p. 552-9.
167. Cooper, A.M., et al., *Disseminated tuberculosis in interferon gamma gene-disrupted mice.* J Exp Med, 1993. **178**(6): p. 2243-7.
168. Akahira-Azuma, M., et al., *Early delayed-type hypersensitivity eosinophil infiltrates depend on T helper 2 cytokines and interferon-gamma via CXCR3 chemokines.* Immunology, 2004. **111**(3): p. 306-17.
169. Gao, D., J. Kasten-Jolly, and D.A. Lawrence, *The paradoxical effects of lead in interferon-gamma knockout BALB/c mice.* Toxicol Sci, 2006. **89**(2): p. 444-53.
170. Feuerer, M., et al., *Self-limitation of Th1-mediated inflammation by IFN-gamma.* J Immunol, 2006. **176**(5): p. 2857-63.

171. Irmler, I.M., M. Gajda, and R. Brauer, *Exacerbation of antigen-induced arthritis in IFN-gamma-deficient mice as a result of unrestricted IL-17 response.* J Immunol, 2007. **179**(9): p. 6228-36.
172. Nakae, S., et al., *Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses.* Immunity, 2002. **17**(3): p. 375-87.
173. Umemura, M., et al., *IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection.* J Immunol, 2007. **178**(6): p. 3786-96.
174. Iwakura, Y., et al., *The roles of IL-17A in inflammatory immune responses and host defense against pathogens.* Immunol Rev, 2008. **226**: p. 57-79.
175. Allison, A.C., *Interactions of antibodies, complement components and various cell types in immunity against viruses and pyogenic bacteria.* Transplant Rev, 1974. **19**(0): p. 3-55.
176. Woodruff, J.F. and J.J. Woodruff, *T lymphocyte interaction with viruses and virus-infected tissues.* Prog Med Virol, 1975. **19**: p. 120-60.
177. Crowle, A.J., *Delayed Hypersensitivity in the Mouse*, in *Advances in Immunology*, F.J. Dixon and G.K. Henry, Editors. 1975, Academic Press. p. 197-264.
178. Pang, T., *Delayed-type hypersensitivity: probable role in the pathogenesis of dengue hemorrhagic fever/dengue shock syndrome.* Rev Infect Dis, 1983. **5**(2): p. 346-52.
179. Cole, G.A., N. Nathanson, and R.A. Prendergast, *Requirement for [Theta]-Bearing Cells in Lymphocytic Choriomeningitis Virus-induced Central Nervous System Disease.* Nature, 1972. **238**(5363): p. 335-337.
180. Leung, K.N. and G.L. Ada, *Cells mediating delayed-type hypersensitivity in the lungs of mice infected with an influenza A virus.* Scand J Immunol, 1980. **12**(5): p. 393-400.
181. Hudson, B.W., K. Wolff, and J.C. DeMartini, *Delayed-type hypersensitivity responses in mice infected with St. Louis encephalitis virus: kinetics of the response and effects of immunoregulatory agents.* Infect Immun, 1979. **24**(1): p. 71-6.
182. Nagarkatti, P.S., M.B. D'Souza, and K.M. Rao, *Use of sensitized spleen cells in capillary tube migration inhibition test to demonstrate cellular sensitization to dengue virus in mouse.* Journal of Immunological Methods, 1978. **23**(3): p. 341-348.
183. Pang, T., P.Y. Wong, and R. Pathmanathan, *Induction and characterization of delayed-type hypersensitivity to dengue virus in mice.* J Infect Dis, 1982. **146**(2): p. 235-42.
184. Rook, G.A. and H.E. Webb, *Antilymphocyte serum and tissue culture used to investigate role of cell-mediated response in viral encephalitis in mice.* Br Med J, 1970. **4**(5729): p. 210-2.
185. Doherty, P.C., *Problems in the experimental analysis of cell-mediated immunity in virus infections.*, in *Infection, immunity*
and genetics. , T.J.L. H. Friedman, and J. E. Prier, Editor. 1978, University Park Press: Baltimore. p. 85-90.
186. Semenov, B.F., *Viruses as nonspecific modulators of immunological reactivity.* Acta Virol, 1981. **25**(2): p. 122-8.
187. Chaturvedi, U.C., et al., *Dengue virus-induced cytotoxic factor suppresses immune response of mice to sheep erythrocytes.* Immunology, 1981. **43**(2): p. 311-6.
188. Chen, J. and X. Liu, *The role of interferon gamma in regulation of CD4+ T-cells and its clinical implications.* Cell Immunol, 2009. **254**(2): p. 85-90.

189. Muranski, P. and N.P. Restifo, *Essentials of Th17 cell commitment and plasticity.* Blood, 2013. **121**(13): p. 2402-14.
190. Hirahara, K., et al., *Mechanisms underlying helper T-cell plasticity: implications for immune-mediated disease.* J Allergy Clin Immunol, 2013. **131**(5): p. 1276-87.
191. Nagafuchi, S., et al., *Delayed type hypersensitivity (DTH) skin reaction to hepatitis B surface antigen (HBsAg) in patients with type B acute and chronic hepatitis.* Clin Exp Immunol, 1985. **61**(3): p. 569-76.
192. Enders, J.F., S. Cohen, and L.W. Kane, *IMMUNITY IN MUMPS : II. THE DEVELOPMENT OF COMPLEMENT-FIXING ANTIBODY AND DERMAL HYPERSENSITIVITY IN HUMAN BEINGS FOLLOWING MUMPS.* J Exp Med, 1945. **81**(1): p. 119-35.
193. Kamiya, H., et al., *Diagnostic skin test reactions with varicella virus antigen and clinical application of the test.* J Infect Dis, 1977. **136**(6): p. 784-8.
194. Galama, J.M., et al., *Measles virus inhibits acquisition of lymphocyte functions but not established effector functions.* Cell Immunol, 1980. **50**(2): p. 405-15.
195. Salmi, A.A., *Cell-mediated immune response to measles virus*, in *Measles and Poliomyelitis: Vaccines, Immunization, and Control*, E. Kurstak, Editor. 1993, Springer Vienna: Vienna. p. 175-185.
196. Fugier-Vivier, I., et al., *Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells.* J Exp Med, 1997. **186**(6): p. 813-23.
197. Acton, Q.A., ed. *Viral Hemorrhagic Fevers—Advances in Research and Treatment*. 2012, Scholarly Editions. 15.
198. Rohde, J.E.-., 1978., *Clinical management of severe dengue.* Trop. Doct. , 1978(8): p. 54-61.
199. Liu, D., et al., *Fine epitope mapping of the central immunodominant region of nucleoprotein from Crimean-Congo hemorrhagic fever virus (CCHFV).* PLoS One, 2014. **9**(11): p. e108419.
200. Saijo, M., et al., *Recombinant Nucleoprotein-Based Enzyme-Linked Immunosorbent Assay for Detection of Immunoglobulin G Antibodies to Crimean-Congo Hemorrhagic Fever Virus.* Journal of Clinical Microbiology, 2002. **40**(5): p. 1587-1591.
201. Wei, P.F., et al., *Serial expression of the truncated fragments of the nucleocapsid protein of CCHFV and identification of the epitope region.* Virol Sin, 2010. **25**(1): p. 45-51.
202. Burt, F.J., et al., *Human defined antigenic region on the nucleoprotein of Crimean-Congo hemorrhagic fever virus identified using truncated proteins and a bioinformatics approach.* J Virol Methods, 2013. **193**(2): p. 706-12.

ÖZGEÇMİŞ

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3. Unvanı **: Ar. Gör.**

4. Öğrenim Durumu **: Yüksek Lisans**

Derece	Alan	Üniversite	Yıl
Lisans	Molekülerbiyoloji ve Genetik	Viyana Üniversitesi	2007-2013
Yüksek Lisans	Biyoteknoloji	Bezmiâlem Vakıf Üniversitesi	2013
Doktora			

5. Akademik Unvanlar

6. Yönetilen Yüksek Lisans ve Doktora Tezleri

6.1. Yüksek Lisans Tezleri

Kırım Kongo Kanamalı Ateşi Virüsü (KKKAV) Kelkit Suşu Nükleoproteinine Karşı Oluşan Gecikmiş Tip Hipersensitivite Cevabının Araştırılması

6.2. Doktora Tezleri

7. Yayınlar

7.1. Uluslar arası hakemli dergilerde yayınlanan makaleler

7.2. Uluslar arası bilimsel toplantılarda sunulan ve bildiri kitabında (Proceeding) basılan bildiriler.

7.3. Yazılan Uluslar arası kitaplar veya kitaplarda bölümler.

7.4. Ulusal hakemli dergilerde yayınlanan makaleler

7.5. Ulusal bilimsel toplantılarda sunulan bildiri kitabında basılan bildiriler

7.6 Diğer Yayınlar

8.Projeler

BAP - 'Kırım Kongo Kanamalı Ateş Virüsü Kelkit Suşu Nükleoproteinine karşı oluşan gecikmiş tip hipersensitivite cevabının araştırılması' (Araştırmacı)

TÜBİTAK - Kırım Kongo Kanamalı Ateş Virüsüne Özgü Serolojik Tanı Sistemlerinin Gelistirilmesi (Araştırmacı)

9.İdari Görevler

10.Bilimsel Kuruluşlara Üyelikleri

11.Ödüller

12.Son iki yılda verdiği lisans ve lisansüstü düzeyindeki dersler

Akademik Yıl	Dönem	Dersin Adı	Haftalık Saati		Öğrenci Sayısı
			Teorik	Uygulama	

*** İşaretli dersler, yüksek lisans dersleridir.**