Pathogenesis of Dengue: Subversion of Innate Immunity

A thesis submitted by

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I, Michela Mazzon, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

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Abstract

Dengue viruses (DENV) are mosquito-borne flaviviruses that cause a severe febrile illness, and sometimes a potentially lethal syndrome called dengue haemorrhagic fever (DHF). Despite decades of effort, the global resurgence of dengue is testament to the inadequacy of current control measures. Dengue has become an immense international public health concern: WHO estimates that there are 50-100 million dengue infections and 500,000 cases of DHF hospitalised each year. Thus dengue has a major economic impact in the developing world through loss of healthy life and utilisation of constrained health resources.

Global dengue control is likely to require a combined approach based on the development of successful strategies for immunization and antiviral drugs, as well as vector control. Better understanding of DENV pathogenesis presents new opportunities for design of rationally attenuated vaccine candidates and antiviral therapies. This thesis focuses on understanding a critical step in DENV pathogenesis: evasion of human innate immune responses mediated by interferon. A lentiviral vector system was developed to express dengue non-structural (NS) proteins in human cells, and we used this to show that expression of dengue NS5 alone inhibited IFN-α, but not IFN-γ, signalling. The IFN-α signalling cascade is blocked downstream of Tyk2 phosphorylation: NS5 binds to the transcription factor STAT2 and inhibits its phosphorylation. The polymerase domain of NS5 is sufficient to block IFN-α induced signal transduction, and inhibition does not require NS5 nuclear translocation. We finally tested several hypotheses to explain

why STAT2 degradation occurs in both DENV-infected and replicon-containing cells, but not when NS5 is expressed alone.

The most important conclusion from this work is that DENV NS5 is a potent and specific type I IFN antagonist. The results of this study are an important step in defining the molecular pathogenesis of dengue, and provide clues to potential new approaches to combat this disease.

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Abbreviations

ADE: Antibody Dependent Enhancement

APC: Allophycocyanin

APS: Ammonium Persulfate

ATP: Adenosin Triphosphate

bp: base pair

BHK: Baby Hamster Kidney

BSA: Bovine Serum Albumin

C/EBP: CCAAT Enhancer Binding protein

CBP: CREB Binding Protein

CIP: Calf Intestine Phosphatase

CK2: Casein Kinase 2

CRD: Carbohydrate Recognition Domain

CREB: cAMP Response Element Binding

CRM: Chromosome Region Maintenance

$C_{T:}$ Threshold cycle

Da: Dalton

DC: Dendritic Cell

DC-SIGN: Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin

DDBP: Damage-specific DNA Binding Protein

ddH_2O: double distilled water

DENV: Dengue Virus

DF: Dengue Fever

DHF: Dengue Haemorrhagic Fever

DMEM: Dulbecco`s Modified Eagle Medium

DMSO: Dimethyl sulfoxide

dsRNA: double strand RNA

DSS: Dengue Shock Syndrome

DTT: Dithiothreitol

ECL: Enhanced Chemiluminescence

EDTA: ethylenediaminetetraacetic acid

EGTA: Ethylene Glycol Tetra acetic Acid

eIF2α: Eukaryotic translation initiation factor 2A

ELISA Enzyme-Linked Immunoadsorbent Assay

Em: Emerald

EMCV: Encephalomyocarditis Virus

ER: Endoplasmic Reticulum

FACS: Fluorescence Activated Cell Sorting

FcR: Fc Receptor

FCS: Fetal Calf Serum

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GFP: Green Fluorescent Protein

GMP: guanosine monophosphate

GPI: Glycosylphosphatidylinositol

GRP: Glucose Regulated Protein

HBSS: Hank's Buffered Salt Solution

HBV: Hepatitis B Virus

HCMV: Human Cytomagalovirus

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV: Human Immunodeficiency Virus

HLA: Human Leukocyte Antigen

hnRNP: Heterogeneous nuclear ribonucleoprotein

HRP: Horseradish Peroxidase

HSP: Heat Shock Protein

HSV: Herpes Simplex Virus

IFN: Interferon

IL: Interleukin

IFNAR: IFN-α Receptor

IP: Immunoprecipitation

IRF: Interferon Regulatory Factor

ISG: Interferon Sensitive Genes

ISGF: Interferon Stimulated Gene Factor

ISRE: Interferon Stimulated Response Element

IκB: Inhibitor of NFκB

JAK: Janus Kinase

JEV: Japanese Encephalitis Virus

kb: kilobase

KO: Knock Out

LB: Luria Broth

LGTV: Langat Virus

LPS: Lipopolysaccharide

LTR: Long Terminal Repeat

MESV: Murine Embrionic Stem Cell Virus

min: minute

MLV: Murine Leukemia Virus

MN: Mannose

MOI: Multiplicity of Infection

MTase: Methyltransferase

MX: Mixovirus resistence gene

NDV: Newcastle Disease Virus

NES: Nuclear Export Signal

NFκB: Nuclear Factor κB

NGC: New Guinea C

NK: Natural Killer

NLS: Nuclear Localisation Signal

NS: Non-Structural

OAS: 2'-5'-oligoadenylate synthetase

OD: Optical Density

ORF: Open Reading Frame

PAGE: Poly Acrylammide Gel Elecrophoresis

PAMP: Pathogen-Associated Molecular Pattern

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PE: Phycoerythrin

pfu: plaque forming unit

PKR: Protein Kinase R

PMSF: Phenylmethanesulfonylfluoride

PPT: Poly Purine Tract

PRR: Pattern Recognition Receptor

PVDF: Polyvinylidine Fluoride

PVP: Polyvinylpyrrolidone

Rbx: Ring finger protein

RdRp: RNA dependent RNA polymerase

RER: Rough Endoplasmic Reticulum

rpm: rotations per minute

RPMI: Roswell Park Memorial Institute

RRE: Rev Responsive Elements

RSV: Respiratory Syncytial Virus

RT: Reverse Transcription; Real Time

RTPase: RNA Triphosphatase

SDS: Sodium Dodecyl Sulfate

SFFV: Spleen Focus-Forming Virus

SH2: Src Homology

SOCS: Suppressor Of Cytokine Signalling

TAE: Tris Acetate EDTA

TBEV: Tick Borne Encephalitis Virus

TBS: Tris Buffered Saline

TEMED: Tetramethylethylenediamine

Tm: Melting temperature

TMB: Tetramethylbenzidine

TNF: Tumor Necrosis Factor

Tyk: Tyrosin Kinase

Ubi: Ubiquitin

UTR: Untranslated Region

UV: Ultraviolet

VSV: Vesicular Stomatitis Virus

WHO: World Health Organization

WNV: West Nile Virus

WPRE: Woodchuck hepatitis virus Post-transcriptional Regulatory Element

YFV: Yellow Fever Virus

Chapter 1.

INTRODUCTION

1.1 EVOLUTION AND SOCIAL IMPACT OF DENGUE VIRUS

The dengue viruses (DENVs) constitute a group of mosquito-borne flaviviruses, characterised by a positive single-strand RNA genome surrounded by nucleocapsid and lipid envelope. DENVs are one of the main causes of arboviral diseases in tropical and subtropical countries, and 3.5 billion people worldwide are currently at risk for DENV infection (Kyle and Harris 2008). WHO currently estimates that there may be 50 million DENV infections worldwide each year, with 500,000 people hospitalised for dengue haemorrhagic fever (DHF), the most severe form of dengue disease (WHO 2009). Dengue and dengue haemorrhagic fever, fact sheet N˚117, March 2009). DENV is responsible for more than 24,000 deaths per year, mainly amongst children under 15 years old (Gibbons and Vaughn 2002), and since the 1950s, the incidence of DHF has increased over 500-fold (Kyle and Harris 2008). The increasing incidence and severity of dengue episodes go along with failure of mosquito containing measures and with the unavailability of vaccines and antiviral therapies. After more than 50 years of research, dengue remains an alarming public health problem, with high economic and social impact, weighing heavily on the financial and human resources of developing countries.

1.1.1 Epidemiology of dengue

There is no consensus about when dengue first appeared in the human population. Descriptions of an illness clinically compatible with dengue fever

(DF) can be found in a Chinese Medical Encyclopaedia dating back to 992 A.D (Gubler 1998). Late in the XVIII century, a disease strongly resembling dengue was causing intermittent epidemics in Asia and America, but it was only during the XIX and XX centuries that the disease widely spread in tropical and subtropical regions (Hayes and Gubler 1992; Monath 1994). After the Second World War, a new dengue-associated disease, DHF, was reported in endemically infected areas in Southeast Asia (HAMMON *et al.* 1960). The first well documented outbreak of DHF occurred in Manila in 1953-1954 (HAMMON *et al.* 1960), followed by a larger outbreak in Bangkok in 1958 (Halstead 1980b); since then, DHF has become endemic in all countries in Southeast Asia (Holmes and Twiddy 2003). Despite intensive mosquito control measures, DHF now represents a significant health problem in the Americas (Guzman *et al.* 1984a; Guzman *et al.* 1984b). Although major epidemics are less commonly reported in Africa, due to the extremely poor surveillance the presence of dengue in this continent is not clear (Holmes and Twiddy 2003).

Dengue epidemics are strongly correlated with demographic and societal changes. In the last 60 years the population in the tropics has grown rapidly, leading to uncontrolled urbanisation. In most large cities, inadequate management of water and waste has provided additional breeding sides for the mosquito vectors, promoting co-circulation of multiple virus strains (Gubler 1998; Gibbons and Vaughn 2002). In 2004, Cummings and colleagues provided evidence that urban environments promote the generation of new epidemic strains, demonstrating the existence of epidemic waves of dengue that originate in Bangkok and spread throughout Thailand every three years (Cummings *et al.* 2004). Further, while in

the past sporadic introductions of new strains were brought only by sea travel, air travel now enables infected people to act as proper human vectors, importing viruses into non-endemic areas (Rosen 1977; Gubler 1998; Gibbons and Vaughn 2002).

In the future, global warming is expected to shift the spectrum of many infectious diseases, especially those transmitted by insects. Even though there is no consensus about the impact of global warming on dengue diffusion, the distribution of the *Aedes* mosquito, the main vector of DENV, is strictly regulated by climate: should the planet become warmer, DENV is likely to spread beyond the current subtropical areas. A 2˚C increase in temperature would also lengthen the mosquito lifespan, resulting in more infected vectors for a longer period of time (Kyle and Harris 2008).

1.1.2 Phylogeny of DENV

Amongst the mosquito-borne flaviviruses, DENV falls into a distinct serogroup that is phylogenetically related to the Japanese encephalitis serogroup and yellow fever virus (YFV) (Kuno *et al.* 1998).

The most recent common ancestor of DENV serogroup is estimated to have originated about 1000 years ago (Twiddy *et al.* 2003). Today there are four distinct serotypes of DENV: DENV-1, DENV-2, DENV-3 and DENV-4. Within each serotype, a group of DENVs "having no more than 6% sequence divergence over a chosen interval" is defined as a genotype (Rico-Hesse 1990). Based on sequences of the envelope (E) gene or of the E-NS1 boundary, DENV-1 is further divided into four to five genotypes, DENV-2 into six, DENV-3 into four, and DENV-4 into two (reviewed in Kyle and Harris 2008).

DENV was originally a sylvatic pathogen mainly infecting non-human primates (Wang *et al.* 2000). When the human population was sufficiently large to sustain viral transmission, sporadic outbreaks in humans could occur following encroachments into the forest habitat. The first strains able to infect humans probably became extinct once the supply of susceptible hosts was exhausted, and this has probably been the case for most virus strains causing dengue outbreaks before urbanisation (Wolfe *et al.* 2001; Twiddy *et al.* 2003).

Some hypotheses suggest that the four serotypes evolved within a single population due to natural selection: according to these theories, distinct serotypes would constitute an evolutionary advantage because of the antibody-dependent enhancement phenomenon (ADE, see section 1.3.2.3), which actively promotes secondary infection by different serotypes. However, if genetic diversity were mainly dependent on ADE, than the virus would be expected to be subject to continual immune selection pressure: studies on natural selection in DENV show that this is not the case and it is now believed that the four serotypes have evolved independently, and that ADE is more likely to be a consequence rather than the cause of their existence (Holmes and Twiddy 2003). Over the past 200 years, genetic diversity within each serotype has risen dramatically, and understanding the rate at which such diversity is changing and whether specific determinants exert selection pressure on DENV evolution could be critical to predict changes of virulence and to design more efficient control strategies.

1.2 STRUCTURE AND REPLICATION OF DENGUE VIRUS

1.2.1 Transmission cycle

DENV replication and spread is perpetuated by a circular transmission cycle involving humans and *Aedes* species mosquitoes (*Aedes aegypti, Aedes albopictus, Aedes polynesiensis*) (Smith 1956). The mosquito remains infected for life, but the virus is only known to cause illness in humans (Gibbons and Vaughn 2002). *Aedes* mosquitoes are dispersed worldwide between latitudes 35ºN and 35ºS. They live in close proximity to humans, and feed preferentially on human blood. Differently from *Anopheles* mosquitoes -the malaria vector-, *Aedes* are daytime feeders and their bite is imperceptible; they rest indoors and this maximises human-vector contact, and also minimises contact with insecticides (Gibbons and Vaughn 2002; Weaver and Barrett 2004).

Aedes aegypti probably spread from jungles of Africa throughout the rest of the world via slave and trading ships between the seventeenth and the nineteenth centuries (Smith 1956; Bosinger *et al.* 2009). *Aedes albopictus* is a secondary vector of DENV in Southeast Asia, the Western Pacific and, increasingly, in Central and South America (Gratz 2004). In recent decades *A. albopictus* has spread in North America, probably from Japan, and its range stretches farther North than *A. aegypti* (Hawley *et al.* 1987; Gratz 2004). Eggs are resistant to subfreezing temperatures and this raises the possibility that *A. albopictus* could mediate a re-emergence of dengue in the United States or in Europe (Hawley *et al.* 1987).

DENV replicates in the salivary glands of adult female mosquitoes, reaches the blood stream, and through it it gains access to different tissues (nervous system, gut, epidermal cells, ovary). Infected mosquitoes transmit the virus to humans by biting: since DENV infects organs controlling feeding-associated activities, infected mosquitoes take longer to complete a blood meal compared with uninfected ones, and as the slightest movement interrupts feeding, several people may be bitten in a very short period of time (Platt *et al.* 1997).

Once in the human host, DENV replicates efficiently and at very high rate, reaching high enough titres to be transmitted to another mosquito through a blood meal.

1.2.2 Target cells of DENV infection in human

Several cell types have been shown to support DENV replication *in vitr*o, but the target of DENV infection *in vivo* is still a matter of speculation. Mosquito vectors release DENV in the skin during a blood meal and several studies have shown that dendritic cells in the epidermis and dermis are the first cells encountering the virus and able to support viral replication (Wu *et al.* 2000; Libraty *et al.* 2001). Even though for a long time monocytes and macrophages have been suggested to be the main target of DENV infection (Halstead *et al.* 1977a), Wu and colleagues showed that at a multiplicity of infection (MOI) of 0.2, 25-40% of immature monocyte-derived DCs are infected *in vitro* with DENV; in contrast, exposure of macrophages from the same donor resulted in 1-2% of infected cells, while neither purified B lymphocytes nor T lymphocytes were infected (Wu *et al.* 2000). Immature DCs are highly permissive to DENV

infection, whereas mature DCs are relatively resistant (Wu *et al.* 2000). Different expression of DENV receptor DC-SIGN (see section 1.2.3.1) may provide an explanation for this, since immature DCs express higher DC-SIGN density on their surface than mature DCs.

After infection by DENV-2 New Guinea C (NGC) strain, monocyte-derived DCs provide an efficient replication site for the virus (Ho *et al.* 2001). 24 hours after infection, virus particles can be detected in cytoplasmic vesicles, vacuoles and endoplasmic reticulum (ER), where they induce hypertrophy of the rough ER (RER), as well as swollen mitochondria. After 48 hours, virus particles are found outside the cell (Ho *et al.* 2001).

DCs have been called "sentinels" of the immune system, as they capture and process microbial products and finally present them to the effector cells of the adaptive immune response (Palucka 2000). Several studies suggest that the activation of DENV-infected immature DCs is blunted compared with the surrounding uninfected cells, with lower expression of maturation markers compared with bystander cells (Libraty *et al.* 2001) and reduced capacity to stimulate T-cells in co-culture (Palmer *et al.* 2005). Impaired activation of infected DC suggests a possible mechanism of evasion of the immune system, but the exact role of DC in DENV pathogenesis is not clear and further studies are required.

Liver dysfunction in DENV infection has been correlated to DHF severity (Wahid *et al.* 2000) and several lines of evidence show that DENV directly invades the liver (Kuo *et al.* 1992; Hilgard and Stockert 2000). Liver cells have been suggested to be one of the major sites of viral replication (Thepparit and

Smith 2004), but besides the identification of some moieties involved in DENV entry, little information is available on the interaction between DENV and hepatocytes.

1.2.3 DENV life cycle

The major steps in the DENV life cycle can be briefly summarised as follows (Figure 1.1):

- DENV virions attach to the host surface and enter the cell in endocytic vesicles;
- conformational changes induce fusion between the viral membrane and the endocytic vesicle, and the viral genome is released into the cytoplasm;
- the positive-sense RNA is translated into a polyprotein, which is co- and post-translationally modified to generate 3 structural and 7 non-structural (NS) viral proteins;
- viral RNA is replicated, providing both new templates for replication and genomes for packaging into new virions;
- virions assemble on the ER surface, mature in the Golgi, and are released via exocytosis outside of the cell.

1.2.3.1 Fusion and entry

DENV infectious particles consist of an external glycoprotein shell and an internal host-derived lipid bilayer, which surrounds the RNA genome and the capsid proteins (core). The glycoprotein shell consists of 180 copies each of the envelope glycoprotein E, which has been shown to be essential to viral infection

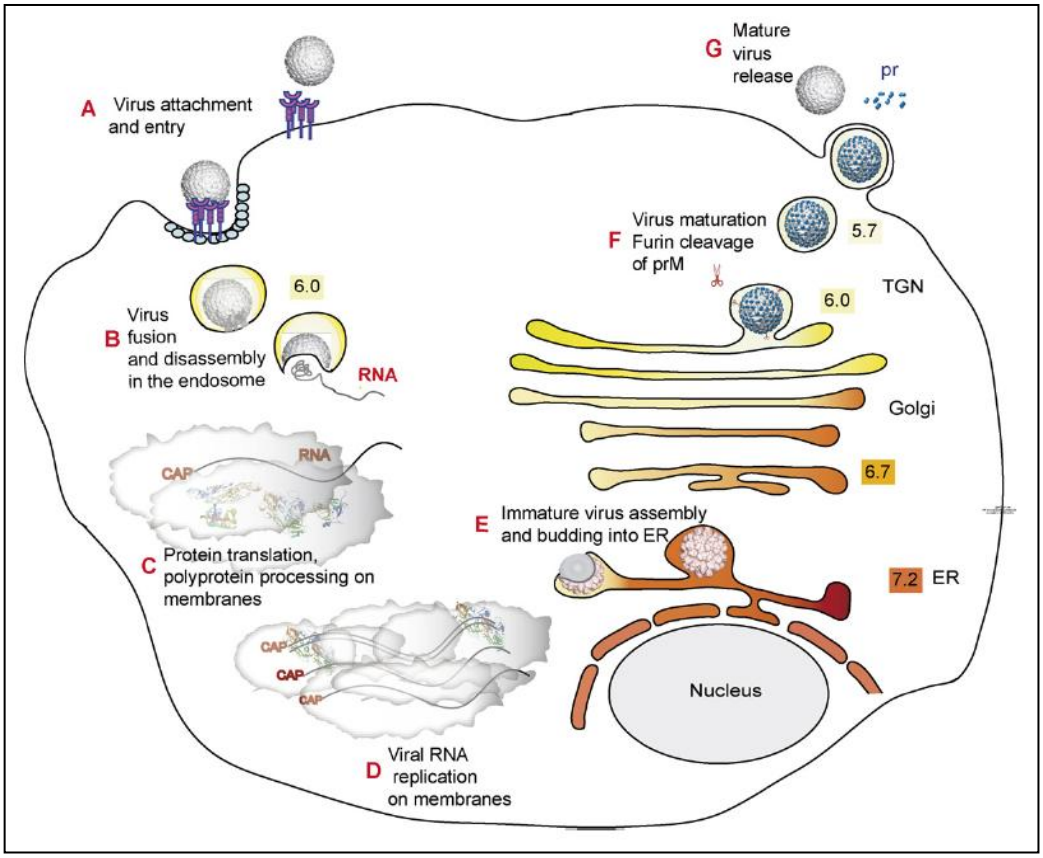


Figure 1.1|

Schematic diagram of DENV replication cycle. After binding, the DENV virion is internalised in an endosomal vesicle. Fusion between viral and endosomal membranes is followed by release of the RNA genome in the cytoplasm. The positive strand RNA can be immediately translated into a polyprotein and processed into individual proteins. Some of these proteins are involved in virus RNA replication and subsequent production of new virions. Structural proteins and RNA assemble in new virion particles in the ER and mature in the Golgi network. Mature virions are released via exocytosis (from Perera *et al.* 2008).

(Perera and Kuhn 2008; Yu *et al.* 2008).

The cell entry mechanism of DENV is still under investigation. The first contact between DENV infectious particles and the cell surface is mediated by the interaction between DENV envelope glycoprotein E and cellular proteins or carbohydrate molecules on the host surface (Hung *et al.* 1999). DENV E protein is organised in dimers. Each monomer is composed of three β-barrel domains (Figure 1.2, A-B). Domain I is the central structural domain, domain II contains the fusion peptide, critical for the release of DENV nucleocapsid into the cytoplasm after endocytosis, and domain III is an immunoglobulin-like domain containing putative receptor-binding sites (Crill and Roehrig 2001).

The literature abounds with descriptions of putative ligands on different host cells and for different DENV serotypes and it is possible that DENV exploits different molecules/mechanisms to enter different cell types. Heparan sulphates (Hilgard and Stockert 2000; Germi *et al.* 2002), Heat Shock Protein 70 (HSP70) and Heat Shock Protein 90 (HSP90) (Reyes-Del Valle *et al.* 2005; Cabrera-Hernandez *et al.* 2007), GRP78/Bip (Jindadamrongwech *et al.* 2004), CD14 (Chen *et al.* 1999), and laminin receptor (Thepparit and Smith 2004) have all been implicated in viral entry, but only DC-SIGN and the mannose receptor (MN) have been shown to play an active role. The four serotypes of DENV have all been shown to bind C-type lectin receptors. **DC-SIGN** (DC Specific ICAM-3 Grabbing Non-integrin) is a membrane protein on DC surface, carrying an external mannose-binding C-type lectin domain. DC-SIGN carbohydrate-recognition domains (CRD) on DENV E protein bind mannose residues within DC-SIGN on DCs (Tassaneetrithep *et al.* 2003; Navarro-Sanchez *et al.* 2003) and the

interaction is sufficient for viral infection. Lozach and colleagues (Lozach *et al.* 2005) showed that internalisation of DC-SIGN is not necessary for DENV infectivity and probably DC-SIGN does not function as a specific receptor but allows virus attachment on cell surface. A second type of C-type lectin receptor, the **mannose receptor** (MR), is also recognised by all DENV serotypes (Miller *et al.* 2008). Since the MR is constitutively internalised, the authors suggest that, differently from DC-SIGN, the MR could work as a proper DENV receptor rather than just as an attachment molecule (Miller *et al.* 2008).

A different mechanism of viral entry exploits Fcγ receptors (FcγR) on the surface of cells of the immune system: this phenomenon, called antibody-dependent enhancement (ADE) (Halstead *et al.* 1970) has been suggested to be a critical determinant of DHF in secondary infections, and it will be described more extensively in section 1.3.2.3. Interestingly, Boonnak *et al* (Boonnak *et al.* 2008) showed that mature DC infectability is higher in ADE conditions, but when sufficient levels of DENV receptors are expressed, ADE-independent mechanisms of cell entry are preferentially used.

Attachment of DENV on the cell surface is followed by endosomal uptake (Modis *et al.* 2004). Recent studies indicate that DENV enters the cell via clathrin-mediated endocytosis. A recent study performed on African green monkey kidney cells shows that virus particles bind and move along the cell surface in a diffusive manner -either as virus-receptor complexes, or rolling over multiple receptors along the cell surface- and then associate to clathrin-coated pits. Shortly after, the clathrin spot matures into a clathrin vesicle and delivers the

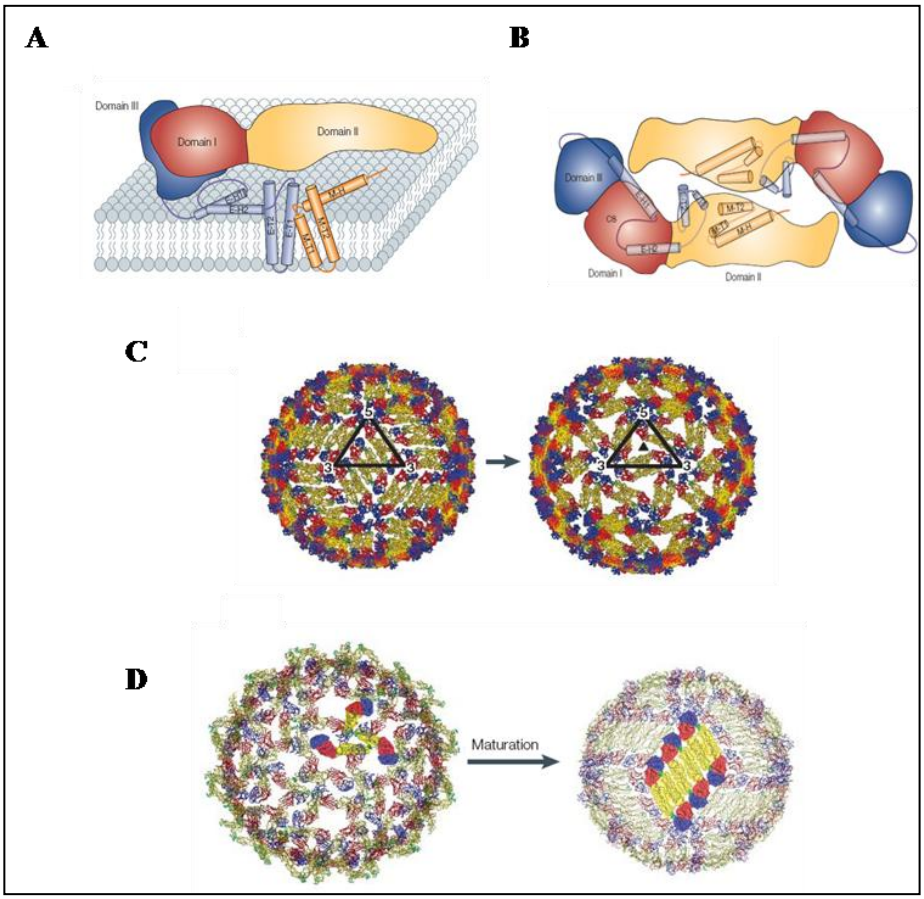


Figure 1.2|
Organisation, membrane fusion and maturation of DENV E protein.
A-B. Diagrams of DENV E protein ectodomains, side **(A)** and top **(B)** view**.** E-H1, E-H2 and E-T1, E-T2 (in blue) represent the stem (H) and transmembrane (T) helices of the E protein; M-H1, M-H2 and M-T1, M-T2 (in orange) represent the stem and transmembrane helices of the M protein. **C.** Rearrangement of DENV virions during membrane fusion. The E protein dimers on the mature virion (*left*) undergo a rearrangement to form the fusogenic intermediate structure (*right*). The solid triangle indicates the position of a quasi three-fold axis, while the arrows indicate the direction of the E rotation (adapted from Mukhopadhyay *et al.* 2005). **D.** Maturation process of DENV virion. The E proteins, organised in trimers in the immature virion (*left*) rearrange into dimers to form the mature viral particle (*right*).

virus to early endosomes in the cytoplasm (van der Schaar *et al.* 2008).

After mediating virus attachment to the cell surface and internalisation, the next major role for DENV E protein is the release of the nucleocapsid into the cytoplasm. Low pH in the endosome causes the dissociation of the E dimers into monomers, and their irreversible re-association into trimers (Figure 1.2 C). In the new conformation, the three fusion peptides, previously buried between domain I and III, are finally exposed on the top of the trimer and can penetrate the endosomal membrane (Modis *et al.* 2004).

Fusion between virion and endosomal membranes results in release of the viral nucleocapsid into the cytoplasm. What happens to the nucleocapsid immediately after is not known, and the precise mechanism of release of the RNA genome is still under investigation.

1.2.3.2 Genome organisation

The DENV genome (Figure 1.3, A) consists of a positive single-stranded RNA molecule of about 11kb, containing a 5` untranslated region (UTR; ~100 nucleotides), a single open reading frame (ORF) encoding the viral polyprotein, and a 3` UTR (~400 nucleotides). A 7-methyl guanosine cap structure covers the 5` end but, unlike cellular mRNAs, the DENV genome is not 3` polyadenylated (Bartenschlager and Miller 2008). Both the 5`UTR and the 3` terminal region of the 3` UTR contain complementary cyclisation sequences which play critical roles in the replication of the negative strand mRNA molecule, as described in section 1.2.3.4 (Henchal and Putnak 1990; Khromykh *et al.* 2001; Alvarez *et al.* 2005).

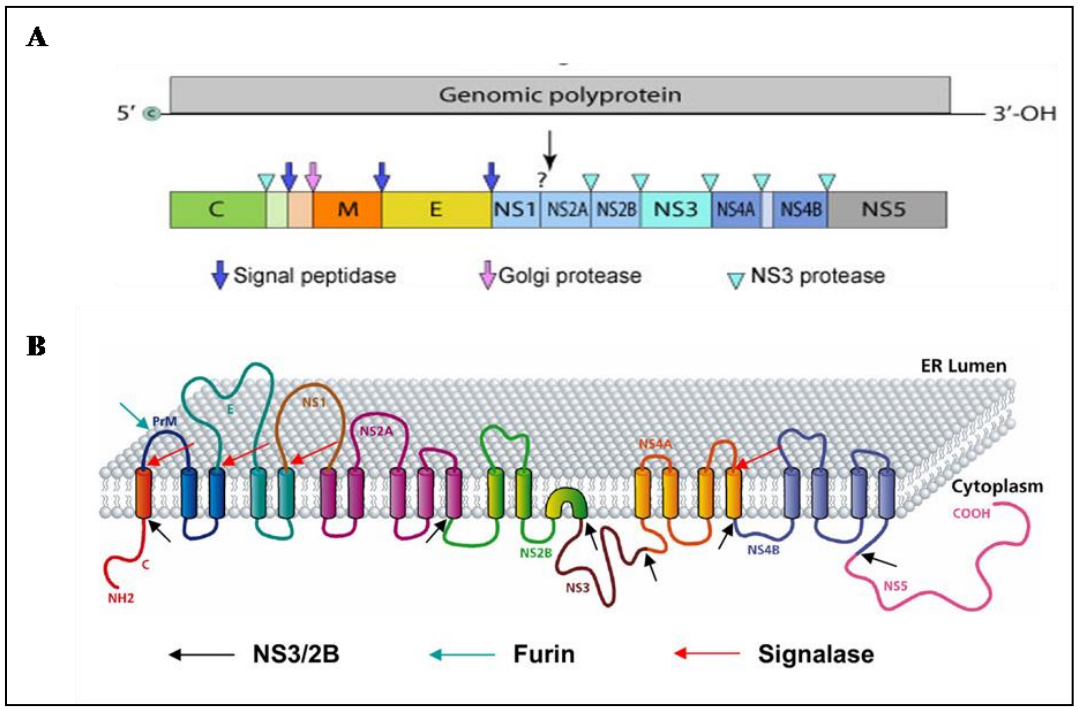


Figure 1.3|

Schematic representation of DENV genome (A) and predicted membrane topology of DENV polyprotein (B). The ~11 kb genome of DENV is translated into a single polyprotein which traverses the ER membrane at several positions. PrM, E, NS1 and part of NS4A and NS4B are thought to localise to the ER lumen via hydrophobic signal sequences, whereas the remaining proteins are thought to localise on the cytoplasmic side of the ER. Arrows represent the polyprotein cleavage sites recognised by viral or host proteases, as indicated by different colours.

1.2.3.3 Genome translation

Being positive sense, DENV RNA genome can be directly translated. When the RNA-ribosome complex encounters the hydrophobic signal sequence at the C-terminus of the capsid protein, the polyprotein is directed to the endoplasmic reticulum (ER). Signal sequences within the nascent polyprotein translocate NS1 and the ectodomain of prM and E into the lumen of the ER; the C protein, NS3 and NS5 localise in the cytoplasm (Figure 1.3, B) and NS2A, NS2B, NS4A and NS4B remain predominantly transmembrane. The anchoring process regulates the start of the proteolytic processing (Markoff *et al.* 1994). Processing of the polyprotein occurs before the translation is completed and is carried out by both host proteases in the lumen of the ER, and the viral protease NS3 and its cofactor NS2B (Falgout *et al.* 1991; Falgout and Markoff 1995). Between C and prM, prM and E, and between NS4A and NS4B, the proteolytic cleavage is mediated by host enzyme signal peptidases, while the serine protease domain of NS3 cleaves the polyprotein between NS2A and NS2B, NS2B and NS3, NS3 and NS4A, NS4B and NS5, and some other residues within its own C-terminus domain (Falgout *et al.* 1991; Zhang *et al.* 1992).

1.2.3.4 Genome replication

About three hours after infection, RNA replication can be detected in the perinuclear region of infected cells, in association with smooth membranes. Positive strand RNA viruses replicate in close association with virus-induced intracellular membranous structures: these structures provide a scaffold for the replication complex and increase the local concentration of all components

required (Bartenschlager and Miller 2008). An interesting hypothesis suggests that these membranes may also prevent the activation of double strand RNA (dsRNA)-induced host defence mechanisms and interferon production (Bartenschlager and Miller 2008). In DENV infected cells membrane alterations seem to be actively induced by the non-structural protein **NS4A** (Miller *et al.* 2007), which would also anchor the replication complex to such membranes (Lindenbach and Rice 1999).

The RNA-dependent RNA polymerase (RdRp) of DENV **NS5** binds to a stem-loop structure in the 5` UTR and it is transferred to the transcription initiation site at the 3` end of the genome by 5`-3` long range interactions (Filomatori *et al.* 2006) (see section 1.2.3.2). Cyclisation of the viral genome is therefore crucial for viral replication and it may be a control mechanism to ensure that only full length templates are amplified (Bartenschlager and Miller 2008). The replication starts with the synthesis of a negative strand RNA. The replicative product, called *replicative form,* is a full-length, double-stranded RNA. The negative strand of the replicative form functions as a template for the generation of new positive RNA strands; the partially double stranded RNA formed during the elongation of the nascent positive strain is called *replicative intermediate*. The helicase domain of DENV **NS3** helps the dissociation of the nascent RNA strand from its template and the relaxation of RNA secondary structures (Gorbalenya *et al.* 1989). The newly produced positive strand is then released from the replicative intermediate and either attaches to ribosomes to undergo a new translation cycle, or assembles into new virions in the ER (reviewed in Bartenschlager and Miller 2008).

DENV proteins **NS1**, **NS2A** and **NS4B** have also been suggested to take part in viral replication: NS1 is assumed to act at an early stage of viral replication, but its precise function is not known, NS2A has been suggested to anchor the viral

replicase to cellular membranes and to recruit the RNA template (Mackenzie *et al.* 1998; Bartenschlager and Miller 2008), and NS4B might help the dissociation between the viral RNA and the helicase NS3 (Umareddy *et al.* 2006). The role of DENV NS proteins will be further discussed in section 1.2.4.

Finally, after RNA replication, a cap is added at the 5` terminus of the positive strand RNA molecule. The addition of a cap is a three stage process involving both **NS3** and **NS5**. The RNA-5`-triphosphatase (RTPase) activity of NS3 is responsible for the cleavage of the 5`-triphosphate terminus and for the subsequent addition of GMP to the 5`-diphosphate (Bartelma and Padmanabhan 2002); the methyltransferase domain of NS5 is responsible for the methylation of the 5`GMP at the $N^7$ position first, and then at the ribose 2`O (Egloff *et al.* 2002).

1.2.3.5 Virion assembly and release

Particle formation is a coordinated process involving the membrane-associated capsid proteins C and the prM-E heterodimer in the ER. The membrane-bound capsid proteins (C) are organised in dimeric structures which constitute the building blocks for the assembly of the nucleocapsid (one molecule of RNA and multiple C proteins). The basic, positively charged composition of the nucleocapsid suggests that the C proteins may function like histones (Ma *et al.* 2004).

The nucleocapsid and the prM-E heterodimers assemble in the ER, and the complex acquires an envelope from the intra-cytoplasmatic membrane (Henchal and Putnak 1990; Mukhopadhyay *et al.* 2005). Immature particles display 60 prominent and irregular spikes on their surface, each spike consisting of a trimer of prM-E heterodimers. The prM proteins cap the fusion peptides on the E

proteins and protect the virus from premature fusion before release (Yu *et al.* 2008; Li *et al.* 2008). Depending on the pH of the cellular environment, the immature particle can exist reversibly in either "spiky" (pH 7) or "smooth" (pH 6) forms. During the maturation process, due to the low pH-conditions in the *trans* Golgi network, the prM proteins expose a furin cleavage site and the pr peptides are irreversibly cleaved. As a consequence, the trimer is disrupted and the E proteins rearrange in the characteristic and irreversible mature conformation, with 90 heterodimers lying flat on the viral surface (Figure 1.2, D) (Mukhopadhyay *et al.* 2005). The pr peptides, which are still associated to the M-E complex, dissociate only when the virion is released into the neutral pH of cellular environment (Yu *et al.* 2008; Li *et al.* 2008). Immature virions are highly stable and relatively inert, but the final cleavage step makes the virus more labile and flexible for the subsequent penetration of the host cell. The dimers formed by the E proteins organise in parallel groups of three, with the putative receptor-binding domain protruding from the surface, ready for binding cellular receptors.

Release of virus from infected cells occurs via secretory exocytosis: the vesicles fuse with the plasma membrane of the host and mature virions are released outside of the cell (Henchal and Putnak 1990).

1.2.4 DENV non-structural proteins

The precise function of most of the non-structural proteins of DENV is still obscure and many effects of viral infection have not been correlated to specific proteins yet. Structural information is available for NS3/NS2B and NS5 only. Diverse functions have been attributed to each DENV NS protein, but only few

observations have been reproduced and validated. In spite of little information, the literature suggests that most NS proteins are able to perform multiple functions, in order to sustain efficient proliferation and, at the same time, to successfully evade the main cellular mechanisms of antiviral defence.

1.2.4.1 NS1/NS2A

NS1 is a glycoprotein of 40-46 kilodalton (kDa). Newly synthesised NS1 appears in the lumen as a monomer but it dimerises after 20-40 minutes without intervention of other proteins (Falgout *et al.* 1989). The N-terminal of NS1 is preceded by a localisation signal of 24 hydrophobic amino acids that leads the protein to the endoplasmic reticulum (ER) and is then cleaved. The localisation signal is followed by a group of charged residues, and by a second hydrophobic sequence of 14 amino acids acting as a stop-transfer (Falgout *et al.* 1989). The correct localisation of NS1 in the ER is critical for the subsequent attachment of two N-linked high mannose carbohydrate moieties; in the Golgi one of the two carbohydrates is trimmed and processed to a complex form (Jacobs *et al.* 2000).

NS1 is found intracellularly, on cell surface and, as soluble form, it is also released extracellularly. The role of **intracellular NS1** is still unclear: its association with the RNA genome (Mackenzie *et al.* 1996) and with hnRNP C1/C2 (human heterogenous nuclear ribonucleoprotein), involved in mRNA biogenesis (Noisakran *et al.* 2008), together with its co-localisation with other DENV NS proteins involved in viral replication (Mackenzie *et al.* 1998), suggests a role for NS1 in RNA replication, as mentioned in section 1.2.3.4.

Transmembrane NS1 has been observed to mimic cellular mechanisms of signal transduction. NS1 can be anchored to the host membrane via a glycosyl-

phosphatidylinositole (GPI) anchor. The N-terminal of **NS2A** can act as GPI anchor signal for NS1, and internal cleavages within NS2A occurring during the processing of the viral polyprotein determine whether NS1 will acquire the GPI anchor or not (Jacobs *et al.* 2000). The precise role of GPI anchors remains uncertain, but they are implicated in signal transduction and confer on proteins the ability to transfer between plasma membranes of different cells. Jacobs and colleagues showed that binding of anti-NS1 antibodies to NS1 proteins anchored on the cell surface initiates a signal transduction cascade leading to tyrosine phosphorylation of a number of cellular proteins. The precise consequences of such activation on DENV life cycle are not known, but the authors suggest that signal transduction by NS1 could promote cellular activation and in turn increase production of viral progeny (Jacobs *et al.* 2000).

Up to 10 μg/ml of a soluble hexameric form of NS1 can be found in the blood of DENV infected people (Alcon-LePoder *et al.* 2005). Whether **extracellular NS1** has any specific role is not clear, but soluble NS1 can activate to completion the complement system, inducing cell damage and vascular leakage in DHF (Avirutnan *et al.* 2006). The role of NS1 and of antibodies elicited against NS1 in DENV pathogenesis will be further discussed in section 1.3.3.

The precise role of **NS2A** remains unclear. Besides the role mentioned above in helping NS1 correct localisation in the ER and the acquisition of a GPI anchor, NS2A has been suggested to take part in viral replication (see section 1.2.3.4), but its precise function has not be identified.

1.2.4.2 NS2B/NS3

NS3 is a 69 kDa multifunctional protein with protease, helicase and capping

activities. The **protease domain** localises at the N-terminus of NS3 and is required for the processing of DENV polyprotein. **NS2B** is required for the optimal activity of NS3, as its central region completes the substrate-binding site of NS3 (Erbel *et al.* 2006). The catalytic triad (His-51, Asp-75, Ser-135) is buried between β-barrel structures and hydrolysis occurs after a pair of basic residues (Lys-Arg, Arg-Arg, or Arg-Lys), followed by a small side chain amino acid (Gly, Ser, or Ala) (Luo *et al.* 2008). The substrate specificity of NS2B/NS3 has not been extensively studied but Shafee *et al* (Shafee and AbuBakar 2003) suggest that cleavages may occur also on substrates other than the viral polyprotein. In this work apoptosis is observed in cells transfected with a functional NS2B/NS3 complex: this suggested that cleavage of apoptosis initiators or antagonism of apoptosis inhibitors by DENV NS3 might activate effector caspases and induce apoptosis.

The C-terminus portion of NS3 (amino acids 170-619) performs different related activities, including the NTPase/helicase activity and the RTPase activity (section 1.2.3.4). The **helicase** separates the nascent RNA strands from the template and assists replication initiation by unwinding RNA secondary structures in the 3`UTR (Gorbalenya *et al.* 1989). The energy required is provided by the **nucleotide-triphosphatase (NTPase)** activity, which hydrolyse NTP γ-phosphates. The **RNA-5`-triphosphatase (RTPase)** is involved in the first step of the RNA capping described in section 1.2.3.4 (Bartelma and Padmanabhan 2002). During RNA replication, NS3 interacts with the hypophosphorylated form of DENV NS5, and such interaction stimulates both the NTPase and the RTPase (Yon *et al.* 2005).

1.2.4.3 NS4A

NS4A is one of the least characterised proteins of DENV. The last 23 amino acids in the C-terminus of NS4A (2K fragment) act as a signal for the translocation of NS4B into the ER lumen, and are then removed by a host signalase (Miller *et al.* 2007). NS4A is the smallest of DENV proteins, highly hydrophobic and therefore tightly associated with cellular membranes. The first 49 amino acids at the N-terminus are exposed in the cytoplasm, while four highly hydrophobic transmembrane regions anchor NS4A to ER-derived cytoplasmic dot-like structures (Miller *et al.* 2007). As mentioned in section 1.2.3.4, NS4A seems to be responsible for the generation of such cytoplasmic membrane structures, particularly following removal of the 2K fragment (Miller *et al.* 2007). As outlined in section 1.2.3.4, these same structures also contain dsRNA and DENV NS proteins involved in viral replication, suggesting that NS4A might re-organise cellular membranes in order to create the subcellular structures required for DENV replication (Miller *et al.* 2007).

1.2.4.4 NS4B

NS4B is a hydrophobic non-structural protein of 248 amino acids (27 kDa) (Miller *et al.* 2006). Immediately after synthesis, NS4B is found in perinuclear regions of the cell in a pattern resembling the ER, and later on large NS4B foci are visible all throughout the cytoplasm. NS4B localises in the ER membrane, directed by the 2K fragment at the C-terminus of NS4A. The N-terminal region lies in the ER lumen while the C terminus is cytoplasmic; the rest of the protein is constituted of transmembrane hydrophobic domains (Miller *et al.* 2007). Yeast two-hybrid assays and immunoprecipitation studies have shown the association between NS4B and the NS3-NS5 complex (Umareddy *et al.* 2006). Full length

NS4B has been shown to interact with the C-terminal region of NS3 encompassing the helicase motif. As mentioned in section 1.2.3.4, this observation suggests a role for NS4B in viral replication, by helping the dissociation of the NS3 helicase from the RNA, and enabling binding of another duplex. Alternatively, NS4B might hold the separated RNA strand apart as the replication complex moves along the duplex (Umareddy *et al.* 2006).

Together with NS4A and NS2A, NS4B has also been suggested to take part in IFN antagonism (Munoz-Jordan *et al.* 2003). Since the evasion of the IFN response by DENV constitutes the focus of this thesis, the role of NS4B and other non-structural proteins in the inhibition of IFN will be further discussed later.

1.2.4.5 NS5

NS5 (104 kDa) is the biggest non structural protein of DENV and the most conserved amongst flaviviruses. Across the four DENV serotypes, NS5 shares a minimum of 67% amino acid sequence identity (Yap *et al.* 2007).

Based on structural and biochemical studies, two functional domains have been identified. The N-terminal domain (amino acid residues 1-260) is an **S-adenosyl methionine methyltransferase** (MTase) which takes part in DENV genome capping, catalysing the sequential binding of two methyl groups from an S-adenosyl methionine to the 5' GMP of any new molecule of viral mRNA (section 1.2.3.4) (Egloff *et al.* 2002; Zhou *et al.* 2007). The C-terminal region is the viral **RNA-dependent RNA polymerase** (RdRp) which synthesises a transient double-stranded RNA intermediate and then additional plus-strand genomic RNAs. The crystal structure of DENV RdRp catalytic domain has been determined (Yap *et al.* 2007). The architecture of NS5 RdRp resembles the typical pattern of known

polymerases, consisting of fingers, palm and thumb. Between the amino acids 320 and 405, two **nuclear localisation sequences** (NLS) have been mapped, both recognised by the importin system (Forwood *et al.* 1999; Johansson *et al.* 2001; Brooks *et al.* 2002; Pryor *et al.* 2007). Transport into the nucleus of proteins bigger than 45 kDa requires intrinsic targeting signals recognised by the importin complex. The importing system starts with the binding of β-importin to the β–NLS; this induces the recruitment of importin-α and its conformational change. α/β-importin binds α/β-NLS and the target protein is translocated into the nucleus in a GTP-dependant process (Brooks *et al.* 2002). While DENV-1 carries only one NLS, DENV-2, DENV-3 and DENV-4 have a bipartite NLS (320-368; 369-389): the first cluster seems to be critical for the interaction with NS3 (residues 312-332 are almost completely conserved between all strains) while the second (residues 369-389) is critical for nuclear localisation and carries the typical pattern of three basic clusters of most NLSs (K371-K372; K388-K389; R401-K402) (Brooks *et al.* 2002; Pryor *et al.* 2007). It has been shown that NS5 contains also a nuclear export signal (NES; amino acids 327-343) that uses the cellular CRM1 exportin complex to translocate from the nucleus to the cytoplasm (Pryor *et al.* 2006). Such a mechanism seems to prevent nuclear accumulation of NS5 but the consequences and the biological significance of this shuttling between nucleus and cytoplasm have not been elucidated.

Cellular localisation of NS5 seems to be dependent on its phosphorylation status. A low phosphorylated form localises in the cytoplasm, where it interacts with DENV NS3 and takes part in the replication of the viral genome; a high phosphorylated form dissociates from NS3 and translocates into the nucleus (Kapoor *et al.* 1995; Johansson *et al.* 2001). Even though phosphorylation induces

NS5 nuclear translocation, protein kinase CK2-mediated phosphorylation of Thr-395 in the NLS seems to inhibit nuclear targeting, probably through a cytoplasmic retaining mechanism (Forwood *et al.* 1999). NS5 activity in the nuclear compartment is not entirely clear, despite some hypotheses suggesting a role for nuclear NS5 in regulation of gene expression at different stages of infection. In 2005 Medin and colleagues (Medin *et al.* 2005) suggested a role for NS5 in the over-expression of IL8. IL8 is a cytokine produced by virtually all cells in response to LPS, TNF, and virus infection. Besides neutrophil recruitment, its role is controversial as it can both enhance viral infection by antagonising interferon and itself act as an antiviral molecule. IL8 concentration is high in the blood of DHF patients, and it may be involved in increased endothelial permeability (Bosch *et al.* 2002). Medin`s work shows that *in vitro* infection of human myeloid or endothelial cells by DENV induces secretion of IL8. This effect was attributed to the NS5-mediated activation of specific cellular factors, such as the CAAT/enhancer binding protein (C/EBP), and to a lesser extent to NF-κB. A later study suggests that the induction of IL8 observed by Medin and colleagues is more likely to be due to the cytoplasmic form of NS5 (Pryor *et al.* 2007). In this work increased IL8 secretion is observed only when nuclear NS5 is reduced. This observation suggests that NS5 has dynamic control of IL8 production and after initial induction mediated by cytoplasmic NS5, nuclear translocation of NS5 reduces IL8 production (Pryor *et al.* 2007; Rawlinson *et al.* 2009). However, a correlation between NS5 regulated production of IL8 and DENV pathogenesis has not been proved and the exact role of NS5 in the nucleus has not been elucidated.

1.3 DENGUE AND DHF: CLINICAL MANIFESTATIONS AND RISK FACTORS

1.3.1 Dengue clinical manifestations

DENV infection can either be asymptomatic or induce undifferentiated fever, dengue fever (DF), or dengue haemorrhagic fever (DHF) (reviewed in Henchal and Putnak 1990). Undifferentiated fever usually follows primary infection and it is indistinguishable from other common viral infections. **DF** may occur during primary or secondary infections: symptoms are sudden high fever, headache, arthralgia, myalgia, anorexia, abdominal pain and sometimes macular papular rash. Recovery is usually uneventful but may be prolonged in adults. **DHF** normally follows secondary DENV infections. DHF in infants may occur also in primary infections, and pre-existent maternal non-neutralising antibodies have been suggested to be in part responsible for this (see section 1.3.2.3). DHF begins with a febrile phase, haemorrhagic phenomena, and generalised constitutional symptoms. This first phase can either be followed by full recovery, or progress towards a second more severe phase, characterized by plasma leakage, tachycardia, hypotension and sometimes pleural effusions and ascites. Bleeding may occur from any site and especially from the gastrointestinal tract. Platelet count is $<100 \cdot 10^9$/l and a generalised leukopenia as well as abnormal coagulation profiles can be observed. Depending on its severity, DHF can be further divided into four grades, the last and most severe is characterised by profound shock (Dengue Shock Syndrome, DSS). Surviving patients undergo an uneventful and short period of convalescence (Henchal and Putnak 1990).

1.3.2 Risk factors for DHF

The reasons for different disease outcomes following viral infection are not completely known and more work is required to understand the precise patho-physiology of dengue disease. In 2000 Vaughn and colleagues (Vaughn *et al.* 2000) showed that DENV peak titre is between 100 and 1000-fold higher in DHF than in DF patients: this suggests a strong correlation between viral replication and disease severity. This observation has been further confirmed by subsequent studies (Libraty *et al.* 2002; Guilarde *et al.* 2008). Success of viral replication early in infection depends upon both viral and host factors. Furthermore, several studies have proved the existence of a strong epidemiological correlation between DHF and secondary infections (see below), suggesting that pre-existing immunity can act as risk factor for DHF.

1.3.2.1 Viral factors

Whether some serotypes are more pathogenic than others remains an open question, but a correlation has been observed between some DENV serotypes and disease severity or increased fitness in the context of host immunity (Kyle and Harris 2008). In secondary infections, DHF is most commonly associated with DENV-2, followed by DENV-1 and DENV-3 (Sangkawibha *et al.* 1984; Balmaseda *et al.* 2006); DENV-4 seems to be the least clinically severe, even if it can on occasions induce DHF in secondary infection (Nisalak *et al.* 2003). Also the particular sequence of infecting DENV serotypes can influence the severity of the disease: DENV-2 and DENV-4 have been associated with increased disease severity in secondary infections, while DENV-1 and DENV-3 with more severe disease in primary infections (Harris *et al.* 2000; Vaughn *et al.* 2000; Nisalak *et*

al. 2003; Balmaseda *et al.* 2006).

Southeast Asia seems to be a source of viral diversity, generating a multitude of strains which are often more virulent and successful than the existing ones, and displacing more benign genotypes (Zanotto *et al.* 1996). Much evidence suggests that DENV-2 strains generated in Southeast Asia are associated with DHF, while strains generated in America are not (Rico-Hesse *et al.* 1997; Diaz *et al.* 2006). In particular it has been reported that the Thai DENV strains of the Asian genotype replicate to higher titres in human monocyte-derived macrophages and dendritic cells, suggesting that the success of the Southeast Asia DENV-2 strains is due to a more efficient replication in human cells, and a more efficient transmission by vector mosquitoes (Pryor *et al.* 2001; Cologna and Rico-Hesse 2003).

Higher virulence has been shown also for DENV-3 genotype III, which has been associated with an increase in DHF: the decisive factor was identified as a clade replacement event, suggesting that increased viral diversity is leading to the emergence of virus clades associated with DHF (Messer *et al.* 2003; Kyle and Harris 2008). Following the analysis of 11 DENV-2 of the American and Southeast Asian genotype, Leitmeyer and colleagues suggest that the amino acid 390 of the E protein and specific sequences within the 3` NTR and the 5` NTR might be determinants of DHF (Leitmeyer *et al.* 1999); however, genetically distinct viruses have not been consistently associated with severe or mild dengue, highlighting once again the complexity of dengue disease.

1.3.2.2 Human factors

DHF has been correlated to age and ethnicity. Children under 15 years old are more at risk of developing DHF, probably because of increased capillary fragility

and decreased tolerance for micro vascular damage (Gamble *et al.* 2000). Certain populations and ethnic groups also show increased tendency to develop DHF: Africans and people of African descent for instance have been suggested to have some genetic polymorphisms that confer partial protection against the most severe forms of dengue (Coffey *et al.* 2009). One hypothesis suggests that the nature and magnitude of the immune response generated by HLA-class I presentation of DENV antigens could also contribute to immunopathogenesis (Stephens *et al.* 2002; Simmons *et al.* 2005; Mongkolsapaya *et al.* 2006; Sierra *et al.* 2007). Selected HLA alleles have only been significantly associated with DF or DHF caused by certain DENV serotypes (Stephens *et al.* 2002); however, no single allele has been associated with dengue disease and some ethnic groups show no consistent HLA association pathway (Coffey *et al.* 2009). Associations between HLA alleles, DENV genotypes and DENV disease are complex and require further investigation.

1.3.2.3 Pre-existing immunity and ADE

In secondary infections, the presence of non-neutralising pre-existing immunity against different DENV serotypes is also recognised as one of the main factors contributing toward the development of DHF. In response to primary infection, protective immunity to the infecting serotypes is believed to last a lifetime (Halstead and O'Rourke 1977; Guzman *et al.* 2007). Differently, complete cross-protective immunity against a secondary infection is present for only 1-2 months after the primary infection (Sierra *et al.* 2002). After 9 months, heterologous DENV infections represent a risk factor for the development of DHF (SABIN 1950). This has been attributed to an immunological phenomenon known as

antibody-dependant-enhancement (ADE) (Halstead *et al.* 1970). During second infections with a different DENV serotype, antibodies binding to non-neutralising viral epitopes or circulating at non-neutralising concentrations fail to clear the virus and in fact enhance DENV infections. DENV-antibody complexes bind to the Fc receptors (FcR) on cell surface and are efficiently internalised, providing an alternative entry route to the cell and contributing to higher viral load (Halstead and O'Rourke 1977; Halstead *et al.* 1977b). The ADE model provides an elegant explanation for increased viral burden following secondary infections and it also suggests that circulation of maternal antibodies might be responsible for the high frequency of cases of DHF in infants. ADE, extensively described *in vitro*, has been reproduced in IFN receptor-deficient mouse models (Williams *et al.* 2009) and suggests that the risk of ADE must be taken into account in the design of vaccines against DENV. However, no evidence of ADE has so far been reported in DENV infected patients and more studies are required to clarify the importance of this phenomenon in DHF pathogenesis.

1.3.3 Pathogenesis of DHF

In spite of the strong correlation between virus titre and disease severity, it has been observed that the most severe symptoms of DHF do not coincide with viral peak, but in fact develop as viraemia is controlled (Halstead 1980a). Furthermore, studies on the molecular mechanism of plasma leakage in DHF have shown that increased vascular permeability is not induced by the virus directly, but can in part be explained by a dysregulated adaptive immune response.

Vascular endothelial cells, lining the inner lumen of blood vessels, constitute the

primary blood-tissue barrier controlling flux of nutrients, microcirculatory dynamics, and permeability of micromolecules. TNF-α, IFN-γ, IL6, IL8, and several other proinflammatory cytokines control endothelial permeability and diapedesis of neutrophils, extravasation of erythrocytes, and infiltration of perivascular mononuclear cells. Skin biopsies from patients with DHF show that blood vessels are not damaged by DENV infection and the endothelial junctions are also intact; in fact, DHF patients display a complex **cytokine secretion** profile, which has been associated with increased capillary permeability and plasma leak (reviewed in Chaturvedi *et al.* 2005; Fink *et al.* 2006; Basu and Chaturvedi 2008).

In response to DENV infection, both T cells and macrophages are activated and they have been shown to contribute to high release of proinflammatory cytokines (Kurane *et al.* 1989; Green *et al.* 1999); however whether they only help virus clearance or they are involved in dengue pathogenesis is still a subject of debate. In secondary infections, the reactivation of pre-existing memory T-cells specific against a different DENV serotype has also been suggested to contribute towards dengue pathogenesis, phenomenon described as "**original antigenic sin**" (Mongkolsapaya *et al.* 2003). Original antigenic sin occurs when memory T cells are highly specific for antigens encountered during the primary infection, but have low binding affinity to new antigens from heterologous serotypes. These cells proliferate quicker than naïve high-affinity clones, and their faster re-activation not only does not undermine virus replication, but may instead contribute to immunopathogenesis through cytokine release and tissue damage (Gagnon *et al.* 1999; Mongkolsapaya *et al.* 2003).

Further studies are required to assess the role of the **complement** system in dengue disease. Avirutnan and colleagues have observed accelerated complement consumption during shock in patients with DHF (Avirutnan *et al.* 2006; Avirutnan *et al.* 2008). Further investigation has shown a correlation between the amount of membrane-associated or soluble NS1, the concentration in the blood of final products of complement activation as SC5b-9, and increased vascular permeability. The authors suggest that expression of NS1 on infected cells may result in binding of heterotypic, non-neutralising antibodies and complement attack. Moreover, when associated to the cell membrane, SC5b-9 can trigger cellular reactions leading to production of cytokines involved in endothelial permeability (Avirutnan *et al.* 2006).

Anti-DENV NS1 antibodies have also been suggested to contribute to dengue pathogenesis by cross-reacting with cell surface antigens on endothelial cells and platelets. This phenomenon is also known as antigenic mimicry. Lin and colleagues showed that binding of anti-NS1 antibodies to endothelial cells is responsible for NF-κB signalling activation and increased levels of cytokines and chemokines; furthermore, anti-NS1 stimulation has been correlated to increased expression of adhesion molecules on endothelial cells, and therefore increased lymphocyte adhesion (Lin *et al.* 2005). According to Wang and colleagues, anti-NS1 antibodies would also be responsible for the drop of platelet count observed in DF and especially in DHF patients (Wang *et al.* 1995). Chen and colleagues suggest that anti-NS1 antibodies recognise protein disulfide isomerase on platelets thereby inhibiting platelet aggregation (Chen *et al.* 2009). However, the precise mechanism is unclear and the importance of anti-NS1 antibodies in determining dengue disease outcome has not been established.

1.4 PROGRESS AND SETBACKS IN VACCINE AND ANTIVIRAL DRUG DEVELOPMENT

More than sixty years after the isolation of DENV, no effective vaccine is commercially available. Theoretically, DENV infections display some characteristics that make the development of a vaccine potentially achievable: the virus causes only acute infection with a short period of viraemia, and individuals who have recovered from DENV infection are immune to second challenges with the same serotype. Several immunogenic epitopes have been identified on both structural and non-structural proteins and antigen delivery does not present major obstacles. However, DHF pathogenesis is not completely clear and every effort to design a vaccine has been further compromised by the lack of adequate animal models. The main challenge in the design of a vaccine against DENV is to separate enhancing and neutralising immunity, since the immune response to DENV infection, while protecting from dengue disease, also appears to be a major risk factor towards DHF (see above). To prevent antibody enhancement phenomena and re-activation of a potentially harmful heterotypic memory response, an effective vaccine should provide protection against all four DENV serotypes simultaneously, and induce a robust and long-lasting immune response. Economic cost-benefit issues must be taken into consideration, and repeated dosing should be kept to a minimum. In recent years the number of different approaches to develop a vaccine against DENV has been unprecedented, with several candidates currently in clinical trial (Chaturvedi *et al.* 2005; Wilder-Smith and Deen 2008).

1.4.1 Live vaccines

1.4.1.1 Live-attenuated vaccines

Live-attenuated vaccines are promising because of low costs of production and because they induce a powerful and long lasting B and T cell immune response, even though the possibility of reversion and spread of vaccine strains must be carefully taken into consideration. Two live-attenuated vaccines are currently in clinical trial. The Mahidol University in Bangkok together with the Sanofi-Pasteur developed candidate live-attenuated vaccines by attenuating the four DENV serotypes through serial passages in primary dog kidney cells (serotypes 1, 2 and 4), or primary African green monkey kidney cells (serotype 3), and the four monovalent candidates were finally combined in a single tetravalent formulation. After a single dose vaccination, seroconversion was observed in all volunteers. The main limit was the high reactogenicity of this formulation, variable among the four serotypes and particularly high for serotype 3 (Kitchener *et al.* 2006). A different live-attenuated vaccine was formulated by The Walter Reed Army Institute of Research together with GlaxoSmithKline, by passaging all four DENV serotypes in primary dog kidney cells. After vaccination with the tetravalent formulation, high seroconversion and moderate reactogenicity were observed (Sun *et al.* 2003).

The US National Institute of Allergy and Infectious Diseases have used reverse genetic techniques to introduce defined attenuating deletions. Tetravalent formulations incorporate a Δ30 deletion in the 3` UTR in wild-type DENVs (Blaney, Jr. *et al.* 2006). Phase I trials showed low level viraemia and acceptable immunogenicity for a live attenuated DENV-4 vaccine candidate (Durbin *et al.*

2005). The same institute is also working on chimaeric viruses expressing the prM-E region of DENV-1, DENV-2 or DENV-3 into DENV-4Δ30. If needed, the level of attenuation of each vaccine can be further increased, introducing additional well established attenuating mutations (Blaney, Jr. *et al.* 2006).

1.4.1.2 Chimaeric vaccines

Successful experience with the chimaeric JEV/YFV vaccine raised optimism that such a formulation could have been used for DENV as well. ChimeriVax™ from Sanofi-Pasteur, currently in phase III of clinical trial, uses YFV 17D backbone and replaces the structural protein genes prM and E with the homologous proteins from DENVs. RNA transcripts are electroporated into *Vero* cells and progeny viruses are purified to produce the vaccine. Preclinical studies on monovalent formulations and on a tetravalent mixture of monovalent chimaeric viruses show that the ChimeriVax™ is replication competent, genetically stable and not-virulent upon 20 passages in *Vero* cells (Guirakhoo *et al.* 2002). The vaccine was shown to be attenuated, efficacious, safe and unlikely to be transmitted by arthropod vectors (McGee *et al.* 2008). However, anti-DENV immune responses are limited to prM and E, while responses to the NS proteins are probably an important component for life-long immunity (Rothman *et al.* 1993).

Chimaeric vaccines are much attenuated formulations and provide strong and long-lasting immunity. However, recent evidence has shown vaccine-related reactions in response to YF vaccine in some individuals who develop acute viscerotropic disease (Monath 2007); in these same individuals high viraemia ($\geq 10^5$ pfu/ml) has been documented, suggesting higher risk of recombination

events in case of dual infection with wild type flaviviruses (Seligman and Gould 2004; Seligman and Gould 2008).

1.4.2 Inactivated vaccines

After inactivation with formalin, DENV-2 retains its immunogenicity in mice and rhesus monkeys, inducing high titres of neutralising antibodies (Putnak *et al.* 1996). The main advantage of inactivated vaccines is the impossibility of reversion, but multiple doses are required and costs remain the main limit of such formulation.

1.4.3 Recombinant subunit vaccines

Many B and T cell epitopes have been mapped on DENV structural and non-structural proteins and these epitopes can be expressed in protein subunit vaccines. The extracellular portions of the E protein of each DENV serotype expressed in *Drosophila* cells have been used as a subunit vaccine by Hawaii Biotech Inc., showing high immunogenicity in mice and monkeys when administered with appropriate adjuvants (Clements *et al.* 2010). Further attempts have been made by fusing the first 395 amino acids of the DENV-2 E protein with the HBV (Hepatitis B Virus) surface antigen as a potent adjuvant. This hybrid was shown to elicit immune response against both components of the hybrid protein *in vivo* (Bisht *et al.* 2002). Scaling-up remains the major problem for recombinant vaccines, and powerful adjuvants are needed. Attempts have also been made by injecting purified recombinant DENV-2 NS1 protein in mice, but antibody production is not protective (Feighny *et al.* 1992).

1.4.4 Antiviral treatments

At present, no antiviral treatment is available against DENV infection. The treatment is only to alleviate symptoms and rehydration in case of hypotension is the main aid to survival. Corticosteroids or drugs decreasing capillary permeability do not reduce mortality in children with DHF/DSS (Leyssen *et al.* 2000).

Rational design of drugs aims at targeting viral mechanisms critical for DENV survival, limiting propagation of the infection. Preventing capping, for instance, has been shown to destabilise viral progeny, and also compounds able to inhibit the viral methyl-transferase have been studied. Pharmaceutical groups are mainly working on the RNA-dependent RNA polymerase and the viral protease/helicase, but no drug is currently available and not all mechanisms involved in DENV replication and pathogenesis are known (reviewed in Leyssen *et al.* 2000).

Deeper knowledge of viral infection and of the immune system response might be the key to identify more specific and effective ways to stop DENV. In particular, as outlined above, high viral replication at early stages of infection is known to be a determinant of dengue outcome (Vaughn *et al.* 2000); it is therefore logical to think that the rational development of attenuated vaccines or antiviral drugs could be greatly helped by better understanding viral and host factors involved at this critical stage of infection.

1.5 DENV INHIBITION OF INNATE IMMUNITY

As outlined in the previous chapters, the most severe forms of dengue disease are associated with high viral titres. Both host genetic determinants and virus characteristics contribute to viral replication, but rapid replication during the short viraemic period of acute infection can be achieved only if innate immunity, which represents the first line of host defence against pathogens, is delayed or inhibited. The more efficient the evasion of innate immunity, the higher will be the viral titre, and the more severe the disease outcome.

Emerging data show that DENV, like many other flaviviruses, has developed several mechanisms to inhibit the innate immune system, in order to systematically suppress the main components of the early immune response and quickly reach high viral titres. Innate immunity seems to have exerted stronger selective pressure than adaptive immunity, since adaptive immunity clears the virus at later stages of infection, when the viral titre has peaked already, and it has probably already been transmitted from human to mosquito.

DENV efficiently inhibits cellular response to type I IFN, and recent work has shown that it also prevents the activation of Natural Killer (NK) cells. Better understanding of these mechanisms is the main focus for this thesis, and they will be discussed in detail below.

1.5.1 DENV and NK cells

NK cells are an important component of the innate immunity, but their role in controlling flavivirus infections is not clearly defined. NK recognition of target

cells does not require receptor rearrangement or clonal expansion, so NK cells respond rapidly to infection (Lodoen and Lanier 2005). Resting mature NK cells constitutively express transcripts for inflammatory cytokines, and contain pre-formed cytolytic mediators like granzymes and perforines stored in intracellular granules (Lanier 2008). NK cell activation culminates in the exocytosis of such granules, lysis of infected target cells, and release of cytokines, primarily IFN-γ (Lodoen and Lanier 2005). NK cells display both activating and inhibitory receptors and activation of NK cells depends on the integration of these opposite signals. Activating receptors mainly recognise glycoproteins induced by cellular stress or pathogen-encoded ligands; inhibitory receptors recognise MHC class I molecules. MHC class I are expressed on the surface of all nucleated cells, and this prevents NK cytolytic activity on cells recognised as "self" (Lanier 2008). Due to the rapid and powerful response of NK cells, the role of inhibitory receptors is crucial.

Following DENV infection in mice, NK cell activity is transiently increased (Shresta *et al.* 2004a), and early activation of NK cells in humans has been associated with mild clinical disease, suggesting that high NK cell activation may be associated with a more favourable prognosis (Azeredo *et al.* 2006). According to Azeredo and colleagues, the majority of NK cells from DENV infected patients display early markers of activation during the acute stage of the disease, and intracellular cytotoxic granules are also up-regulated early. A recent study has shown that DENV E protein binds the NKp44 activating receptor on NK cells, inducing activation (Hershkovitz *et al.* 2009). Therefore, evasion of NK recognition might be critical to the establishment of a successful viral infection. A number of studies have shown that flaviviruses up-regulate expression of MHC

class I molecules on the cell surface in an IFN-independent manner (reviewed in Lobigs *et al.* 2003). Uncleaved C-prM structural protein has been implicated, suggesting that MHC-I up-regulation may be an incidental consequence of virus assembly rather than a proper evasion strategy (Lobigs *et al.* 2004). Differently, Hershkovitz et al (Hershkovitz *et al.* 2008) have shown that expression of DENV NS proteins only, in the context of DENV replicon, is sufficient to enhance membrane expression of MHC-I and therefore reduce susceptibility to NK lysis. These studies suggest that a complex balance exists between DENV-induced triggering of NK cell activation and subversion of NK-mediated killing by enhanced MHC-I expression on infected cells (Hershkovitz *et al.* 2008).

While preventing activation of NK cells, up-regulation of MHC class I molecules on the cell surface also increases cell susceptibility to cytotoxic T cells. This could provide potential disadvantages to the virus, but while adaptive immunity represents a relatively late event in infection, NK activation would jeopardise early stages of viral replication. As suggested above, it is likely that DENV evolution may have been affected by selective pressure of innate rather than adaptive immunity, and therefore that neutralisation of early NK cells rather than T cells might have been the priority (Hershkovitz *et al.* 2008).

1.5.2 DENV and type I IFN

The IFN system provides the earliest and most potent line of defence against many pathogens. The IFN response induces apoptosis and growth inhibition of infected cells, and regulates the immune response. The IFN family is composed of two main classes of related cytokines: type I IFN (including IFN-α and IFN-β)

which mounts a robust response against viruses, and type II IFN (IFN-γ) which also exhibits antiviral activity but is mainly regarded as a powerful immunomodulatory cytokine (Bonjardim *et al.* 2009). A third less well-known class, type III IFN, regulates the antiviral response and cell proliferation, and it has been proposed to be the ancestral antiviral system of vertebrates (Sadler and Williams 2008; Bonjardim *et al.* 2009).

Experiments with knock-out mice lacking IFN-α/β or IFN-γ receptors or both show that a functional response to IFN-α/β is critical in controlling the early viral load in the first stages of DENV infection; in contrast, the IFN-γ response provides partial resistance against DENV-induced disease and limits viral spreading in the periphery, but it is less important in controlling viral load (Shresta *et al.* 2004b). Several studies have now shown that DENV is capable of inhibiting type I IFN response. This thesis further investigates the molecular mechanisms of such inhibition. Therefore the type I IFN system is the main focus of the following sections.

1.5.2.1 The type I IFN system

1.5.2.1.1 Viral induction of type I IFN

Recognition of different viral components by specific host receptors, called Pattern Recognition Receptors (PRR), activates multiple distinct routes that trigger induction of the type I IFN. The importance of each route depends upon the specific virus, the infected cell and the stage of infection. IFN production can be induced by different viral components, from viral genome (RNA or DNA) or replication intermediates (dsRNA), to viral proteins, or simply following viral entry (Bonjardim *et al.* 2009). In response to PRR signalling, cytoplasmic IRF-3

(IFN Regulatory Factor-3) is phosphorylated, dimerises and exposes a nuclear localisation signal (NLS) which allows its translocation to the nucleus. The NF-κB (Nuclear Factor κB) inhibitor IκB, which holds NF-κB in the cytoplasm, is also phosphorylated and this induces dissociation from its substrate and subsequent ubiquitination and proteasome-mediated degradation. The NLS on NF-κB also becomes accessible and NF-κB translocates to the nucleus. IRF-3, NF-κB, and a third component, the c-jun/ATF-2 heterodimer, assemble on the IFN-β promoter and aid the recruitment of CREB-binding protein (CBP)/p300 which in turn promotes the assembly of the transcription machinery. The assembly of the transcription machinery on the IFN-β promoter induces transcription of IFN-β. Positive feedback models have been proposed in which the IFN-β produced feeds back onto cells and induces the synthesis of at least three other transcription factors, IRF-1, IRF-7 and IRF-9, which in turn, in the presence of a continued infection, enhance the transcription of "primary IFN genes" (IFN-β genes) and allow the transcription of "secondary IFN genes" (IFN-α genes) (reviewed in Randall and Goodbourn 2008).

1.5.2.1.2Type I IFN signalling

In spite of differences in induction, IFN-α and IFN-β are the main components of the type I IFN sub-family and they signal through the same pathway (Figure 1.4) (reviewed in Stark *et al.* 1998; Randall and Goodbourn 2008). Interaction of IFN-α/β molecules with two different receptor subunits, IFNAR1 and IFNAR2c, forces the two subunits together and induces their dimerisation.

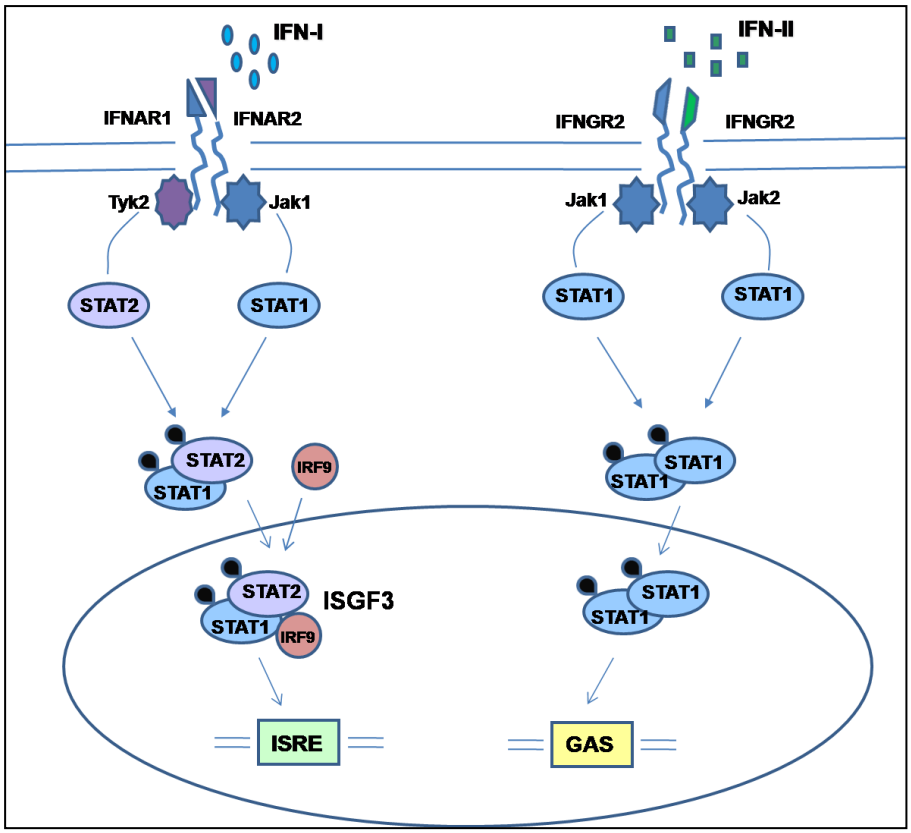


Figure 1.4|

Type I and type II IFN signalling cascade. After activation at receptor level, phosphorylation of STAT transcription factors induces their dimerisation and translocation into the nucleus, where they activate transcription of specific IFN-regulated genes.

The two receptor-associated kinases JAK1 (associated with IFNAR2c) and Tyk2 (associated with IFNAR1) are now close enough to phosphorylate and activate each other (Gauzzi *et al.* 1996). Once activated, JAK1 and Tyk2 phosphorylate some critical residues on IFNAR1. STAT2 binds IFNAR2c also before IFN induction and it is weakly associated with STAT1, but only IFNAR1 phosphorylation creates a docking site for STAT2 on IFNAR1. STAT2-IFNAR1 association brings STAT2 close enough to Tyk2 to allow STAT2 phosphorylation on tyrosine 690. Finally, phosphorylated STAT2 provides the docking site that orients STAT1 for phosphorylation on tyrosine 701 (Li *et al.* 1997). Once phosphorylated, the two STATs heterodimerise and dissociate from the receptor. This creates a novel NLS and, simultaneously, inactivates the constitutive NES of STAT2. The heterodimer and the associated transcription factor IRF-9 form the transcription complex ISGF3 and translocate into the nucleus by interaction with the nuclear importin system (Reich and Liu 2006). A recent study suggests that the assembly between the STAT heterodimer and IRF-9 is coordinated at the receptor, following acetylation of IFNAR2 after IFN binding (Tang *et al.* 2007).

Once in the nucleus, the transcription complex ISGF3 binds to specific DNA sequences called ISRE (Interferon-Stimulated Regulatory Elements) and induces the expression of more than 300 genes that specify the antiviral state. Amongst those genes some encode enzymes that directly block viral replication, such as protein kinase R (PKR) which prevents recycling of the eukaryotic translational initiation factor 2 (eIF2α), halting translation initiation; or the 2`5`-oligo-adenylate synthetase (OAS), which bind to activate the RNAse L and degrade cellular and viral RNA; or the MX GTPases, that recognise virus nucleocapsid structures and restrict their localisation within the cell, preventing viral

replication. No single gene is pivotal and for any virus a subset of genes is probably required to establish an antiviral state and prevent viral replication (reviewed in Randall and Goodbourn 2008; Sadler and Williams 2008).

Besides its potent antiviral effect at early stages of infection, type I IFN is also known as an important immunological modulator. Type I IFN bridges innate and adaptive immunity by enhancing clonal expansion of CD8+ T cells (Tough *et al.* 1996) and B cells (Le Bon *et al.* 2006), by activating NK cells (Biron and Brossay 2001), and by regulating differentiation and maturation of DC cells (Luft *et al.* 1998).

Even thought the importance of the type I IFN response in preventing viral spreading is well established, recent works are showing that rapid and efficient control of the IFN signalling is as critical as its activation. Prolonged activation of IFN-α can exert deleterious effects on the host immune system through several mechanisms, including elevated serum levels of pro-inflammatory cytokines (Stacey *et al.* 2009), apoptosis of uninfected cells, and generalised immune activation due to defects in the T cell selection and thymopoiesis (Bosinger *et al.* 2009). Recent work has shown that SIV-infected sooty mangabeys, a monkey species that is a natural SIV host, do not develop pathogenesis. Differently, infected rhesus macaques, in spite of similar levels of viral replication and early immune response activation, develop pathogenic infection (Bosinger *et al.* 2009). The difference can be explained by the presence in sooty mangabeys of lower levels of immune activation during the chronic phase of infection, and by reduced production of IFN-α (Bosinger *et al.* 2009; Jacquelin *et al.* 2009). Also, by using lymphocyte-deficient hosts, Kim and colleagues (Kim *et al.* 2007) also suggest

that in the absence of T cells the innate immune response can be a direct cause of death due to uncontrolled release of proinflammatory cytokines. This suggests that, while the type I IFN response is critical to activate T cells, the presence of active mechanisms of immune regulation exerted by T cells is also crucial to temper the initial innate response (Kim *et al.* 2007).

1.5.2.1.3Viral evasion of type I IFN

Millions of years of co-evolution between hosts and pathogens have resulted in the acquisition by most viruses of mechanisms to evade the host immune system. Different viruses inhibit different steps of the IFN pathway, either by interfering with host-cell gene expression and/or protein synthesis, or by inhibiting IFN production, signalling, or IFN-regulated antiviral pathways (Levy and Garcia-Sastre 2001). To circumvent the antiviral response, viruses often use a combination of these strategies, such as inhibition of both IFN production and signalling (Randall and Goodbourn 2008).

Inhibition of IFN production allows viruses to spread to non-infected cells before full establishment of an antiviral state. Viruses can minimise production of IFN by using replication strategies that limit generation of pathogen-associated molecular patterns (PAMPs) recognised by PRRs, by inhibiting PRRs, or by interference with specific critical points in the IFN-induction cascade (Randall and Goodbourn 2008).

Since IFN can still be produced by uninfected cells, inhibition of IFN signalling can be even more efficient than inhibition of IFN production. Several viruses have developed different strategies to block IFN signalling, and there are examples of viral proteins blocking each step of the IFN signalling cascade, as exemplified in

Table 1.1.

Virus	Target
HCMV	JAK1/IRF-9 reduction (Miller 1998) (Miller *et al.* 1999) STAT1/STAT2 sequester (Paulus *et al.* 2006)
HSV-1	JAK1/STAT2 reduction (Chee and Roizman 2004) SOCS3 up-regulation (Yokota *et al.* 2004)
PIV-2	STAT2 degradation (Andrejeva *et al.* 2002)
Nipah virus, Hendra virus	STAT1/STAT2 sequester (Rodriguez *et al.* 2003; Rodriguez *et al.* 2004)
RSV	STAT2 degradation (Elliott *et al.* 2007)
Adenovirus	STAT1/IRF-1 reduction (Look *et al.* 1998) CBP/p300 sequester (Look *et al.* 1998)
Ebola virus	Inhibition of STAT import (Reid *et al.* 2007)

Table 1.1|
Mechanisms of inhibition of type I IFN signalling by different viruses.

1.5.2.1.4 Flavivirus inhibition of type I IFN

Most (or possibly all) flaviviruses have evolved mechanisms to inhibit IFN-mediated signal transduction (Best *et al.* 2005), but the exact mechanisms remain elusive. Besides differences between flaviviruses, the picture is further complicated by the employment of different cell lines and expression systems by different investigators. The literature abounds in contradictory reports and very few observations have been reproduced and validated. **West Nile virus** (WNV) has been reported to inhibit the phosphorylation of STAT1, STAT2, Tyk2 and JAK1; four non-structural proteins (NS2A, NS3, NS4A and NS4B) have been implicated (Guo *et al.* 2005), but no mechanism has been described. Differently, Laurent-Rolle and colleagues, have recently identified NS5 of the virulent strain WNV NY99 as a potent antagonist of type I IFN signalling (Laurent-Rolle *et al.*

2010). **Japanese encephalitis virus** (JEV) blocks Tyk2 phosphorylation and this has been attributed to the non-structural protein NS5 (Lin *et al.* 2004; Lin *et al.* 2006). **Langat virus** (LGTV) NS5 has also been shown to be involved in IFN inhibition and specific regions have been mapped. Best and colleagues suggest that the IFN inhibition by LGTV may be the consequence of a direct interaction between LGTV NS5 and the IFN receptor, but the mechanism remains unclear (Best *et al.* 2005; Park *et al.* 2007). The methyltransferase domain of **tick borne encephalitis virus** (TBEV) NS5 has also been reported to be critical to IFN inhibition: Werme and colleagues suggest that it allows NS5 localisation at cell membrane, where the block of the IFN signalling seems to take place (Werme *et al.* 2008).

All these studies suggest that IFN inhibition occurs at early stages of the signalling cascade, and that different viruses within the same genus may have developed different mechanisms to antagonise the IFN response. However, several questions remain unanswered and more studies are required for a clear understanding of flavivirus mechanisms of evasion.

1.5.2.2 DENV inhibition of type I IFN

Inhibition of type I IFN response in infected cells by DENV has been observed in several circumstances. However, not only is the mechanism unclear, but also conflicting and dubious observations have been reported. Different studies disagree both on the steps of the signalling cascade antagonised by DENV (STAT1 (Munoz-Jordan *et al.* 2003), STAT2 (Jones *et al.* 2005), Tyk2 (Ho *et al.* 2005)) and on the viral proteins involved (NS4A, NS4B, NS2A (Munoz-Jordan *et al.* 2003; Munoz-Jordan *et al.* 2005)), and only few observations have been

reproduced by different investigators. Even though it is possible that DENVs have developed more than one strategy to evade the IFN response at different stages of the signalling cascade, the lack of reproducibility and consistency suggests that such diversity in the literature is more likely to be due to experimental variability.

The pathogenicity of DENV despite high concentrations of circulating IFN in patients was reported many years ago (Kurane *et al.* 1993), suggesting some malfunction in the IFN system. The first study addressing the possibility that DENV might escape cellular responses to IFN was published by Diamond and Harris in 2001 (Diamond and Harris 2001). The work mainly investigated how IFN prevents DENV-2 replication, but the authors observed that such inhibitory effect occurred only when IFN treatment preceded viral infection; differently, when cells were treated only a few hours after infection, a significant percentage of the IFN antiviral effect was lost. Even though the existence of an active mechanism of inhibition was considered, Diamond and Harris proposed an alternative hypothesis. According to this model, as soon as a short polypeptide is synthesised from DENV RNA, membrane-anchor signals in the C protein recruit the ribosome-RNA complexes to the ER: if cells are treated with IFN before the infection, some unidentified IFN-induced molecules prevent RNA translation before the translocation to the ER; conversely, if IFN treatment follows infection, the complexes, protected by the ER, become inaccessible to the IFN-induced molecules (Diamond and Harris 2001). This hypothesis could explain the inefficacy of late IFN administration, but has never been demonstrated experimentally; instead, there is growing evidence that DENV actively antagonises the IFN response. In 2003, Muñoz-Jordan and colleagues showed that the IFN response was inhibited in DENV infected cells and by measuring the

replication of the IFN-sensitive virus NDV (Newcastle Disease Virus)-GFP after IFN-β treatment in different cell lines expressing individual protein/s of DENV-2, the group suggested that DENV NS2A, NS4A and NS4B are all IFN antagonists (Munoz-Jordan *et al.* 2003). NS4B in particular demonstrated the strongest inhibitory properties, but the effect was further magnified by the co-expression of NS2A and NS4A. In this work, inhibition of STAT1 phosphorylation in response to both IFN-β and IFN-γ was reported in cells expressing NS4B and the authors suggested that DENV NS4B inhibits IFN signalling by preventing STAT1 activation. In 2005, the same group reported the importance of the N-terminal sequence of NS4B and of the proteolytic cleavage between NS4A and NS4B for IFN inhibition (Munoz-Jordan *et al.* 2005). Moreover, the authors suggested that the same inhibitory function of NS4B is conserved in WNV and YFV (Munoz-Jordan *et al.* 2005). This is the only preceding work investigating the role of specific DENV proteins in IFN antagonism, and it is widely cited in the literature. However, the phenotype described in these studies has been contradicted by others, potentially questioning the validity of the observations: even though inhibition of STAT1 phosphorylation has been observed by others (Ho *et al.* 2005; Jones *et al.* 2005), inhibition of type II IFN has never been reproduced, suggesting that inhibition of STAT1 phosphorylation is more likely to be the consequence of upstream blocks rather than direct effect of DENV antagonism.

DENV-2 inhibition of IFN signalling was investigated in primary monocyte-derived DC by Ho and colleagues (Ho *et al.* 2005). This study does not address the role of individual DENV proteins in IFN antagonism, but investigates which step of the signalling cascade pathway is targeted by DENV. According to this work, up to 24 hours post-infection, the basal levels of all proteins involved in

type I IFN signalling are unaltered and DENV-2 is reported to block the phosphorylation of Tyk2.

Jones and colleagues used both DENV-2 infected and replicon containing cells: since the replicon expresses only NS proteins, the role of structural components or viral entry in the phenomena observed can be excluded. The most interesting observation in this study is that in both DENV infected and replicon containing cells, cellular levels of STAT2 are greatly reduced; this suggests that STAT2 reduction could be the mechanism by which DENV inhibits the IFN response (Jones *et al.* 2005). Even though in Ho`s work no STAT2 reduction could be observed, later studies by Umareddy and colleagues reproduced Jones` observation (Umareddy *et al.* 2008). It is possible that reduced STAT2 expression was not observed in Ho's study because the analysis was performed at a relatively early time-point (24 hours).

In spite of several inconsistencies, taken together these studies have made some steps forward toward the understanding of DENV inhibition of type I IFN. These studies show that DENV actively inhibits IFN signalling and the inhibitory effect is probably specific for type I IFN. **The focus of the work in this thesis was to elucidate the precise mechanism of inhibition of type I IFN signal transduction by DENV-2 and to identify the non-structural protein/s that mediate/s this effect.** A precise link between IFN antagonism and dengue pathogenesis has not been proved, but a relationship between IFN response, virus replication and viral pathogenesis has been shown for WNV. In particular WNV virulence has been linked to control of JAK-STAT signalling pathway and to

overall resistance to IFN response (Keller *et al.* 2006; Tobler *et al.* 2008). Therefore, as outlined above, evasion of the innate immunity seems critical to high viral replication and therefore severe disease. Better understanding of how DENV evades the IFN response would therefore represent a crucial advance in understanding dengue pathogenesis.

Chapter 2.

MATERIALS AND METHODS

2.1 MOLECULAR CLONING

2.1.1 High-Fidelity Polymerase Chain Reaction (PCR)

Amplification of long templates for successive cloning requires high accuracy and speed. Phusion™ High-Fidelity DNA Polymerase (Finnzymes, New England BioLabs) incorporates a processivity-enhancing domain to a *Pyrococcus*-like enzyme, which possesses a 5`-3` DNA polymerase activity and a 3`-5` exonuclease ("proofreading") activity: such a combination allows an error rate of $4.4 \cdot 10^{-7}$ in HF Buffer (50-fold lower than Thermus aquaticus DNA polymerase, and 6-fold lower than Pyrococcus furiosus DNA polymerase), and it is suitable for amplification of long amplicons such as 7.5 kb.

In this work we employed High-Fidelity PCR whenever high accuracy in template amplification was required, i.e. any time we amplified an insert from the replicon or other plasmid for subsequent cloning into the lentiviral vector.

2.1.1.1 Primer design

When possible, depending on the specific sequence to amplify, primers were designed in order not to be complementary to each other and with roughly similar annealing temperatures.

To aid with the primer design, primer sequences were checked on the web-based program Primer3, available at http://frodo.wi.mit.edu/primer3.

Primers used for cloning are listed in Table 2.1. Primers *Lentivector Forward (F)* and *Lentivector Reverse (R)* were exclusively used for sequencing.

Primer name	Primer sequence
NS1/NS2A For	AGGATCC**CACCATG**AGCACCTCACTGTCTGTGTCA
NS1/NS2A Rev	GCGCGGCCGC**TTA**CCTTTTCTTGTTGGTTCTTGAAAG
NS2B/NS3 For	TAGGATCC**CACCATG**AGCTGGCCACTAAATGAGG
NS2B/NS3 Rev	GCGCGGCCGC**TTA**CTTTCTTCCAGCTGCAAACTC
NS4A For	TAGGATCC**CACCATG**TCCCTGACCCTGAACCTAAT
NS4A Rev	TAGCGGCCGC**TTA**TCTCTGCTTTTCTGGTTCTGG
NS4B For	AGGATCC**CACCATG**ACACCCCAAGATAACCAATTG
NS4B Rev	TAGCGGCCGC**TTA**CCTTCTCGTGTTGGTTGTGT
NS5 For	AGGATCC**CACCATG**GGAACTGGCAACATAGGAGAG
NS5 Rev	TAGCGGCCGC**CTA**CCACAGGACTCCTGCCT
NS5-FLAG Rev	TAGCGGCCGC**CTA**CCCTTTGTCATCGTCGTC
MTase 260 Rev	TAGCGGCCGC**CTA**GCTTCCGAGGTCTACATCTGG
Pol 270 For	AGGATCC**CACCATG**GAGATACCAAACCTAGACATAA
Pol 405 For	AGGATCC**CACCATG**AATGCAGCCTTGGGGGCC
NLS 320 For	AGGATCC**CACCATG**AATGCCTCCGGGAACATAGTG
NLS 405 Rev	TAGCGGCCGC**CTA**GCTTCTCACCTTTCTTGT
Lentivector F	*AAAGAGCTCACAACCCCTCA*
Lentivector R	*CAAATTTTGTAATCCAGAGGTTGA*

Table 2.1|
Primers employed for PCR amplification of DENV NS cassettes and for sequencing. The nucleotide sequences recognised by restriction enzymes are underlined; the Kozak sequence+start codon (forward primers, *For*) and the stop codons (reverse primers, *Rev*) are in bold. *Lentivector F* and *Lentivector R* map within the lentiviral vector, upstream and downstream of the cloning site respectively, and were used for DNA sequencing.

2.1.1.2 High-Fidelity PCR

To avoid DNA contamination, PCR reactions were set up in a separate designated area. Separate micro-pipettes, reagents, equipment, laboratory coats and sterilized pipette tips were used.

Negative PCR controls in which the DNA template had been replaced by ddH_2O were included: should these give positive results, then the experiment and the current batch of reagent aliquots were discarded.

Accordingly to manufacturer`s instructions, the following reagents were mixed as in Table 2.2:

Component	Volume	Final Concentration
ddH_2O	Up to 50 μl	
5X Phusion GC Buffer	10 μl	1X
10 mM dNTPs	1 μl	200 μM each
25 μM Primer Forward	1 μl	0.5 μM
25 μM Primer Reverse	1 μl	0.5 μM
DNA template	X (10-50 ng)	
Phusion DNA polymerase	0.5 μl	0.02 U/ml

Table 2.2|
Reagent mix for High Fidelity Phusion PCR

PCR reactions were performed in the thermal cycler I-cycler (BIO-RAD), preheated to 95˚C, as described in Table 2.3.

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	98˚C	30 seconds	1
Denaturation Annealing* Extension	98˚C X˚C 72˚C	10 seconds 30 seconds 30 sec/kb	30
Final Extension	72˚C 4˚C	10 minutes hold	1

Table 2.3|
Cycling conditions for High Fidelity Phusion PCR. *An annealing temperature equal to the melting temperature (Tm) of the lower Tm primer has been used. The Tm of each primer has been calculated using the nearest-neighbor method on the Finnzymes` website (www.finnzymes.com). When necessary, a temperature gradient has been used in order to find the optimal annealing temperature.

2.1.2 Agarose gel

PCR products, amplified plasmids, and DNA digestions were analysed on agarose gel in order to test the presence and the correct size of the DNA of interest.

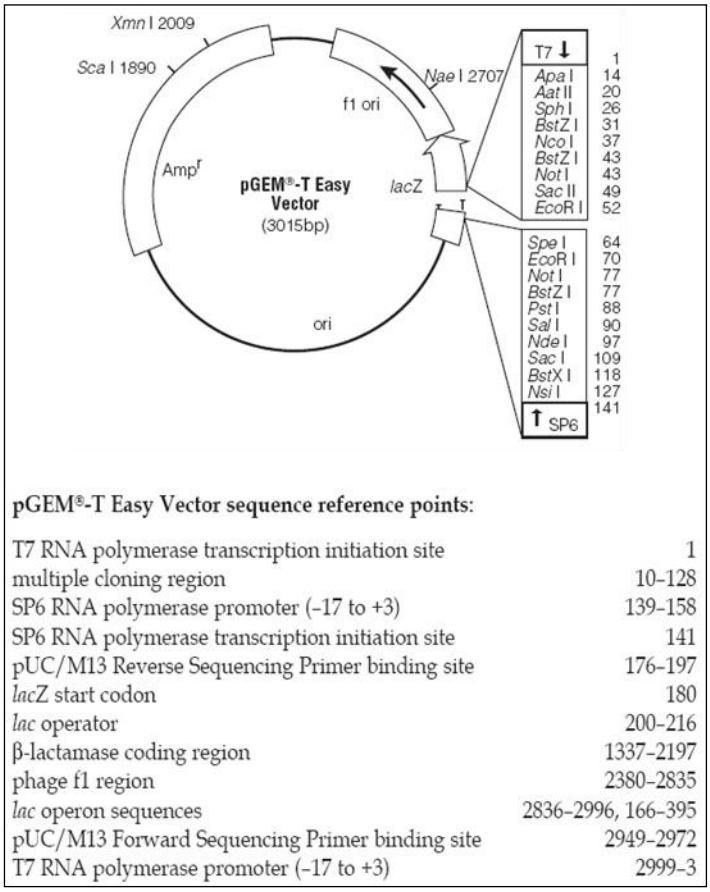
All gels were prepared dissolving 1 g of agarose (Melford) in 100 ml of 1X TAE buffer, diluted from 50X TAE stock [1L: 242 g Tris base (Trizma), 57.1 ml glacial acetic acid and 100 ml 0.5M EDTA, pH 8.0], and adding 5 μl of 10 mg/ml Ethidium Bromide (Sigma).

1X loading dye [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF in 40% (w/v) sucrose solution] was added to 50-200 ng of DNA before loading. 0.5 μg of molecular ladder (1 kb or 100 bp, NEB) were loaded as reference.

Gels were run at 5 V/cm until the bromophenol blue front in the loading dye had migrated at a sufficient distance. Gels were then visualized using a UV trans-illuminator (Epi Chemi II darkroom, UVP Laboratory Products) and photographed if required (Software: Labwork 4.6).

2.1.3 Subcloning in pGEM®-T Easy Vector System

The pGEM®-T Easy Vector System is a convenient system for cloning PCR products. DNA amplified by PCR has flat extremities that make particularly difficult the cloning into a vector; even when the DNA template is flanked by restriction sites, enzymatic digest of linear fragments is particularly difficult, especially close to the DNA extremities. Inserting the PCR product into a subcloning vector is a common strategy to facilitate the activity of restriction



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Figure 2.1|
Map of the pGEM®-T Easy Vector and sequence reference points.

enzymes, and sometimes to insert or substitute restriction sites in the subcloned DNA.

The pGEM®-T Easy Vector (Figure 2.1) carries 3`-T overhangs at both ends of the linearised vector: 3`-Ts at the insertion site prevent re-circularisation of the vector and provide compatible overhangs for PCR products flanked by deoxiadenosines (dATP), improving the efficiency of ligation.

The multiple cloning region is located within the α-peptide coding region of the enzyme β-galactosidase: insertional inactivation of the α-peptide allows recombinant clones to be immediately identified by colour screening in indicator plates. In this work, subcloning in pGEM®-T Easy vector has been used every time we amplified a construct by PCR, for following cloning into the lentiviral

vector.

2.1.3.1 A-tailing

In order to match the 3`-T overhangs of the pGEM®-T Easy Vector cloning site, PCR products must carry A-overhangs. Thermostable DNA polymerase with proofreading activity generates blunt ends during PCR amplification, but these fragments can be modified by insertion of an A-tail at both sides of the fragment by incubating for 30 minutes at 70˚C the purified (see section 2.1.3.2) PCR products with a Taq DNA polymerase in excess of dATP.

The A-tailing reaction mix was prepared as in Table 2.4.

Component	Volume
10X Taq Polymerase Buffer	1 µl
dATP (1 mM)	2 µl
$MgCl_2$ (25 mM)	0.6 µl
GoTaq Flexi DNA Polymerase	1 µl
Purified PCR product	5.4 µl

Table 2.4|
Reagent mix for A-tailing reaction.

2.1.3.2 PCR purification

The addition of a poly-A tail would be prevented by the proof-reading activity of the High Fidelity DNA polymerase in the reaction. Therefore, all products were purified from PCR reagent by using the QIAquick® PCR purification kit (Qiagen), following manufacturer`s instructions.

Briefly, each PCR sample were mixed with Binding Buffer and loaded in a small silica column: the high salt concentration provided by the Binding Buffer allows DNA products between 100 bp and 10 kb to absorb to the column, while

contaminants pass through. Impurities were washed away by an ethanol containing buffer, and pure DNA was eluted with water.

2.1.3.3 Ligation in pGEM®-T Easy Vector

Ligations were performed following manufacturer`s instructions; reagents were mixed as shown in Table 2.5.

Reaction Component	Standard reaction	Positive control	Background control
2X rapid ligation	5 µl	5 µl	5 µl
pGEM®-T Easy Vector (50ng)	1 µl	1 µl	1 µl
PCR product	X µl*	-	-
Control insert DNA	-	2 µl	-
T4 DNA ligase (3 Weiss units/ml)	1 µl	1 µl	1 µl
Nuclease free water	Up to 10 µl	Up to 10 µl	Up to 10 µl

Table 2.5|
Reagent mix for cloning into pGEM®-T Easy vector.
*A volume corresponding to 1:1 or 3:1 insert:vector ratio has normally been used. To calculate the appropriate amount of insert to include in the ligation reaction we used the following equation: [(ng of vectors x kb size of insert)/kb size of vector] x insert:vector molar ratio = ng of insert.
Size of vector pGEM®-T Easy = ~3 kb.

The reactions were incubated at 4˚C overnight to increase the number of transformants.

2.1.3.4 Transformation

Max Efficiency® DH5α™ competent cells have been used for transformations with pGEM®-T Easy Vector, following manufacturer`s instructions. Briefly, 50 µl of competent cells were aliquoted into pre-chilled tubes and 1 µl of the ligation reaction was added. Cells were incubated 30 minutes on ice, heat-shocked 30

seconds in a 37˚C water bath, and placed back on ice for 2 minutes.

450 µl of room temperature LB medium were added and the samples were incubated at 37˚C, 225 rpm for 1 hour. Half the volume was spread onto LB plates containing 100 µg/ml ampicillin and prepared by adding 15 µl of 50 µg/ml X-gal, and 3 µl of 1 mM IPTG onto the plates 1 hour before using. Plates were incubated at 37˚C overnight. As successful insertion into pGEM®-T Easy Vector interrupts the coding sequence of β-galactosidase, recombinant clones can be identified by colour screening, with clones containing PCR products generating white colonies.

2.1.4 DNA preparation

2.1.4.1 Mini-DNA preparations (minipreps)

Minipreps were employed when only a small amount of DNA was required, i.e. for screening of positive colonies after transformation. On average, 5 white colonies per plate where selected and grown overnight at 37˚C, 225 rpm, in 5 ml of LB medium containing 100 µg/ml of ampicillin. The following day DNA was isolated using Promega Wizard® *Plus* SV Minipreps DNA Purification System, according to manufacturer`s instructions. Briefly, 3 ml of bacteria culture were harvested by centrifugation at maximum speed in a tabletop centrifuge. Pellet was resuspended in Cell Resuspension Solution and lysed by Cell Lysis Solution. After short incubation, Alkaline Protease Solution was added to inactivate endonucleases and other proteins released during the lysis. Lysis was stopped by adding Neutralisation Solution. Bacteria lysate was centrifuged at maximum speed in a microcentrifuge for 10 minutes, and the cleared lysate transferred to a silica-membrane column. The column was washed with a Column Wash solution

containing ethanol and the DNA eluted in nuclease-free water. Eluted DNA was quantified and assessed for purity by NanoDrop (ND-1000 NanoDrop® Spectrophotometer; software: ND-1000 V3.2.1).

2.1.4.2 Midi-DNA preparation (midipreps)

When higher amounts of DNA were required, Promega PureYield™ Plasmid Midiprep System were used, following manufacturer`s instructions. The main differences compared with the miniprep system are the possibility of processing larger volume of bacteria culture (50-100 ml), and therefore obtaining larger amounts of purified DNA. Eluted DNA was quantified and tested as described above.

2.1.5 Enzymatic Digestions

Enzymatic digestions are used in order to generate compatible ends in vectors and inserts before ligation, or to check the correct size of the selected clones.

In this work restriction digests were used:

- to extract the inserts from the pGEM®-T Easy Vector;
- to linearised the lentiviral backbone;
- to test the presence and the correct size of the insert, after cloning in pGEM®-T easy or in the lentiviral vector.

Restriction endonucleases employed in this study:

- BamHI (NEB, Buffer BamHI, BSA): cloning site

- NotI (NEB, Buffer 3, BSA): cloning site
- AscI (NEB, Buffer 4): cloning site for the MESV "leader sequence" (see chapter 3, section 3.3.3.1)
- ScaI (NEB, Buffer 3): to break the pGEM®-T Easy backbone when the insert had a similar size to the pGEM®-T Easy itself, in order to help the separation on gel.

1 unit (U) of enzyme is defined as the amount of enzyme required to digest 1 μg of DNA in 1 hour at 37˚C. The required amount of DNA was mixed with the appropriate dilution of the specific 10X Restriction Buffer recommended, the appropriate dilution of 100X BSA when required, and with sufficient amount of enzyme.

To generate compatible ends for cloning, between 5 and 10 μg of DNA from a midiprep were digested in a final volume of 100 μl. To check the correct size of the inserts only 1 μg of DNA was digested, in a final volume of 50 μl. When double digestion was required, but no restriction buffer was compatible with both enzymes at the same time, restrictions were performed subsequently, heat inactivating the first enzyme (when possible) and substituting the restriction buffer by ethanol precipitation of the DNA (see below).

2.1.6 Ethanol precipitation of DNA

Ethanol precipitation of DNA was used to replace the restriction buffer in sequential digestions, when the efficiency of the second restriction enzyme was suboptimal in the buffer used for the first restriction digestion.

To precipitate DNA, 1/10 volumes of 3 M sodium acetate (pH 4.8) were added, followed by 2.5 volumes of cold ethanol. The solution was mixed thoroughly and placed at –80ºC for at least 30 minutes. The tube was then spun at 12,000 g for 15 minutes at 4ºC. The supernatant was aspirated and the pellet washed once with 70% ethanol, air-dried and resuspended in the desired buffer.

2.1.7 Insert preparation

Inserts were extracted by double digestion from the pGEM®-T Easy Vector. Digested DNA was run on agarose gel in order to separate the linearised fragments. All digested DNA volume was loaded on 1% agarose gel and run until the loading dye has reached the bottom end of the gel. The gel was then exposed to UV light for the shortest time possible in order not to damage the DNA and a thin slice of gel containing the fragment of interest was cut and carefully trimmed. DNA was purified from agarose as described in section 2.1.9.

2.1.8 Backbone preparation

2.1.8.1 Dephosphorylation of linearised DNA fragments

During a ligation reaction the 5’ end phospho-group and the 3’ hydroxyl-group become the substrate for the T4 DNA ligase resulting in a phosphodiester bond. This could generate recircularisation of the vector, preventing the insertion of the transgene of interest. Therefore, especially when the extremities of the vector are blunt, or when high background is observed in the absence of the insert, dephosphorylation of the vector is required. By using the calf intestinal alkaline phosphotase (CIP, NEB), the 5’ phosphate ends of the DNA vector are removed,

and the vector is unable to self-ligate.

After double digestion, the reaction was performed resuspending the DNA in 1X NEB buffer 3 at a concentration of 0.5μg/10μl, and adding 0.5U of enzyme per 1 μg of DNA. The reaction was incubated at 37ºC for 1 hour.

2.1.9 Isolation of DNA from agarose gel

The procedure to purify DNA from gel depends on the size of the fragment: for fragments longer than 10 kb we used QIAEX II Agarose Gel Extraction kit (Qiagen), while for shorter fragments we used QIAquick Gel Extraction kit (Qiagen), following manufacturer`s instruction. Both kits are based on solubilisation of the agarose and selective absorption of the DNA onto silica gel in the presence of high salt, but the QIAEX II Agarose Gel extraction kit, optimised for larger DNA fragments (up to 50 kb), employs silica gel particles, while the QIAquick Gel Extraction kit, optimised for fragments up to 10 kb, employs silica gel membranes. Washing steps remove impurities and DNA is eluted in water.

2.1.10 Cloning in lentiviral vector

2.1.10.1 Ligation

Ligation between the BamHI-NotI linearised, dephosphorylated, and gel purified lentiviral vector, and the genes excised from pGEM®-T Easy were performed following the instruction for the Rapid DNA Ligation Kit (Roche). Briefly, 50 ng of lentiviral backbone and a 3:1 insert:vector ratio were diluted in 1X DNA Dilution Buffer, in a final volume of 10 μl. 10 μl of T4 DNA Ligation Buffer were added to the DNA mix, followed by 1 μl of T4 DNA Ligase. The

reaction was incubated 10 minutes at room temperature.

2.1.10.2 Transformation

Transformation of DH5α cells was performed as described above (see section 2.1.3.4), using 1-2 μl of ligation mix.

Colonies were screened by miniprep (section 2.1.4.1) followed by double digestion BamHI-NotI (section 2.1.5), and correct clones were amplified by midiprep (section 2.1.4.2).

2.1.11 DNA sequencing

DNA from selected clones was sequenced by The Sequencing Service of the University of Dundee.

Primers mapping shortly upstream (*Lentivector F*, see table 2.1) and downstream (*Lentivector R*, see table 2.1) of the cloning site were used to check the correct insertion of sequences into the lentiviral vector.

2.2 PRODUCTION OF LENTIVIRAL VECTORS

2.2.1 Virus production

Generation of lentiviral vectors was performed by triple transfection of 293T cells with three different plasmids: the lentiviral vector containing the gene of interest, a second plasmid coding for the VSV-G envelope, and a third plasmid (pCMVR8.91) coding for viral enzymes required for reverse transcription and integration. Supernatant containing virus particles was collected and cleared after

48 and 72 hours and concentrated by ultracentrifugation. Each virusprep correspond to the supernatant collected from four 10 cm tissue culture dishes; virus preparations were scaled up as needed, taking into consideration the amount of virus required and the average yield of the specific lentivirus, often depending on the size of the transgene.

2.2.1.1 Cell plating

On the first day, 293T cells were washed, trypsinised, counted and $1.3 \cdot 10^6$ cells/plate were seeded in 10 ml of complete DMEM (10% FCS, 1% Gln). Cells were incubated at 37˚C, 10% CO_2 till the following day.

On the following day media was replaced (10 ml) 2 hours before transfection.

2.2.1.2 Transfection

Transfections of 293T cells were performed using FuGENE®6 Transfection Reagent (Roche). FuGENE is a blend of lipids and other components that forms complexes with DNA and transports it into animal cells. Benefits of FuGENE over other transfection systems are the high transfection efficiency of 293T and no cytotoxicity.

200 µl of OPTIMEM were aliquoted into 4 eppendorf tubes and 18 µl of FuGENE were added to each tube, without allowing the transfection reagent to touch the side or the bottom of the tubes. The liposomes were left for 15 minutes at room temperature. In the meantime, the DNA mix was prepared as follows (Table 2.6):

Component	Volume
pCMVR8.91 plasmid	6 μg
VSV-G plasmid	6 μg
Lentiviral vector	9.2 μg
Water	Up to 200 μl

Table 2.6|
DNA mix for triple transfection of 293T cells.

50 μl of DNA mix were added to each eppendorf containing FuGENE, and the mix was incubated at room temperature to allow the formation of the DNA-liposome complexes. After at least 30 minutes, the complexes were added to the cells and incubated at 37˚C, 10% CO_2 overnight.

The following day the media was replaced.

2.2.1.3 Supernatant collection

The supernatant was collected at 48 and 72 hours, and cleared from detached cells and cell debris by low speed centrifugation (5 minutes, 300 g), followed by filtration (0.45 μm filter). Cleared supernatant was kept in the fridge at 4˚C until ready to be concentrated.

2.2.1.4 Virus concentration

After the last collection, the supernatant was pooled together and kept cold. Maintaining the temperature low is critical along all the procedure. Therefore viral supernatant, tubes, rotor, centrifuge, buckets must be kept refrigerated all the time. The supernatant was aliquoted into sterile pre-chilled centrifuge tubes and ultracentrifuged (Sorvall Discovery 100SE) at 4˚C for 2 hours, at 25,000 rpm.

After centrifugation, the supernatant is removed by inversion and the tubes kept upside-down to drain all the media from the walls. 50 μl of complete media (DMEM, 10% FCS) were added to each tube and the virus resuspended by pipetting. The tubes were left on ice for 10 minutes to allow complete resuspension of the virus and finally all volumes were pooled together in a pre-chilled eppendorf tube. The eppendorf was spun in a table top microcentrifuge (13,000 rpm for 1.5 min) to remove bubbles, and the virus aliquoted in 20 μl aliquots in sterile pre-chilled eppendorf tubes.

Viruses were stored at -80˚C.

2.2.2 Virus titration

Each virusprep was quantified by measuring the efficiency of transduction of different amounts of virus on 293T cells. 293T cells are known for their high permissivity to viral infection, allowing the approximation that 1 cell is infected by 1 viral particle. 200,000 293T cells/well were seeded into six wells of a 24-well plate in a volume of 400 μl. One aliquot of virus was defrosted on ice and in the meantime 100 μl of complete media were aliquoted into 6 eppendorf tubes. To each tube the following volumes of virus were added: 0 μl; 0.3 μl, 0.5 μl; 1 μl; 2 μl; 5 μl, and the content of each tube was transferred onto the cells. Cells were incubate at 37˚C for 72 hours and GFP expression was measured by flow cytometry, calculating the percentage of positive cells compared to the untransduced cells.Virus concentration was calculated by using the following equation:

Virus particles/μl = [200,000 · (% positive cells / 100)] / volume of virus added

2.2.3 Transduction

$10 \cdot 10^5$ cells were washed, resuspended in 400 μl of complete media, and seeded in a 24 well plate. The volume of virus needed to transduce the desired number of cells was calculated using the following equation:

$[10 \cdot 10^5$ (= number of cells to transduce) · MOI] / virus titre

where MOI (Multiplicity Of Infection), represent the number of infecting particles per cell.

The corresponding number of vials was defrosted on ice and the calculated volume of virus was added to 100 μl of complete media previously aliquoted in an eppendorf tube. The diluted virus was added onto the cells and the plate incubated at 37˚C for 72 hours. After 24 hours more media was added to the cells. Efficiency of transduction was measured by flow cytometry by comparison with untransduced cells.

2.3 REVERSE TRANSCRIPTION AND REAL TIME PCR

2.3.1 RNA extraction

RNA was extracted from $2 \cdot 10^6$ cells for each sample. Cells were lysed in Trizol® (Invitrogen), a mono-phasic solution of phenol red and guanidine isothiocyanate, which maintains the integrity of RNA while disrupting cells and dissolving cell components. RNA extraction was performed following manufacturer`s instructions; briefly, after resuspension of cell pellet in Trizol®,

chloroform was added, followed by centrifugation, in order to separate the solution into an aqueous phase, containing exclusively RNA, and an organic phase containing DNA and proteins. RNA is recovered from the aqueous phase by precipitation with isopropyl alcohol, and resuspended in RNAse free water.

RNA was quantified by Nanodrop and stored at -80˚C.

2.3.2 DNAse treatment

3 μg of RNA were treated with DNAse, in order to ensure the complete removal of any DNA contamination: 1 μl of 10X DNAse buffer and 3 μl of DNAse (FPLC pure Dnase I, 5000U, Amersham Pharmacia Biotech Inc.) corresponding to 3 units were added to the RNA. RNAse free water was added up to 10 μl and the sample incubated at 37˚C for 30 minutes. The reaction was stopped by adding 1 μl of STOP buffer and incubating at 65˚C for 10 minutes.

2.3.3 cDNA reverse transcription

2 μg of RNA were mixed to 1 μl of Random Primers (Promega), 1 μl of RNAse inhibitor (Promega), and incubated at 70 ˚C for 3 minutes to melt secondary structures within the template, and then immediately on ice to prevent secondary structure re-formation. The following components were then added: 5 μl of dNTPs (40 mM), 5 μl of 5X MLV buffer (Promega), 5.3 μl of RNAse free water, and 1 μl of M-MLV reverse transcriptase enzyme (Promega). The mix was incubated 1 hour at 37 ˚C.

The remaining RNA was used as a negative control, to exclude the presence of

DNA contaminants, and was therefore treated according to the same procedure, but in the absence of M-MLV.

For qualitative experiments aiming at testing the presence of a transcript, both the samples and the corresponding negative controls were amplified by PCR and separated on an agarose gel.

2.3.4 Taq Polymerase Chain Reaction

For PCR screening and control PCRs high fidelity was not required and we employed GoTaq® Flexi DNA Polymerase (Promega). The enzyme is a modified form of Taq DNA polymerase that lacks 5`→3` exonuclease activity.

I this work, Taq PCR was employed in order to confirm the presence of the transcript in K562 after transduction with lentiviruses encoding different DENV NS proteins. For NS1/NS2A, NS4A, and NS4B the same primers used for cloning were employed; for NS2B/NS3 and NS5 internal primers amplifying a shorter region were specifically designed (Table 2.7)

Primer	Sequence
NS2B/NS3 RT Forward	AGGCTGGAAGCTAGAAGGAG
NS2B/NS3 RT Reverse	GTCGATGATTGGAGATCCTG
NS5 RT Forward	ACAAACCACATGGAAGGAGA
NS5 RT Reverse	GTCAGGTGCTGAATGCTTTT

Table 2.7|
Primers for amplification from cDNA of NS2B/NS3 and NS5.

PCR were performed following manufacturer`s instructions, carefully mixing the following components (Table 2.8):

Component	Volume	Final Concentration
ddH_2O	Up to 50 µl	
5X GoTaq® Flexi Buffer	10 µl	1X
$MgCl_2$ Solution, 25 mM	5 µl	2.5mM
10 mM dNTPs	1 µl	200 µM each
25 µM Primer Forward	1 µl	0.5 µM
25 µM Primer Reverse	1 µl	0.5 µM
DNA template	X (10-50 ng)	
GoTaq® DNA Polymerase	0.25 µl	1.25 U

Table 2.8|
Reagent mix for PCR from cDNA.

and using the following conditions (Table 2.9)

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	95˚C	2 minutes	1
Denaturation Annealing* Extension	95˚C X˚C 72˚C	1 minute 1 minute 1 minute/kb	30
Final Extension	72˚C 4˚C	5 minutes hold	1

Table 2.9|
Cycling conditions for PCR from cDNA.
*The annealing temperature was normally 5°C below the calculated melting temperature (Tm) of the primers. The Tm of each primer has been calculated using the nearest-neighbor method on the Finnzymes` website (www.finnzymes.com).

2.3.5 Real time PCR

In this work real-time PCR (RT-PCR) were performed to measure the induction of the type I IFN sensitive genes (ISG) MX1 and OAS1 in response to stimulation

with IFN-α.

Cells were stimulated with 100 IU/ml of IFN-α, and the RNA extracted, DNAse treated and reverse transcribed as described above. SYBR® Green is a DNA-binding dye that incorporates into dsDNA and emits fluorescence only when bound to dsDNA. Maximal excitation and emission are, respectively, at 494 nm and 521 nm. As the fluorescent signal increases proportionally with the increasing amount of amplicon generated in each cycle, the techniques can be used for accurate quantification.

Primers were designed following the same criteria described above but, differently from ordinary primers, RT-PCR primers were specifically designed to amplify internal regions of the genes of interest no longer that 100-200 bp, and having both a melting temperature between 50 and 60˚C. The primers used are listed in Table 2.10 below:

Primer name	Primer sequence
MX1 Forward	AACAACCTGTGCAGCCAGTA
MX1 Reverse	AAGGGCAACTCCTGAGAGTG
OAS1 Forward	ACAGGCAGAAGAGGACTGGA
OAS1 Reverse	GCCAGGAGTCAGGAGACTTG
GAPDH Forward	ACAGTCCATGCCATCACTGCC
GAPDH Reverse	GCCTGCTTCACCACCTTCTTG

Table 2.10|
Primers employed for real time PCR.

2 μl of template were added to a mix constituted of 12.5 μl of SYBR® Green (Qiagen), 9.5 μl of water and 1 μl of primers (10 μM). A sample in which the template had been replaced with water has been used as a negative control, to exclude the presence of contaminants. All samples were analysed in duplicate.

Samples were run in a Rotor-Gene (RG-3000, Corbett Research; software: Rotor-Gene 6), using the following cycling conditions (Table 2.11):

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	95˚C	15 minutes	1
Denaturation	94˚C	15 seconds	35
Annealing	50-60˚C	20-30 seconds	
Extension	72˚C	10-30 seconds	

Table 2.11|
Cycling condition for real time PCR.

To correct experimental variation the experiments were normalised to the constitutively expressed gene GAPDH, coding for an abundant glycolytic enzyme and present in most cells. GAPDH was chosen because its expression is constant among different tissues and because it is not influenced by the experimental treatment. Differences in the expression of GAPDH indicate that the expression of the normaliser is either up or down-regulated, and therefore the experiment must be discharged.

Data analysis

After the run was completed, the threshold cycle (C_T) value (cycle at which a significant increase with an exponential growth of PCR product is first detected) was manually set. The Ct value is inversely proportional to the amount of product present. The threshold should be placed above any baseline activity and within the exponential increase phase (which looks linear in the log transformation).

The Ct of the gene of interest was normalised to the housekeeping gene GAPDH, using the algorithm:

$$Relative\ expression = 2^{(Ct\ GAPDH\ -\ Ct\ Gene\ of\ interest)}$$

2.4 IMMUNOCHEMISTRY TECHNIQUES

2.4.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

2.4.1.1 Sample preparation

Pellet from 3-10·10^5 cells was washed twice in PBS before adding 100 µl of whole cell lysis buffer (2 ml HEPES KOH [1 M], 5 ml NaCl [1 M], 2 ml NP40, 0.5 g Sodium Deoxycholate, 0.2 g SDS, 1 ml Sodium Orthovanadate [100 mM], 1 ml EGTA [100 mM], 10 ml Sodium Fluoride [100 mM], made up to 100 ml with deionised H_2O) containing 10 µl Sigma protease inhibitor cocktail, and 10 µl PMSF [100 mM] freshly dissolved in DMSO. Cell lysate was centrifuged at 13,000 rpm at 4°C for 15 min to remove the nucleic acid pellet. To 20 µl of lysate, 2.5 µl DTT [1 M] and 7.5 µl of gel loading buffer (Invitrogen) were added, and the sample was heated at 70 °C for 10 min, to allow reduction of disulphide bonds.

2.4.1.2 Electrophoresis

Samples were loaded onto BIO-RAD minigels (for 15 ml of 10% acrylamide **running gel**: 6.15 ml H2O; 5 ml 30% acrylamide, 3.75 ml 0.5 M Tris-HCl pH8.8, 150 µl 10% SDS, 75 µl 10% APS, 15 µl TEMED; for 10 ml of **stacking gel**: 6.1 ml H_2O, 1.3 ml 30% acrylamide, 2.5 ml 0.5M Tris-HCl pH6.8; 100 µl 10% SDS, 50 µl 10% APS, 20 µl TEMED), using the following running buffer: 3 g Tris base, 14.5 g Glycine, 1 g SDS, in 1 litre of deionised H_2O.

Gels were run at 200 V until the front of the dye reached the bottom of the gel.

2.4.2 Western blotting

2.4.2.1 Protein transfer

After separation by SDS-PAGE the stacking was gel removed and the running gel was soaked in protein transfer buffer (9 g Tris, 1.46 g Glycine, 100 ml Methanol, for 500 ml of deionised H_2O) for 10 minutes for equilibration, in order to prevent any distortion during the transfer process. A sheet of Hybond PVDF membrane or nitrocellulose membrane (Amersham) was cut to size and equilibrated briefly in transfer buffer. Before equilibration in transfer buffer, PVDF membranes only were activated in methanol. The transfer process was carried out at 15 V for 45 minutes-1 hour in a BioRad Transblot®SD Semy-Dry Transfer cell.

2.4.2.2 Blocking

Following transfer, the membrane was rinsed briefly in TBS. Non-specific binding sites were blocked by immersing the membrane in blocking solution (TBS-Tween 0.2% with 5% PVP and 0.5% FBS; or TBS-Tween 0.2% with 5% non-fat dry milk, depending on the primary antibody) at 4˚C overnight, or at room temperature for 1 hour.

2.4.2.3 Blotting

Blocking is followed by incubation with the appropriate dilution of the primary antibody in blocking solution for an hour at room temperature, or at 4 ºC overnight. After incubation the membrane was rinsed five times in washing buffer

(TBS-Tween 0.2%), alternating quick rinses with longer washings (5 minutes), and then incubated again for 1 hour at room temperature with an appropriate dilution of secondary antibody in blocking solution. The washing steps were repeated as above in TBS without Tween.

Primary antibodies employed in this study:

- anti NS1 (5H5.4): mouse monoclonal (from Paul Young)
- anti NS5: rabbit polyclonal (from Andrew Davidson)
- anti-FLAG M2: mouse monoclonal (Sigma)
- anti-human STAT1: mouse monoclonal (Zymed)
- anti-human phospho-STAT1: mouse monoclonal (Zymed)
- anti-human STAT2: mouse monoclonal (BD Transduction Laboratories)
- anti-human phospho-STAT2: rabbit polyclonal (Upstate Technology)
- anti-phospho Tyk2: rabbit polyclonal (Cell Signaling Technology)
- anti-human phospho-Tyk2: rabbit polyclonal (Cell Signaling Technology)
- anti-human β-actin: rabbit polyclonal (Sigma)

Secondary antibodies employed in this study:

- HRP-conjugated goat anti-mouse (Dako Cytomation)
- HRP-conjugated goat anti-rabbit (Dako Cytomation)

2.4.2.4 Development

The membrane was covered by a mix of ECL or ECL-Plus reagents (Amersham) following manufacturer`s instructions and incubated for 1-5 minutes. The membrane was then carefully drained on tissue, wrapped in clingfilm, and exposed to a sheet of autoradiography film for 2seconds-5minutes before

development (Developer Xograph imaging system CompactX4).

2.4.3 NS5-FLAG Immunoprecipitation

Immunoprecipitation (IP) of tagged proteins is a well established procedure which has been standardised for different tags. FLAG is a short peptide (N-*Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys*-C), which allows fusion proteins to retain their original conformation and function. Its hydrophilic character increases the likelihood that it will be located on the surface of the protein, where it is accessible to the antibody.

To immunoprecipitate NS5-FLAG, we employed the FLAGIPT1 FLAG® Immunoprecipitation Kit from SIGMA, following manufacturer`s instructions. The IP is performed with anti-FLAG® -M2 affinity gel, which is a highly specific monoclonal antibody covalently attached to agarose resin. The high standardisation of this procedure allows efficient binding of FLAG-tagged proteins without need for preliminary steps and calibration.

Briefly, 10^7 cells were washed twice in PBS and then lysed in 1 ml of Lysis Buffer, containing 10 μl of Sigma protease inhibitor cocktail, for 30 minutes at 4˚C, in a roller shaker. The lysate was centrifuged 10 minutes, at 12,000 g, 4˚C, and the supernatant transferred into a chilled test tube.

40 μl of resuspended gel were used for each reaction and washed 3 times with 0.5 ml of 1X Washing Buffer, once with 0.5 ml of Elution Buffer to remove traces of unbound anti-FLAG antibodies, and 3 more times with 0.5 ml of 1X Washing Buffer. 900 μl of cell lysate were added to the resin and the final volume was brought to 1 ml with Lysis Buffer. The remaining 100 μl of whole lysate were

kept as control for following immunoblots.

Two control reactions were performed: a positive control consisting of the immunoprecipitation of a FLAG-BAP fusion protein, and a negative control consisting of blank reagents, without proteins. For the positive control, 1 ml of 1X Wash Buffer and 4 μl of 50 ng/μl (= 200 ng) of FLAG-BAP fusion protein were added to the resin; for the negative control only 1 ml of Lysis Buffer with no proteins was added to the resin.

Samples and controls were gently agitated on a roller shaker for at least 2 hours. After incubation the resin was centrifuged for 30 minutes at 8,200 g and the supernatant, containing all the unbound proteins, was removed. The resin was washed 3 times with 1X Washing Buffer.

Elution was performed with 3X FLAG™ peptide, which provides efficient and gentle elution through direct competition. 3 μl of 5 mg/ml 3X FLAG peptide were added to 100 μl of 1X Washing Buffer. After 30 minutes of gentle shaking at 4˚C, the resin was centrifugated and the supernatant, containing the eluted NS5-FLAG was collected and stored at -20 ˚C, and then used for immunoblots as described above.

2.4.4 NS1 ELISA

A 96 well plate was coated overnight at 4° C with 50 μl of Coating Buffer (1.59 g Sodium Carbonate; 2.93 g Sodium Bicarbonate; 0.2 g Sodium Azide; 900 ml deionised H_2O. Adjust to pH 9.6 with HCl and make up to 1 L), containing α-NS1 antibody (1:1500). The plate was washed 3 times with PBS Tween20, and each well blocked with 150 μl of 1% gelatin in PBS, 0.1% Sodium Azide for 1 hour at

room temperature. The plate was then emptied and washed again 3 times with PBS Tween20.

The samples were serially diluted in PBS Tween20 containing 0.25% gelatin, and together with a standard curve of Dengue NS1 (100-5 ng/ml), left to incubate for 1 hour at 37°C, before washing 3 times with PBS-Tween20. 100 μl of 1H7.4 α-NS1 antibody diluted to 1/1000 in PBS Tween20-0.25% gelatin were added to each well and incubated for 1 hour at 37°C, before washing 3 times with PBS Tween20. 100 μl of 1/1000 dilution of goat α-mouse IgG/Peroxidase conjugate were added to each well and again incubated for 1 hour at 37°C. The plate was then washed three times and 100 μl of TMB substrate solution were added for 15 minutes at room temperature in the dark. The incubation was stopped with 100 μl of 1 M H_2SO_4 and the plate mixed with swirling for 15 seconds. The absorbance at 450 nm was measured in a microplate reader (Tropix TR 717 microplate Luminometer PE applied Biosystems) within 5 minutes after the addition of the stop solution.

2.4.5 Surface Staining and Flow Cytometry

In this work, surface staining was employed whenever we wanted to test the presence of a specific antigen, or alteration on its expression on cell surface. In this work surface staining was used:

-to measure expression of the IFN sensitive antigen HLA-ABC in response to both IFN-α and IFN-γ in different K562 based cell line

-to measure the expression of the type I IFN receptor IFNAR2c

Primary antibodies employed in this study:

- anti-human HLA-ABC (clone W6/32): mouse monoclonal (Dako Cytomation)
- anti-human IFNAR2c: mouse monoclonal (CalbioChem)

Secondary antibodies employed in this study:

- APC-conjugated goat anti-mouse immunoglobulins (Jackson Immunoresearch)
- PE-conjugated goat anti-mouse immunoglobulins (Jackson Immunoresearch)

All stainings were performed on 1-3·10^5 cells. Cells were washed once in HBSS and incubated in 50 μl of blocking buffer (HBSS, 0.1% sodium azide, 5% serum from the same species of the secondary antibody) for 1 hour at 4 ˚C. After incubation, 50 μl of primary antibody at the appropriate dilution in blocking buffer were added, and the sample was incubated 30-45 minutes at 4 ˚C. Negative and isotype controls were incubated without primary antibody. Cells were washed 3 times with blocking buffer in order to remove the unbound primary antibody, and 50 μl of the secondary antibody at the appropriate dilution in blocking buffer were added; the samples were incubated for 20-30 minutes at 4˚C in the dark. Negative staining controls only were incubated without secondary antibody. After incubation, cells were washed 3 times with HBSS to remove unbound secondary antibody, and finally resuspended in 100 μl of 3.8% formaldehyde. Samples were kept at 4˚C in the dark until ready to be analysed by flow cytometry, normally no later than 12 hours.

Controls

Negative control: cells incubated in the absence of primary and secondary antibodies, to test the specificity of the staining and check autofluorescence of cells.

Isotype control: cells incubated in the absence of primary antibody but in the presence of the secondary antibody, to test the specificity of the secondary antibody.

2.5 TRANS-RESCUE ASSAY

$2 \cdot 10^6$ non-adherent cells were treated overnight with 100 IU/ml of IFNα-2a. The following day $1x10^6$ cells were counted and 0.5 pfu/cell of EMCV added in DMEM containing 2% serum for 1 hour at 37°C. Cells were washed and seeded in 6-well plates in DMEM containing 10% serum. After 18 hours, the cells were centrifuged at 6000 rpm for 2 minutes and the supernatants collected. The supernatants were added as a serial dilution onto an A549 monolayer ($2 \cdot 10^5$ cells plated the night before) in a 96-well plate for 1 hour, before being replaced with DMEM containing 10% serum and incubated overnight. The following day, cell viability was determined by staining with methyl violet and reading the absorbance at 570 nm in a plate reader (Tropix TR 717 microplate Luminometer PE applied Biosystems). The OD measured was plotted against the serial dilution of the EMCV: low optical density is index of cell death, while high optical density suggests high viability.

2.6 CELL MAINTENANCE

K562: RPMI 10% foetal bovine serum

Passaged 1:10 every 3-4 days

K562_Replicon: RPMI 10% foetal bovine serum, with 3 ng/ml of puromycin

Passaged 1:10 every 3-4 days

293T: DMEM 10% foetal bovine serum

Passaged 1:10 every 3-4 days

All cells derived from K562 following lentivirus transduction have been cultured as parental K562.

Chapter 3.

DEVELOPMENT OF K562 CELL LINES EXPRESSING INDIVIDUAL DENV NON-STRUCTURAL PROTEINS

3.1 INTRODUCTION

The inhibition of the type I IFN response in DENV infection has been extensively described (see chapter 1, section 1.5.2.2). Our group and others have reported reduced intracellular levels of STAT2, a key component of the type I IFN signalling pathway, in DENV infected cells (Jones *et al.* 2005; Umareddy *et al.* 2008). This suggests that STAT2 reduction is at least one of the mechanisms responsible for the inhibition of the IFN response.

In work published in 2005, our group reported inhibition of type I IFN and reduction of cellular STAT2 levels in human cell lines stably expressing the DENV replicon ΔCprMEPAC2A, a self-replicating RNA-based DENV-2 (New Guinea C strain, NGC) sub-genome in which the genes coding for the structural proteins have been removed (Jones *et al.* 2005). The absence of viral entry and the lack of structural components imply that both IFN inhibition and STAT2 reduction must involve one or more DENV non-structural proteins.

The initial question that we wanted to address in this project was which of the DENV non-structural protein/s was/were responsible for the inhibition of IFN-α and for the reduction of STAT2 levels.

In order to test the roles of the individual DENV non-structural components in the subversion of the type I IFN response, in the first part of this work we aimed to establish five K562 cell lines, each one expressing a different DENV NS protein cassette. K562 are a human chronic myeloid leukaemia cell line, originally

established from a patient in blast crisis (Klein *et al.* 1976). Similarly to monocytes, macrophages and dendritic cells, K562 are cells of myeloid haematopoietic origin, and therefore related to DENV target cells *in vivo* (chapter 1, section 1.2.2). K562 have been extensively used by our group since they efficiently support the replication of the DENV replicon ΔCprMEPAC2A; moreover, since they are incapable of producing type I IFN (Diaz *et al.* 1988), their response to viral infection can be studied in the absence of a complicating IFN autocrine loop.

One potential disadvantage of using K562 cells is that, in common with many other cells of myeloid hematopoietic origin, they are difficult to transfect using conventional mammalian expression vectors and standard molecular biology techniques. In order to overcome the refractoriness of K562 cells to the most common transfection methods, we decided to express DENV NS proteins in the context of a lentiviral vector. The lentiviral vector used in this work is the dual promoter self-inactivating vector pHRSIN-CSGWdINotI_pUb_Em (Figure 3.1), described by Rowe (Rowe *et al.* 2006) and Escors (Escors *et al.* 2008) and kindly given to us by Professor Mary Collins. In order to diminish the risk of vector mobilisation and recombination, the 5` LTR (Long Terminal Repeat) are replaced by heterologous promoting sequences, while the U3 region of the 3` LTR is partially deleted to abolish promoter activity (Zufferey *et al.* 1998). The sequences Ψ/RRE (Rev Response Element) is preserved in order to improve RNA packaging efficiency and nucleocytoplasmic transport, and the 178 base pair fragment cPPT (central PolyPurine Tract) is a cis-acting sequence enhancing the nuclear translocation of the pre-integration complex (Demaison *et al.* 2002). The WPRE (Woodchuck hepatitis virus Post-transcriptional Regulatory Element) was

added to increase the expression of the transgene by increasing the levels of nuclear transcripts and facilitating RNA nuclear export (Zufferey *et al.* 1999; Schambach *et al.* 2000).

The expression of the transgene is regulated by the SFFV (Spleen Focus Forming Virus) promoter, chosen for its high activity in human dendritic cells (Rowe *et al.* 2006), and to overcome the relative inefficiency of the HIV long terminal repeat (LTR) in the absence of Tat protein (Demaison *et al.* 2002), while the Ubi (Ubiquitin) promoter regulates the expression of the reporter gene emerald GFP (Em). The employment of a dual promoter system allows the expression of a fluorescent marker with less likelihood of interference with the transgene.

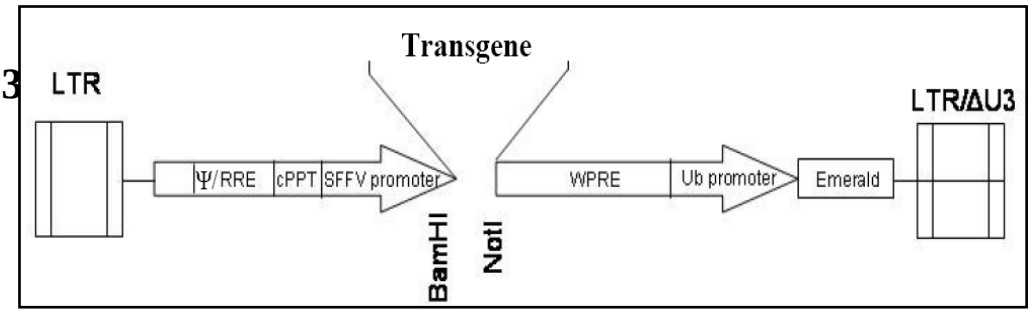


Figure 3.1|
Schematic representation of the lentiviral vector pHRSIN-CSGWdINotI_pUb_Em. *LTR*: long terminal repeats; *RRE*: rev response element; *cPPT*: central poly-purine tract; *SFFV*: spleen focus forming virus; *WPRE*: woodchuck hepatitis virus post-transcriptional regulatory element; *Ub*: ubiquitin. ΔU3: 400-nucleotide deletion in the 3`LTR that abolishes promoter activity.

OBJECTIVES

- To clone separate DENV NS protein cassettes into the dual promoter lentiviral vector pHRSIN-CSGWdINotI_pUb_Em
- to generate five different K562 cell lines, each one stably expressing an individual DENV NS protein cassette, by transducing K562 cells with the corresponding lentiviral vectors
- to validate and optimise the expression of DENV NS proteins in K562 cells.

3.3 RESULTS

3.3.1 Generation of lentiviruses encoding individual DENV NS proteins

3.3.1.1 Design of DENV NS cassettes

In designing the separate NS cassettes to be cloned into the lentiviral vector, we took into account what is known about the functions and localisation of the individual NS proteins in the context of DENV infection. NS2A has been shown to act as a GPI anchor for NS1 signal and to allow the correct localisation of NS1 in the ER (chapter 1, section 1.2.4.1) (Falgout *et al.* 1989; Jacobs *et al.* 2000), and therefore NS1 and NS2A were expressed together. The proteolytic site between NS1 and NS2A in DENV polyprotein is recognised and cleaved by an ER protease (see section 1.2.4.1), generating two individual proteins. NS3 and its co-factor NS2B were also expressed together (chapter 1, section 1.2.4.2) (Erbel *et al.* 2006) and the proteolytic site between the two is cleaved by the proteolytic

complex NS2B/NS3 in an autoproteolytic event. The short 2K fragment localised between NS4A and NS4B was cloned at the N-terminal of NS4B, to allow correct localisation of NS4B in the ER (chapter 1, section 1.2.4.4) (Miller *et al.* 2007), and because this sequence has been reported to play a role the IFN antagonism attributed to NS4B (Munoz-Jordan *et al.* 2003). This short sequence is also known to be cleaved by host proteases. Therefore, all the NS protein cassettes were engineered in order to be finally expressed as individual proteins.

Each cassette (NS1/NS2A, NS2B/NS3, NS4A, NS4B, and NS5) was amplified by PCR from the DNA genome of the DENV replicon ΔCprMEPAC2A: forward and reverse primers were designed in order to incorporate BamHI and NotI restriction sites at the 5` and 3` termini respectively, necessary for subsequent cloning into the lentiviral vector. A Kozak sequence (CACCATG), required for optimal transcription in eukaryotic systems, was inserted immediately downstream of the restriction site in all the forward primers, and a stop codon was incorporated before the restriction site in all the reverse primers (see chapter 2, Table 2.1).

3.3.1.2 Cloning of DENV NS protein cassettes into the lentiviral vector pHRSIN CSGWdINotI_pUb_Em

After high-fidelity PCR amplification, the correct size of each PCR product was confirmed by DNA electrophoresis (Figure 3.2, A). Restriction digest of linear, amplified fragments is inefficient, and therefore each blunt-ended PCR product was first subcloned into the vector pGEM®-T Easy, and then sequentially digested by the restriction enzymes BamHI and NotI. Inserts were separated by DNA electrophoresis, isolated by gel extraction and purification, and finally

cloned into the linearised and dephosphorylated lentiviral vector, as described in chapter 2, section 2.1. Correct sequences and insertions were checked by double digestion BamHI-NotI (Figure 3.2, B) and by DNA sequencing.

For generation of lentiviruses, all lentivector constructs were amplified by DNA midi-preparations.

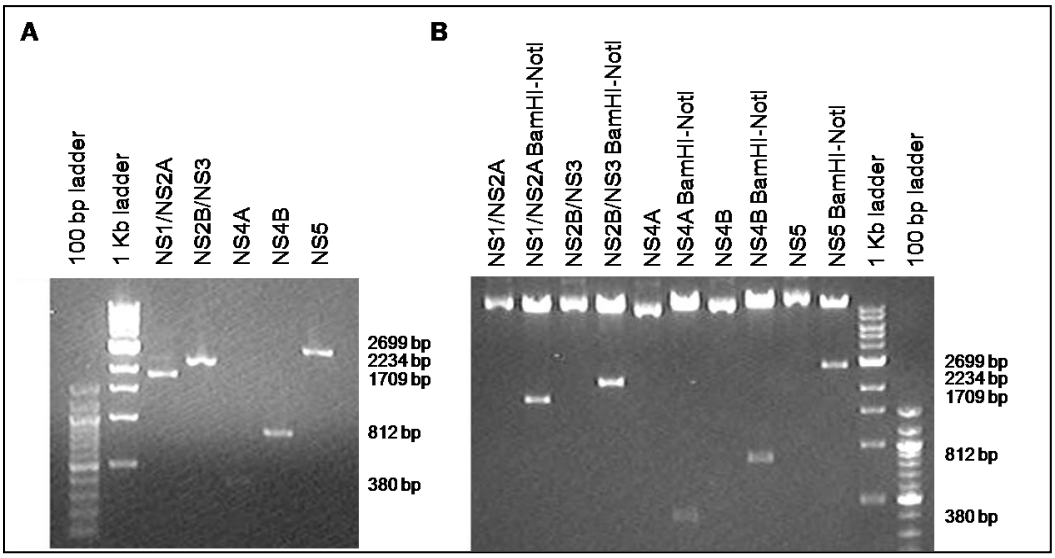


Figure 3.2|

A. Gel electrophoresis of PCR products amplified from the DNA replicon ΔCprMEPAC2A Each cassette (NS1/NS2A, NS2B/NS3, NS4A, NS4B, and NS5) was amplified by High-Fidelity DNA polymerase from the DNA replicon ΔCprMEPAC2A, and the PCR products were analysed by gel electrophoresis. The gel shows the correct size of each fragment and the absence of unspecific bands.

B. Gel electrophoresis of lentiviral vectors containing individual DENV NS protein cassettes, before and after double digestion BamHI-NotI. Each cassette amplified by PCR was subcloned into the pGEM®-T Easy vector, extracted by double digestion BamHI-NotI and cloned into the lentiviral vector pHRSIN-CSGWdINotI_pUb_Em. In order to verify the presence and correct insertion of each cassette, 1 μg of each vector was digested by the restriction enzymes BamHI and NotI and loaded on agarose gel. The picture shows each construct before and after double digestion.

3.3.1.3 Virus preparation

Each virus was prepared by transient transfection of 293T cells with the lentiviral vectors described above and two more plasmids: a multi-deleted packaging plasmid coding for HIV *gag* and *pol* genes, required for vector particle production and efficient transduction of target cells, and the envelope plasmid VSV-G. VSV-G envelope protein binds to target cells via a receptor-independent mechanism, interacting with common phospholipids on the membrane of the target cell. VSV-G confers on the virus a broad tropism and makes the virus particles resistant to ultracentrifugation and long-term storage; furthermore, it directs lentiviral vector entry to an endocytic pathway, which reduces the requirements for viral accessory proteins for infectivity (reviewed in Cockrell and Kafri 2007). Viruses were prepared as described in chapter 2, section 2.2.

Viruses were titrated by infecting 293T cells with sequential dilutions of virus and measuring GFP expression by flow cytometry after 72 hours: depending on size and characteristics of the transgene, virus titres were 10^7-10^8 infecting units/ml.

3.3.2 Development of K562 cell lines expressing individual DENV NS proteins

3.3.2.1 Transduction of K562

The lentiviruses described above were employed to transduce K562 cells. Transduction of K562 was carried out at a multiplicity of infection (MOI) that ensured at least 50% transduced cells and at the same time minimised the number of vector integrating copies, in order to minimise the risk of experimental artefacts

from disruption of host cell genes. In order to assess the optimal MOI to employ, we transduced K562 cells at MOI 1, 4, and 7 with the lentivirus containing the NS1/NS2A cassette. After 72 hours, GFP expression was measured by flow cytometry. As shown in Figure 3.3, at MOI 4 more than 50% of the cells (74%) were GFP positive, and therefore we chose MOI 4 for all the other transductions.

K562 cells were transduced at MOI 4 with each lentivirus (NS2B/NS3, NS4A, NS4B, and NS5) and the expression of the reporter GFP was measured 72 hours later by flow cytometry (Figure 3.4). GFP positive cells were then separated by FACS sorting.

3.3.2.2 Protein expression

Viral integration could be assessed by detection of the reporter transgene GFP, but direct assessment of DENV protein expression was possible by immunoblot only for NS1/NS2A, as no other specific antibodies were available at the time of the experiment. As described in chapter 1 (section 1.2.4.1), DENV NS1 localises both intracellularly and on the cell surface, but it is also secreted from the cell (Mackenzie *et al.* 1996; Jacobs *et al.* 2000; Alcon-LePoder *et al.* 2005). In order to assess whether the protein was correctly expressed, processed and secreted, we tested NS1 expression both by immunoblotting and by ELISA.

For immunoblotting, $5 \cdot 10^5$ K562_NS1/NS2A cells were lysed and proteins were separated by SDS-PAGE. Immunoblot was performed using an antibody specific for NS1. Surprisingly, we could not detect any signal for NS1 from K562 cells transduced with NS1/NS2A lentivirus, despite a strong signal from dengue replicon containing cells, included as a positive control (data not shown). We next tested the presence of NS1 in the supernatant of the same cells by ELISA: only

low levels of extracellular NS1 were detectable (about 200 ng/ml). Taken together, these results suggested that in our lentiviral system DENV NS1 is correctly expressed and processed, but expression levels are low.

3.3.2.3 Transgene expression is diminished over time

High levels of GFP expression were sustained over time, suggesting that the low NS1 transgene expression does not depend on low efficiency of transduction or on insufficient number of integrated copies. We therefore hypothesised that either *(a)* the SFFV promoter might not have been efficient enough to sustain sufficient levels of protein expression in K562, or *(b)* that other translational/post-translational mechanisms, such as transcriptional silencing, could intervene and compromise protein production. In order to test the efficiency of the SFFV promoter, we checked the expression of NS1 by immunoblot shortly after transduction, and we also tested whether integration of more copies of the transgene could overcome poor expression efficiency. We therefore transduced K562 cells at MOI 4, 10, and 20. After 72 hours, we measured GFP expression by flow cytometry. Transduction efficiency was higher than 96% at all MOI (Figure 3.5, A). At higher MOI, the mean fluorescence intensities of GFP were slightly increased (55 at MOI 4, 68 at MOI 10, and 103 at MOI 20), presumably reflecting more lentivector copies integrated in the cell genome. Three days later, cells were lysed and proteins were separated by SDS-PAGE. NS1 expression was tested by immunoblot: as shown in Figure 3.5 B, similar levels of NS1 were detectable in all samples, suggesting that, shortly after transduction, the transgene is expressed and the SFFV promoter is functional in K562 cells.

Finally, to test whether progressive silencing was occurring over time, we

transduced K562 cells at MOI 25 to ensure that all cells were GFP positive and no preferential expansion of un-transduced cells occurred. We then measured both GFP (flow cytometry) and NS1 expression (immunoblot) 72/96 hours after transduction and 1 month later. As shown in Figure 3.6, GFP expression levels were maintained over time (Figure 3.6, A); in contrast, we observed that one month after transduction NS1expression was reduced (Figure 3.6, B).

3.3.3 Enhancement of protein expression over time: cloning of the MESV leader sequence into lentiviral constructs

We therefore hypothesised that long-term stable protein expression could have been impaired by some post-transcriptional mechanism. In order to overcome loss of protein expression over time, we introduced an enhancer element ("leader sequence"; GeneBank accession number: AJ132037) (Hildinger *et al.* 1999) immediately downstream of the SFFV promoter into all the lentivirus constructs containing the NS protein cassettes. The leader sequence, originally isolated from murine embryonic stem cell virus (MESV), constitutes the upstream untranslated region that in retroviruses controls translation of *gag*. The introduction of an intron in the 5` untranslated sequence, containing a minimal splice acceptor oligonucleotide, reduces silencing of eukaryotic genes; moreover, all the aberrant AUG starting codons have been removed in order to abrogate the expression of aberrant proteins (Hildinger *et al.* 1999).

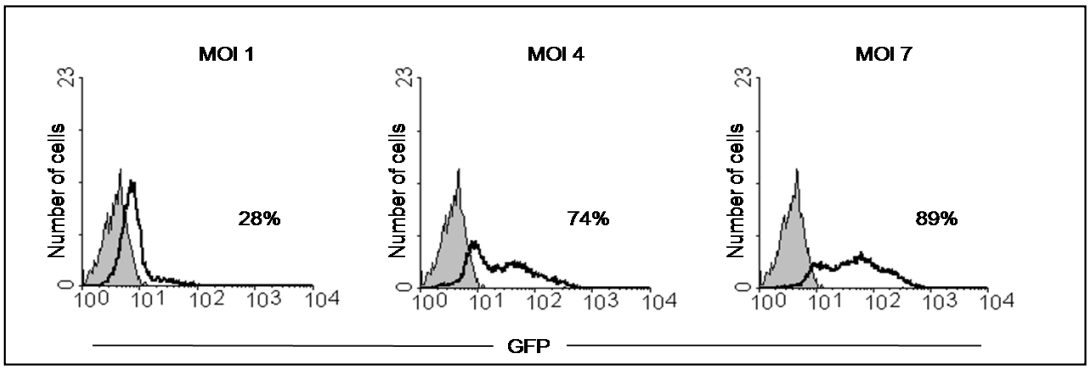


Figure 3.3|

Transduction efficiency of the lentivirus NS1/NS2A at MOI 1, 4, and 7. K562 cells were transduced at MOI 1, 4, and 7. GFP expression (x axes) in transduced cells (black line histograms) was measured by flow cytometry 72 hours after transduction. Untransduced cells (plain gray histograms) were used as a negative control. The percentage of GFP positive cells is calculated by comparison with the negative control.

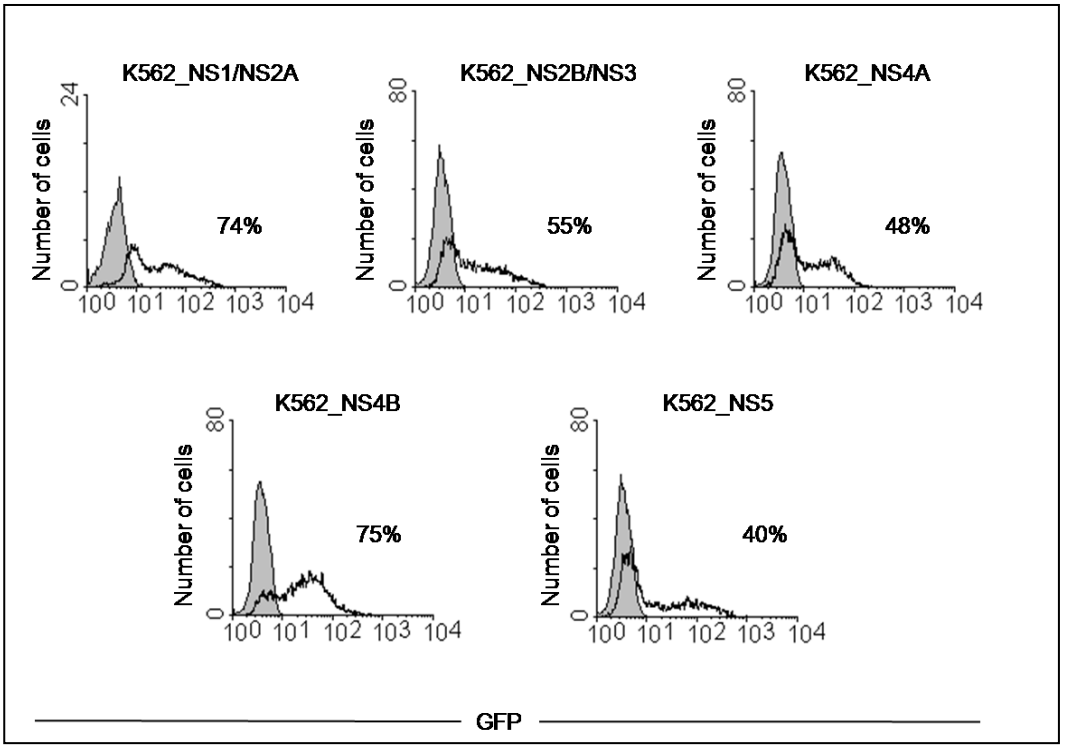


Figure 3.4|

Transduction efficiency of K562 cells, transduced by different lentiviral vectors. K562 cells were transduced at MOI 4 with the lentiviruses NS1/NS2A, NS2B/NS3, NS4A, NS4B, or NS5. GFP expression (x axes) in transduced cells (black line histograms) was measured by flow cytometry 72 hours after transduction. Untransduced cells (plain gray histograms) were used as a negative control. The percentage of GFP positive cells is calculated by comparison with the negative control.

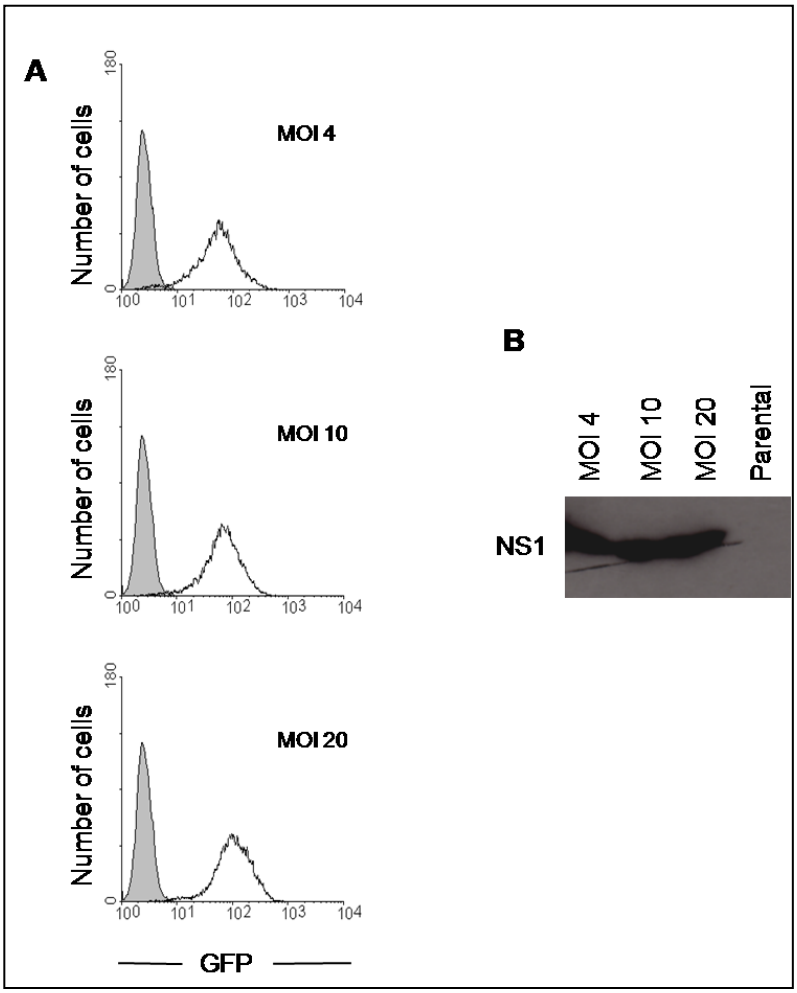


Figure 3.5|

NS1 expression in K562 cells transduced at different MOI of the lentivirus NS1/NS2A. Cells were transduced at MOI 5, 10, and 20 with the lentivirus containing the NS1/NS2A cassette. Expression of GFP **(A)** was measured by flow cytometry 72 hours later (black line histograms). Untransduced cells were used as a negative control (plain gray histograms). Expression of NS1 **(B)** was measured by immunoblot 96 hours later, using an antibody specific for NS1. Parental cells were included as a negative control.

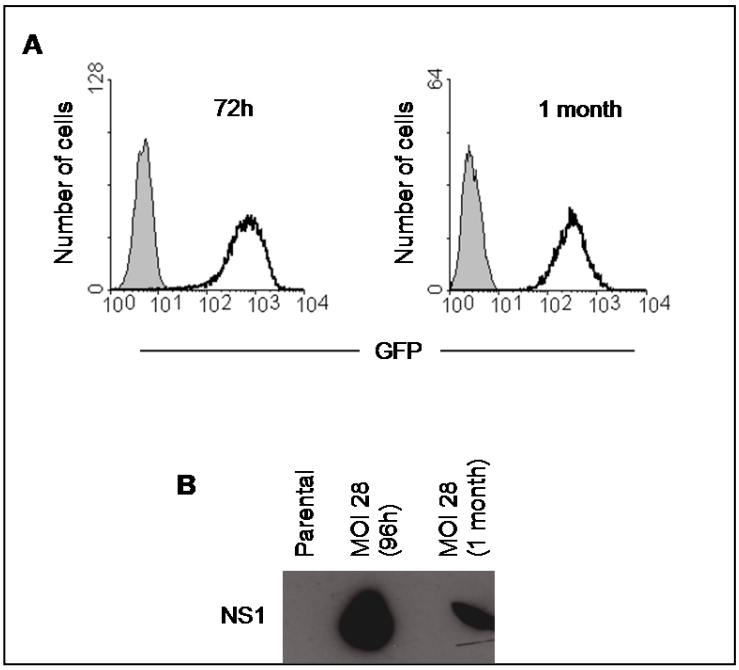


Figure 3.6|
Expression of GFP and NS1 in K562_NS1/NS2A over time. Cells were transduced at MOI 25. GFP expression was measured by flow cytometry 72 hours (left panel) and 1 month (right panel) after transduction. The percentage of transduced cells (~100%) (black line) was calculated by comparison with untransduced parental cells (plain grey histograms) **(A)**. 96 hours and 1 month after transduction, transduced cells were lysed and protein separated by SDS-PAGE. NS1 expression was measured by immunoblot using a specific antibody. Untransduced parental cells were included as control **(B)**.

3.3.3.1 Cloning of the "leader sequence" into the lentiviral vectors encoding DENV NS protein cassettes

The leader sequence was isolated from the lentiviral vector GFPLV (kindly provided by Professor Mary Collins) by double digestion with the restriction enzymes AscI and BamHI, and cloned into all the vectors containing the individual DENV NS protein cassettes, following linearisation and dephosphorylation. The sequence (about 700 bp) localises between the SFFV promoter and the transgene (Figure 3.7).

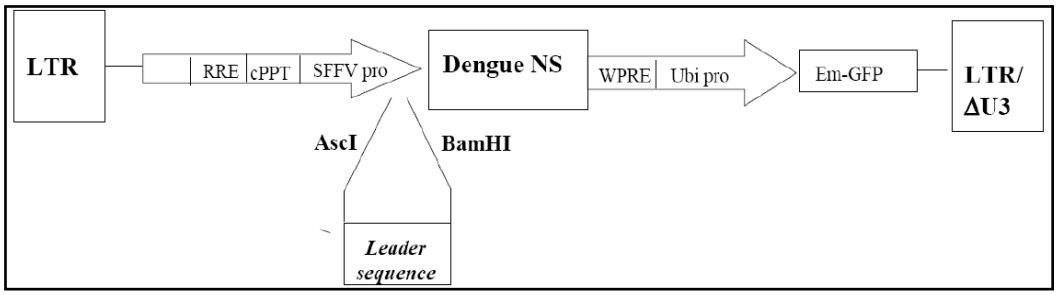


Figure 3.7|
Insertion of MESV Leader sequence into the lentiviral vector pHRSIN-CSGWdINotI_pUb_Em. *LTR*: long terminal repeats; *RRE*: rev response element; *cPPT*: central poly-purine tract; *SFFV pro*: spleen focus forming virus promoter; *WPRE*: woodchuck hepatitis virus post-transcriptional regulatory element; *Ub*: ubiquitin. ΔU3: 400-nucleotide deletion in the 3`LTR that abolishes promoter activity.

Correct insertions into each construct were checked by AscI-BamHI double digestion, followed by DNA sequencing. The selected constructs were amplified by midi DNA preparation and used to prepare the lentiviruses as previously described. We observed that the insertion of the leader sequence did not affect virus titres, which remained at 10^7-10^8 infecting units/ml.

3.3.3.2 Transduction of K562

To assess the efficiency of transduction of the lentiviral vector containing the

leader sequence, we transduced K562 cells at MOI 1, 4, and 10. At 72 hours post-transduction, we measured the expression of GFP by flow cytometry. As shown in Figure 3.8 A, the percentage of GFP positive cells is higher than in cells transduced with the same lentivirus without leader sequence (Figure 3.3), suggesting either higher transduction or, more likely, higher expression efficiency. At 96 hours post-infection cells were lysed and proteins separated by SDS-PAGE, in order to assess by immunoblot the expression of NS1. As shown in Figure 3.8 B, high levels of NS1 are detectable in all samples, with higher expression in cells transduced with higher MOI of the leader sequence lentivector, in keeping with the higher percentage of transduced cells.

In spite of the higher expression efficiency in the presence of the leader sequence, we decided to maintain MOI 4 to transduce K562 cells with all other constructs. GFP expression was measured 72 hours later (Figure 3.9). As already noticed for NS1/NS2A, higher percentage of GFP positive cells was observed when cells were transduced with lentiviruses containing the leader sequence. A further cell line was generated by transduction with an "empty" control, without any transgene after the SFFV promoter (Empty Vector). We did not observe any significant alterations of cell phenotype or viability following lentivirus transduction.

3.3.3.3 DENV NS message and protein expression

Due to the lack of available antibodies specific for each DENV NS protein, we could only assess the presence and transcription of some genes by reverse-transcription PCR (RT-PCR). RNA from transduced cells was extracted, DNAse treated and reverse transcribed, and the cDNA was amplified by PCR using the

same primers employed during the cloning stage for NS1/NS2A, NS4A and NS4B; due to the length of the NS2B/NS3 and NS5 genes, and in order to optimise the efficiency of the reverse transcription process, we employed primers mapping inside the genes, amplifying a smaller internal portion (see chapter 2, section 2.3.4). All the PCR products were analyzed by gel electrophoresis. Figure 3.10 shows that all genes are present and efficiently transcribed. The absence of bands in samples in which the reverse transcription step has been omitted excludes artefacts from DNA contamination and amplification of the integrated DNA gene.

Protein expression could be assessed by immunoblot for NS1/NS2A (as discussed above) and also for NS5, as a new polyclonal antibody had become available. Both proteins were detectable and the expression levels were comparable to those of cells stably expressing the DENV replicon ΔCprMEPAC2A (Figure 3.11). Expression of both proteins was tested by immunoblot at different times after transduction and, in the presence of the leader sequence, we did not observe any significant decreases of protein level over time (data not shown).

GFP positive cells were isolated by FACS sorting. The isolation of a mixed population overcomes the limits of clonal selection, mitigating the risk of experimental artefacts from isolation of an aberrant cell phenotype.

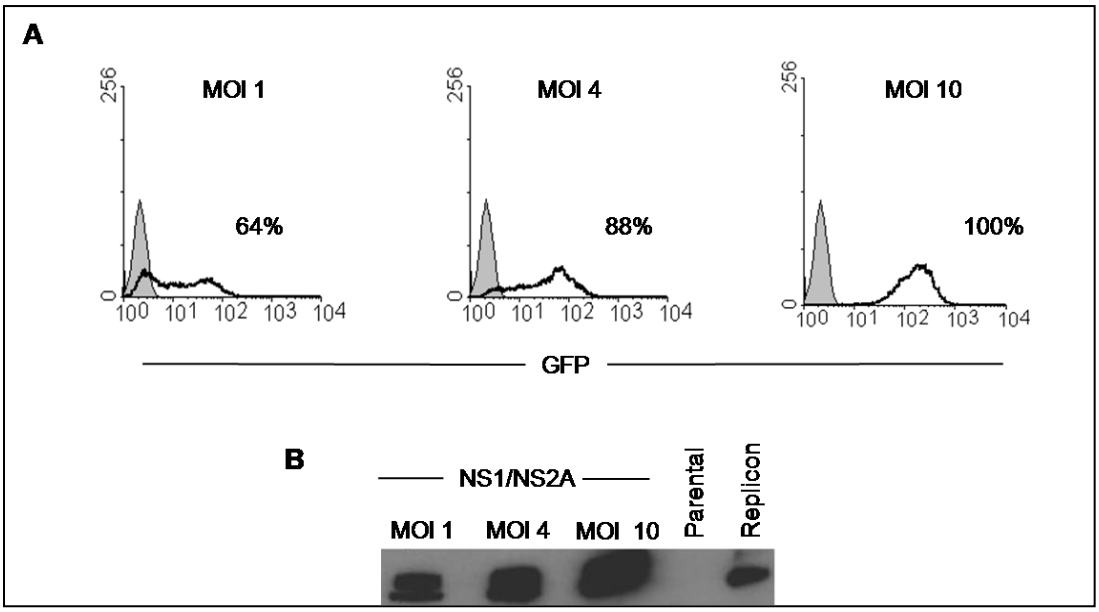


Figure 3.8|

Transduction efficiency of the lentivirus NS1/NS2A containing the leader sequence at different MOI. K562 cells were transduced at MOI 1, 4 and 10. GFP expression (x axes) in transduced cells (black line histograms) was measured by flow cytometry 72 hours after transduction. Untransduced cells (plain gray histograms) were used as a negative control. The percentage of GFP positive cells is calculated by comparison with the negative control **(A)**. 96 hours after transduction, transduced cells were lysed and protein separated by SDS-PAGE. NS1 expression was measured by immunoblot using a specific antibody. Untransduced parental cells and cells containing the DENV replicon were included as a control **(B).**

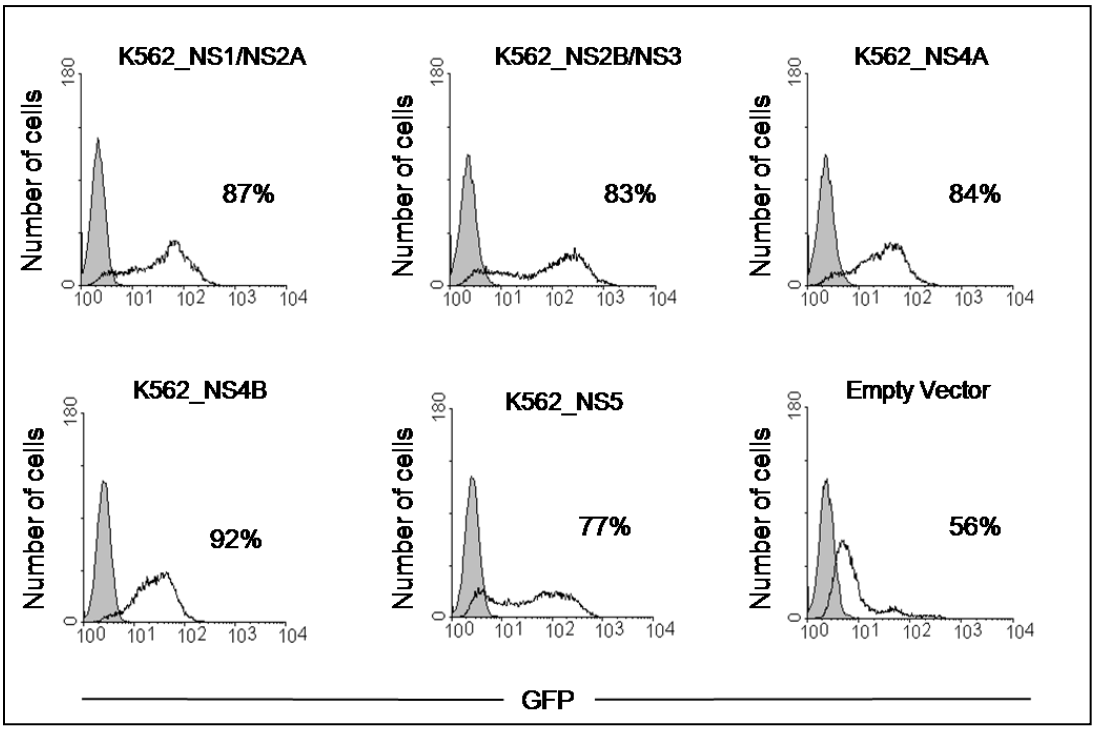


Figure 3.9|

Transduction efficiency of K562 cells transduced by different lentiviral vectors containing the MESV leader sequence. K562 cells were transduced at MOI 4 with the lentiviruses NS1/NS2A, NS2B/NS3, NS4A, NS4B, and NS5, containing the MESV leader sequence. GFP expression (x axes) in transduced cells (black line histograms) was measured by flow cytometry 72 hours after transduction. Untransduced cells (plain gray histograms) were used as a negative control. The percentage of GFP positive cells is calculated by comparison with the negative control.

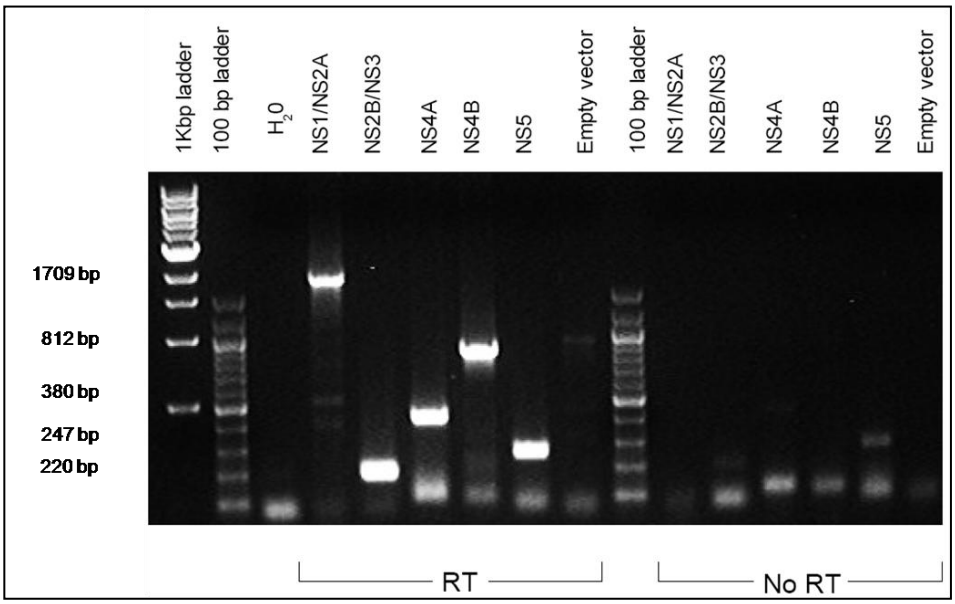


Figure 3.10|

Presence of the NS gene transcripts in K562 cells transduced with the lentiviral vectors NS1/NS2A, NS2B/NS3, NS4A, NS4B and NS5. RNA was extracted from stably transduced cells, treated with DNAse and reverse transcribed (*RT*, left samples) or not (*No RT*, right samples) by the MLV enzyme. cDNA was amplified by PCR using the same primers employed for cloning for NS1/NS2A, NS4A, and NS4B, and primers mapping internal regions for NS2B/NS3 and NS5 (expected sizes, respectively, 220 and 247 bp).

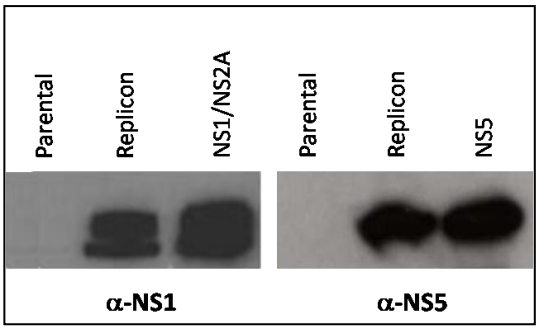


Figure 3.11|
Comparison of NS1 and NS5 protein levels in DENV replicon containing cells and in K562 transduced with the corresponding lentiviral vectors. Equal numbers of cells were lysed and proteins were separated by SDS-PAGE. NS1 and NS5 were detected by immunoblotting using specific antibodies Parental cells were included as a negative control.

3.4 DISCUSSION

Identifying the role of individual viral proteins in the subversion of innate immunity is crucial to understanding DENV pathogenesis. In order to investigate the effects of DENV NS proteins on the type I IFN system, the first part of this project was directed towards the development of five cell lines, based on the human chronic myeloid leukaemia cell line K562, each one individually expressing a different non-structural protein cassette of DENV. We elected the use of a lentiviral vector system in order to overcome the well-known refractoriness of K562 cells to traditional transfection methods, and in anticipation of further experiments in human primary dendritic cells. Mainly because of their efficient mechanisms of cell entry, viral vectors represent the first choice in gene delivery for cells refractory to other transfection systems. Retroviruses are particularly appealing because the DNA is integrated into the genome of target cells, enabling stable and long term expression of the transgene, but cells must be in active proliferation at the time of infection, as the pre-integration complex needs the disruption of the nuclear membrane to translocate into the nucleus. Lentiviral vectors overcome this limitation because the karyophilic lentiviral pre-integration complex is recognised by the cell nuclear import machine. Therefore, lentiviral vectors are capable of efficiently transducing growth-arrested cell lines, terminally differentiated cells, or haematopoietic stem cells (reviewed in Cockrell and Kafri 2007).

The main safety concern about the employment of retroviral vectors is the possibility that the parental pathogenic virus may be reconstituted. Removal or

replacement of structural, accessory and regulatory genes considerably reduces the risk of homologous recombinations, and splitting the structural genes and the *gag-pol* packaging constructs into separate plasmids is one of the most common strategies to maximise the number of recombination events required to reconstitute a replication competent virus. Replication defective lentiviral vectors are commonly generated by transient transfection of a producer cell line with three different plasmids expressing respectively: *1)* the proteins of the *core* and the enzymes required for viral replication, reverse transcription and integration; *2)* the envelope protein, usually from a different virus with the required tropism; *3)* the gene of interest with the *cis*-acting sequences required for the optimisation of packaging, reverse transcription and integration of the transgene into the genome (reviewed in Delenda 2004; Cockrell and Kafri 2007). This is the strategy that we employed in this work. In the lentiviral vector used in this study, the SFFV promoter regulates the expression of the transgene, while a second promoter controls the expression of the transduction marker GFP: this allows indirect assessment of transgene integration without altering conformation, function and localisation of the protein of interest.

Successful cloning of all individual NS proteins into the lentiviral vector pHRSIN-CSGWdINotI_pUb_Em was confirmed by DNA sequencing, but in spite of high efficiency of transduction, we could only detect protein expression in freshly transduced K562; a dramatic reduction of transgene expression was observed over time. Such a phenomenon would compromise consistency and reliability of subsequent studies on the transduced cell lines. Information about stability of gene expression after lentiviral vector integration is limited, but it is known that in wild type HIV infection a proportion of integrated viruses becomes

latent. In particular, long term expression seems to be compromised in primary cells and in haematopoietic stem cells due to their high level of cytidine methylation, a general mechanism used by cells to silence foreign DNA and defend against transposable elements (Cherry *et al.* 2000; Pannell and Ellis 2001). Studies have been carried out on the murine leukaemia virus (MLV) and its derived vectors and they showed that proviral gene expression does not persist, mainly because of epigenetic changes such as *de novo* DNA methylation of CpG sequences and local histone deacetylation, which form repressive chromatin configurations (reviewed in Pannell and Ellis 2001). Mok and colleagues (Mok *et al.* 2007) suggest that there are two forms of vector silencing: immediately after infection, probably dependent on the site of integration, or after long term, due to late epigenetic changes. Besides gene silencing, the literature describes two more factors that can contribute to impaired expression efficiency in retroviral vectors: first, the majority of transcribed RNA is not efficiently processed and licensed for nuclear export (Schambach *et al.* 2000); second, the presence of several aberrant AUG starting codons may start the translation of polypeptides with unknown function that may be toxic for the cells, or simply prevent the transcription of the entire gene (Hildinger *et al.* 1999).

Several studies suggest that expression efficiency can be enhanced by manipulating promoter and enhancer elements. In particular, Hildinger (Hildinger *et al.* 1999) developed a 5` untranslated leader sequence that results in a reduction of silencing and consequent improvement in gene expression. In this work, Hildinger and colleagues examined the architecture of specific retroviruses selected for their high level of protein expression, such as the murine embryonic stem cell virus (MESV): in simple retroviruses, the upstream untranslated region

mimics the translational control of Gag, and therefore the sequence upstream of the *gag-pol* genes was selected as enhancer. Subsequent deletion of all the aberrant AUG starting codons abrogated the expression of aberrant proteins or peptides and optimised the specificity of translation, since no transcripts are lost by recruitment of aberrant reading frames. Further, the introduction of an intron in 5` prevented transcriptional silencing due to cytosine methylation and induced higher levels of transgene mRNA over time. The effect is not dependent on the transgene or on the number of integrated copies of the vector, but can still depend on the cell line.

We therefore decided to insert the same leader sequence described by Hildinger into all lentiviral constructs expressing each individual NS protein of DENV. The presence of the leader sequence, confirmed by DNA sequencing, did not affect viral titre, but ensured higher and more uniform efficiency of transduction and, most important, high and prolonged expression of the transgene over time.

3.5 CONCLUSIONS

In this preliminary part of our work, we developed five K562 cell lines expressing individual DENV NS protein cassettes: K562_NS1/NS2A, K562_NS2B/NS3, K562_NS4A, K562_NS4B, and K562_NS5. We incorporated a leader sequence from MESV into the lentiviral vector, a strategy that resulted in high level and stable expression of DENV NS proteins. Each cell line also expressed the fluorescent marker GFP under control of a separate promoter, enabling positive cells to be sorted by flow cytometry to generate non-clonal populations of cells all expressing the NS protein/s of interest.

Chapter 4.

IDENTIFICATION OF NS5 AS A POTENT IFN-α ANTAGONIST

4.1 INTRODUCTION

DENV is known to inhibit the type I IFN response. As previously discussed (chapter 1, section 1.5.2.2), DENV prevents STAT1 phosphorylation in infected cells, but neither the precise mechanism of inhibition nor the viral proteins involved are clear. In work published in 2005, our group observed reduced levels of STAT2 both in DENV infected cells and in replicon containing cells, and suggested that reduced STAT2 expression explains the inhibition of the IFN–α signalling (Jones *et al.* 2005). Follow-on work in replicon containing cells has shown that STAT2 reduction is probably due to proteasome-mediated degradation: STAT2 mRNA levels were unaffected in the presence of the replicon, and treatment with the proteasome inhibitor bortezomib restored STAT2 levels in DENV replicon containing cells (Mazzon *et al.* 2009). As noted previously in chapter 3, these observations in DENV replicon containing cells, rule out the possibility that STAT2 degradation might be caused by expression of DENV structural proteins or by virus assembly, and instead imply a role for one or more DENV non-structural protein/s. By expressing individual NS protein cassettes of DENV in K562 cells (chapter 3), we were able to test the role of each non-structural component in the inhibition of type I IFN and in STAT2 degradation.

4.2 OBJECTIVES

- To test the role of separate DENV NS protein/s in reducing cellular expression of STAT2
- to test the role of separate DENV NS protein/s in inhibiting the type I IFN response
- to investigate the selectivity of the inhibitory effect on the type I IFN, especially in relation to the type II IFN response
- to test whether NS5 from a different strain of DENV-2 has the same inhibitory effect.

4.3 RESULTS

4.3.1 DENV NS proteins expressed separately do not induce STAT2 degradation

In order to test the hypothesis that one or more DENV NS proteins target/s STAT2 for proteasomal degradation, we tested by immunoblotting intracellular levels of STAT2 in all K562 cell lines described in chapter 3 (K562_NS1/NS2A, K562_NS2B/NS3, K562_NS4A, K562_NS4B, and K562_NS5). As shown in Figure 4.1, in all these cell lines STAT2 is expressed at the same level as in parental cells and cells transduced with empty lentiviral vector. As anticipated, STAT2 levels are markedly reduced in DENV replicon containing cells, which express all the NS proteins together. These results imply that DENV NS proteins expressed separately are incapable of inducing STAT2 degradation.

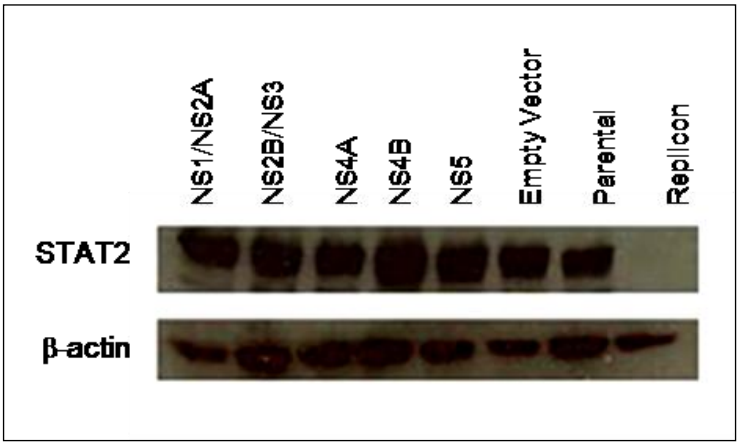


Figure 4.1|
STAT2 is degraded in the presence of DENV NS proteins expressed together but not separately. Comparison of STAT2 levels in K562 cells expressing DENV non-structural protein cassettes. Equal numbers of cells stably transduced with lentiviral vectors as indicated were lysed and proteins separated by SDS-PAGE. Cellular levels of STAT2 were analysed by immunoblotting. Parental K562 cells and K562 cells containing DENV replicons were included as controls.

4.3.2 NS5 inhibits the type I IFN response

The observation that none of the NS protein cassettes expressed separately was sufficient to induce degradation of STAT2 does not exclude the possibility that they may mediate other inhibitory effects on the type I IFN response: it is possible that STAT2 degradation might be the final outcome of a more complex process, or that other completely different mechanisms are involved.

We therefore examined the response to IFN-α in the same cell lines. Cells were stimulated with IFN-α for 6 hours, and then RNA was extracted and induction of the IFN-α-sensitive gene MX1 was measured by quantitative RT-PCR. Figure 4.2 shows the percentage induction of MX1 compared with parental cells: MX1 induction was significantly inhibited only in cells expressing NS5. This inhibition was similar to that observed in replicon containing cells, included as a positive control.

4.3.2.1 NS5 inhibits the dose-dependent induction of ISGs.

Figure 4.2 shows the mean of three independent experiments; high standard deviations associated with some NS proteins suggest biological variability within the system. Therefore, in order to further validate our observations, we measured the activation of MX1 and of a second IFN sensitive gene, OAS1, in response to different concentrations of IFN-α (0, 10, 100, and 1000 IU/ml). After 6 hours from IFN stimulation, we extracted the RNA and measured by RT-PCR the up-regulation of MX1 and OAS1. As shown in Figure 4.3, while both negative controls -parental cells and cells transduced with empty lentiviral vector- respond to IFN-α in a dose-dependent manner, in cells expressing NS5 the up-regulation

of both MX1 and OAS1 is inhibited, as it is in replicon containing cells. At the highest concentration of IFN-α (1000 IU/ml), the antagonistic effect is still significant in cells expressing NS5.

4.3.2.2 The antiviral effect of IFN-α is suppressed in NS5 expressing cells

Inhibition of type I IFN response was also tested at protein level, by measuring the expression of the IFN-inducible molecules HLA-ABC in response to IFN-α. 24 hours after IFN stimulation, we measured HLA-ABC expression on cell surface by flow cytometry. Figure 4.4 shows that, differently from parental cells and cells transduced with empty lentiviral vector, cells containing NS5, as well as cells containing the replicon, do not up-regulate HLA-ABC in response to IFN.

In previous work, a *trans*-rescue assay was performed in order to test whether the presence of DENV replicon affected the general antiviral effect of type I IFN (Jones *et al.* 2005). In order to assess the biological consequences of NS5 inhibition of IFN-α, we compared the antiviral activity of IFN-α in K562 cells expressing NS5 and in K562 cells transduced with empty lentiviral vector. After 24 hour treatment with or without 100 IU/ml of IFN-α, both cell lines were infected by the IFN-sensitive virus ECMV (encephalomyocarditis virus). Viral replication in infected cells was measured by titrating cell supernatant on indicator A549 cells, as described in chapter 2 (section 2.5). Since IFN treatment prevents ECMV replication, persistent viral replication reflects inhibition of the IFN response. As shown in Figure 4.5, pre-treatment with IFN−α of cells transduced with empty vector inhibits ECMV replication; in contrast, when NS5 is expressed, ECMV replication is higher, reflecting reduced antiviral response.

Finally we examined the effect of NS5 and of all other DENV NS proteins on the IFN-α signalling by immunoblotting. After 15 minute stimulation, cells were lysed and proteins separated by SDS-PAGE; immunoblotting was performed with a specific antibody recognising phosphorylated STAT1, a key downstream signalling component of the IFN pathway, which is known to be inhibited in DENV infection. As shown in Figure 4.6, reduction of STAT1 phosphorylation is observed only in cells expressing NS5 and in cells expressing the replicon. Total cellular levels of STAT1 did not significantly differ between the different K562 lines.

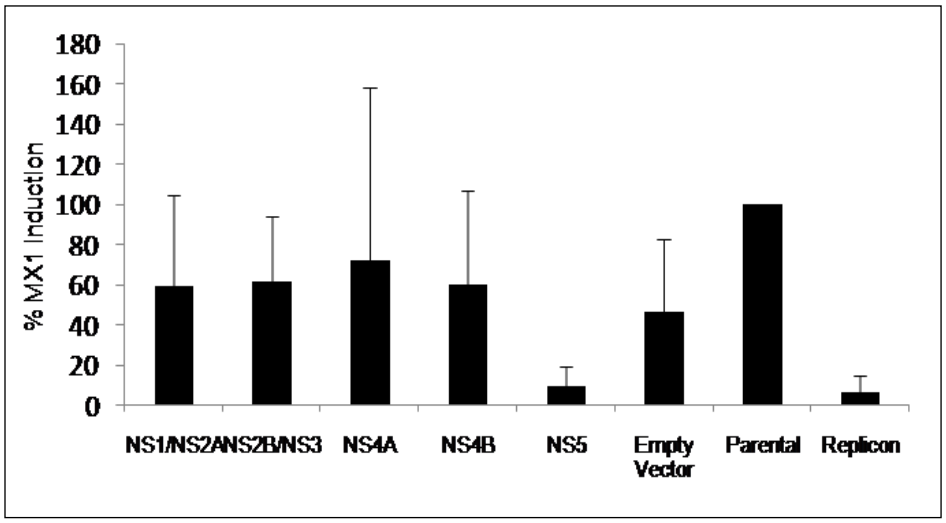


Figure 4.2|

NS5 inhibits the induction of the IFN stimulated gene MX1. K562 cells expressing individual DENV NS proteins as indicated were treated with 100 IU/ml of IFN-α for 6 hours before RNA extraction. MX1 induction was measured by quantitative RT-PCR and normalised to the housekeeping gene GAPDH. The percentage induction of MX1 was compared with parental K562 cells (100%) and the figure shows the mean (+SD) of three independent experiments, each performed in duplicate. Replicon containing K562 were included for comparison.

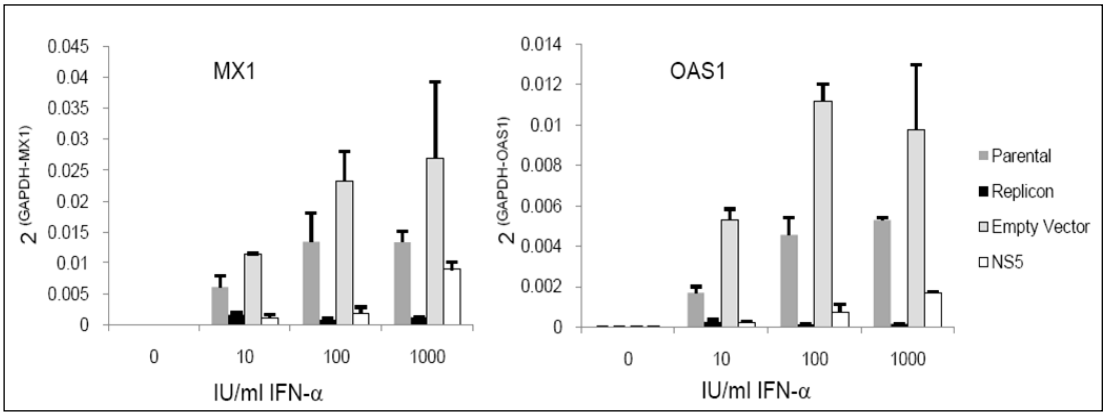


Figure 4.3|

NS5 inhibits the dose-dependent induction of ISGs. Parental K562 cells, replicon-containing K562 cells, K562 cells transduced with empty lentiviral vector and K562 cells expressing NS5 were treated with different concentrations of IFN-α as shown for 6 hours before RNA extraction. MX1 (left panel) and OAS1 (right panel) induction was measured by real time PCR and normalised to the housekeeping gene GAPDH using the formula $2^{(\text{GAPDH-GENE OF INTEREST})}$.

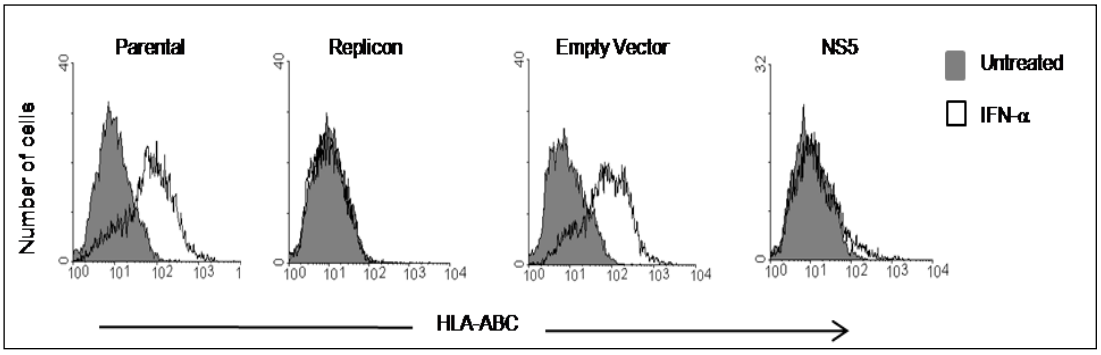


Figure 4.4|

NS5 inhibits cellular responses to IFN-α. NS5 expressing cells and controls as indicated were treated for 24 hours with (open histogram) or without (grey histogram) 100 IU/ml of IFN-α and cell surface expression of the IFN inducible antigen HLA-ABC was measured by flow cytometry.

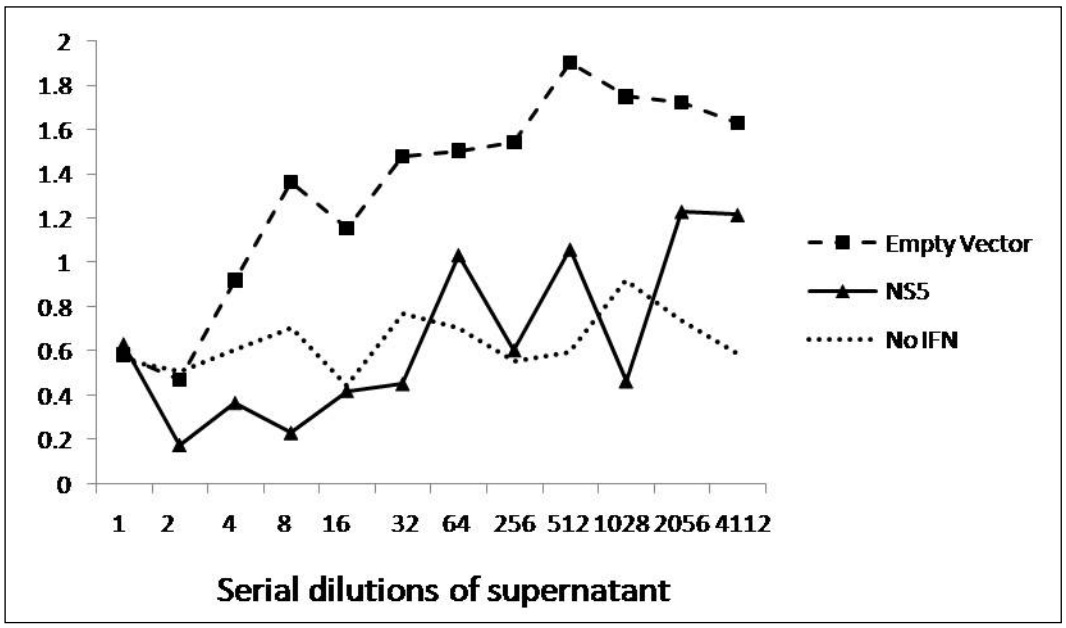


Figure 4.5|

The antiviral effect of IFN-α is suppressed in NS5 expressing cells. K562 transduced with NS5 (solid line) or with empty lentiviral vector (broken line) were treated with 100 IU/ml of IFN-α for 24 hours. The cells were then infected with ECMV and after further 24 hours the supernatants were harvested and serially diluted on confluent A549 cells (x axes). 24 hours later the A549 cells were fixed and stained with methyl violet. The optical density (*O.D.*, y axes) of each well was measured at 570 nm. More ECMV replication results in increased cell death and lower optical density reading.

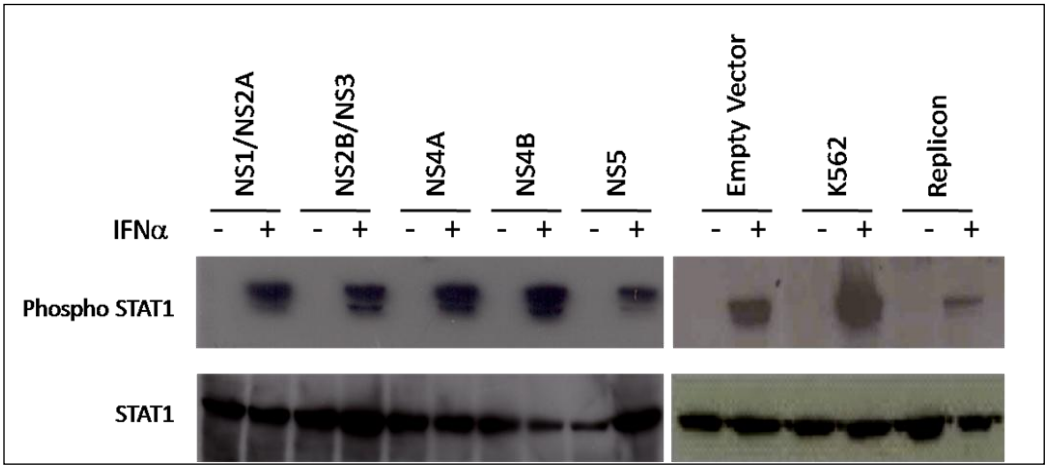


Figure 4.6|

NS5 reduces STAT1 phosphorylation. K562 cells expressing individual DENV NS proteins as indicated were treated with 100 IU/ml of IFN-α for 15 minutes. Equal number of cells were lysed and proteins separated by SDS-PAGE. STAT1 levels and STAT1 phosphorylation were analysed by immunoblotting. Parental K562 were included as a control.

4.3.3 NS5 does not inhibit cellular responses to IFN-γ

Previous work reported that DENV replicon expression specifically blocked IFN-α but not IFN-γ signalling (Jones *et al.* 2005). In order to test the response to IFN-γ in cells containing NS5, we first looked at phosphorylation of the transcription factor STAT1. STAT1 is common to both type I and type II IFN signal transduction pathways, and we show above that its phosphorylation in response to IFN-α is reduced in the presence of DENV NS5 (Figure 4.6). Cells were incubated with or without 100 IU/ml of IFN-γ for 15 minutes. Cells were then lysed, proteins separated by SDS-PAGE and analysed by immunoblotting. As expected, the presence of DENV replicon did not inhibit STAT1 phosphorylation in response to IFN-γ (Figure 4.7, A). Similarly, expression of NS5 alone did not affect STAT1 phosphorylation.

We also confirmed the functionality of IFN-γ response by measuring the expression of the surface molecules HLA-ABC (Figure 4.7, B). Cells were stimulated with or without 100 IU/ml of IFN-γ and 24 hours later the expression of HLA-ABC molecules was measured by flow cytometry. As shown in Figure 4.7 B, and in contrast to the HLA response to type I IFN, up-regulation of HLA-ABC is equivalent in all cell lines, further confirming the specificity of NS5/DENV replicon inhibitory effect on type I IFN. Taken together, these results suggest that DENV antagonism of type I IFN signalling involves components of the signal transduction pathway that are not shared with the type II IFN signalling pathway.

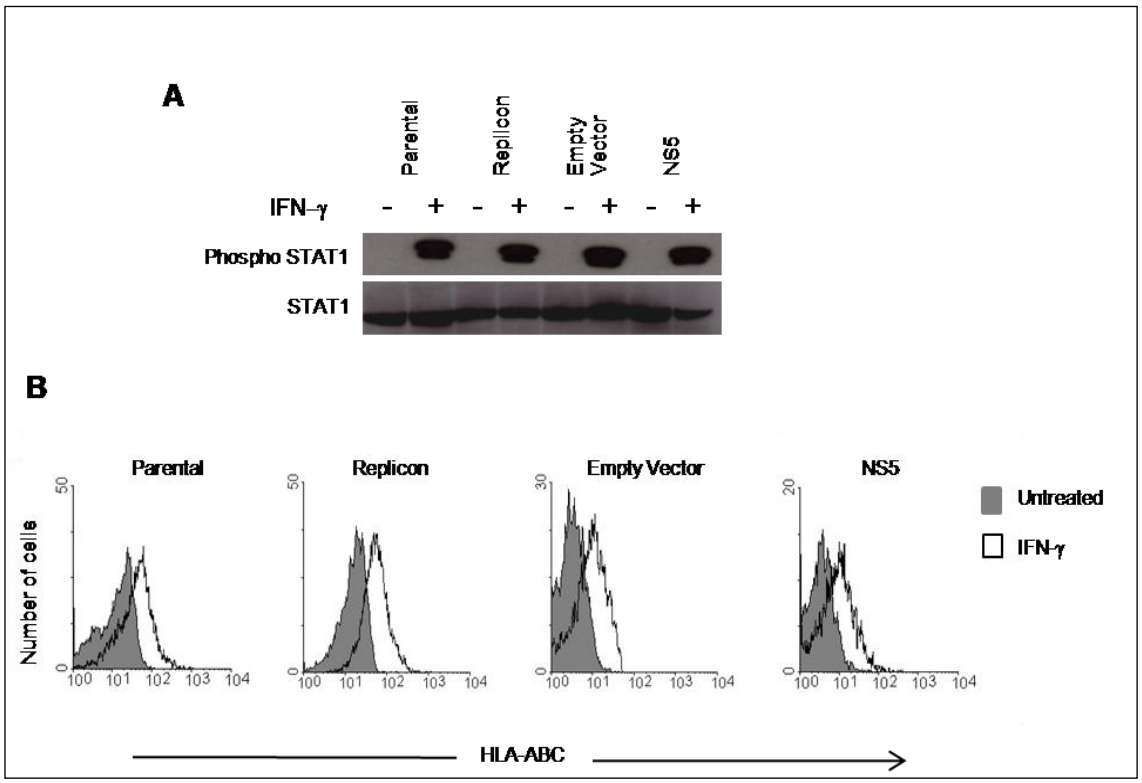


Figure 4.7|

NS5 does not inhibit cellular response to IFN-γ.

A. NS5 does not inhibit STAT1 phosphorylation in response to IFN-γ. NS5 expressing cells were treated with or without 100 IU/ml of IFN-γ for 15 minutes before lysis. Proteins were separated by SDS-PAGE and cellular levels of STAT1 and phosphorylated STAT1 were analysed by immunoblotting. **B.** NS5 does not inhibit up-regulation of HLA-ABC in response to IFN-γ. NS5 expressing cells and controls were treated for 24 hours with (open histogram) or without (grey histogram) 100 IU/ml of IFN-γ and cell surface expression of the IFN inducible antigen HLA-ABC was measured by flow cytometry.

4.3.4 DENV-2 TSV01 NS5 also inhibits type I IFN response

In recent work, Umareddy and colleagues (Umareddy *et al.* 2008) suggested that DENV inhibits type I IFN signalling in a strain-dependent manner. They compared the response to IFN in cells infected with two closely related DENV-2 strains, NGC and TSV01 (Gene Bank AY037116). Phosphorylation of STAT1 and STAT2, as well as the induction of the IFN response element ISG, were all found to be inhibited by NGC but not TSV01 infection.

NGC and TSV01 NS5 proteins differ in only 17 residues across the gene, of which 9 are conservative substitutions. A differential effect of the two NS5 proteins on the cellular response to IFN-α might therefore help identify those residues in NGC NS5 that are critical for IFN subversion. We therefore decided to investigate the effects of TSV01 NS5 on the IFN-α response.

TSV01 NS5 was cloned into our lentiviral vector, following the procedure described in chapter 3. Briefly, TSV01 NS5 was amplified by High-Fidelity PCR from the TSV01 cDNA genome (kindly provided by M. Johansson, Stockholm University), inserting the restriction sites for BamHI and NotI in 5` and 3` respectively, and the amplified product was cloned in the pGEM®-T Easy vector. The insert was finally cloned into the dual promoter lentiviral vector containing the leader sequence described in chapter 3. Correct sequences and insertions were checked by double digestion BamHI-NotI and by DNA sequencing. The construct was used to generate lentivirus and transduce K562 cells. TSV01 NS5 expression was tested by immunoblotting, showing expression levels similar to NGC NS5 (Figure 4.8, A). Positive cells were not FACS sorted because cellular response to

IFN-α was tested by flow cytometry, which allows gating on the GFP positive cells only; cells not expressing GFP (not transduced) were used as an internal control.

Cells were treated with 100 IU/ml of IFN-α for 24 hours and then cell surface expression of HLA-ABC was measured by flow cytometry. K562 expressing NGC-NS5 and K562 transduced with empty lentiviral vector were included as control. As shown in Figure 4.8, differently from cells containing the empty vector, which respond to IFN-α by up-regulating HLA-ABC, IFN-α mediated up-regulation of HLA-ABC in cells expressing TSV01 NS5 was inhibited to a similar extent as in cells expressing NGC NS5.

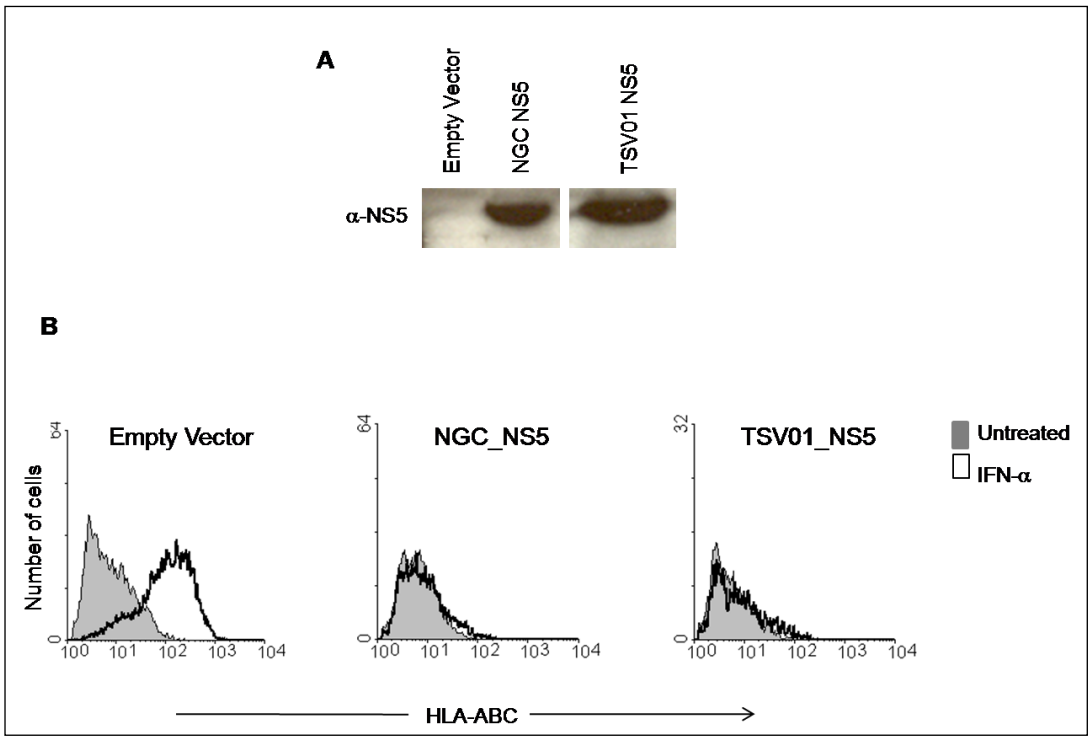


Figure 4.8|

TSV01_NS5 inhibits cellular response to IFN-α

A. NGC_NS5 and TSV01_NS5 are expressed at similar levels in K562 transduced with the correspondent lentiviruses. Equal numbers of cells were lysed and proteins contained in the lysates separated by SDS-PAGE. NS5 was detected by immunoblotting using an anti-NS5 antibody. **B.** TSV01_NS5 expressing cells, NGC_NS5 expressing cells and empty control were treated for 24 hours with (open histogram) or without (grey histogram) 100 IU/ml of IFN-α and cell surface expression of the IFN inducible antigen HLA-ABC was measured by flow cytometry.

4.4 DISCUSSION

Having developed human cell lines stably expressing DENV NS protein/s separately (chapter 3), we investigated which NS protein/s is/are responsible for STAT2 degradation. STAT2 expression was consistently reduced in replicon containing cells, in which all NS proteins are expressed together, but this effect was not replicated in cells expressing individual DENV NS protein cassettes. One possibility is that when expressed in a context other than the replicon, some NS proteins might be differently processed, orientated or compartmentalised, and this might compromise some of their functions. In chapter 3 we have shown that expression levels of NS1 and NS5 in our lentiviral system are comparable to the expression level of the same proteins in cells containing the replicon; however, since we could not test any other NS protein, we cannot exclude the possibility that absence of STAT2 degradation might be due to inadequate levels of protein expression. Another possible explanation is that an untested combination of NS proteins might be required to reduce cellular STAT2 expression, and this possibility will be further investigated in chapter 5. We therefore examined whether NS proteins expressed separately have the capacity to inhibit the IFN response, either through an interaction with STAT2 that is not sufficient to lead to its degradation, or via a completely different mechanism.

Using the cell lines established in chapter 3, we first measured the induction of type I IFN sensitive genes (ISG) in response to IFN-α by RT-PCR. Despite observing some variability between repeated experiments, we observed consistent

inhibition of ISG induction in cells expressing NS5. In view of the biological variability in this experimental system, we performed a series of further experiments to test whether or not NS5 is an IFN antagonist. Dose-response experiments measuring the induction of different ISG in cells treated with increasing concentrations of IFN-α consistently showed that the response to IFN is suppressed in the presence of NS5. Also, pre-treatment of NS5 cells with IFN-α does not prevent replication of the IFN-sensitive virus ECMV, failing to establish an IFN-induced antiviral state. Finally, immunoblot measuring phosphorylation of STAT1, a key component of IFN signalling, showed reduced STAT1 phosphorylation in cells containing NS5. Since in the presence of other DENV NS proteins STAT1 phosphorylation levels were comparable to parental cells and to cells transduced with empty lentiviral vector, this observation further highlights the unique activity of NS5 on the IFN signalling pathway. Taken together, these experiments provide compelling evidence that NS5 is a potent inhibitor of type I IFN and blocks IFN-α mediated signal transduction.

In all our experiments we observed that in the presence of the replicon, IFN inhibition is slightly stronger than in the presence of NS5 only. This might suggest that other mechanisms or more NS proteins may be required to completely block the IFN response. Furthermore, even if a role for the UTRs in DENV protein synthesis has not been shown, we cannot exclude that they might play a role in helping the correct localisation or processing of the DENV polyprotein. In this case, the presence of the UTRs in the replicon might concur to explain the phenotypic differences between NS5 in the context of the lentiviral vector and in the replicon. Degradation of STAT2 observed in the presence of the replicon might itself contribute to a more complete inhibition of IFN signalling, allowing

NS5 recycling and binding of newly synthesised STAT2 proteins.

Potent inhibitory activity is paired with tight selectivity, as the type II IFN signalling pathway, which shares with type I IFN some components of the transduction cascade, is not affected by NS5. Our observations are in disagreement with Muñoz-Jordan`s work (Munoz-Jordan *et al.* 2003), but are in agreement with several other reports (Ho *et al.* 2005; Jones *et al.* 2005; Dejnirattisai *et al.* 2008) showing that DENV infected/replicon containing cells efficiently respond to IFN-γ stimulation.

In two different works, Muñoz-Jordan and colleagues (Munoz-Jordan *et al.* 2003; Munoz-Jordan *et al.* 2005) report that DENV NS2A, NS4A and NS4B are all involved in the inhibition of type I IFN. Although we do not definitively rule out the involvement of these and/or other NS proteins, we could not observe any significant or consistent inhibition of the IFN-α response in cells expressing proteins other than NS5. Our findings bring DENV in line with studies of other flaviviruses, in which NS5 has been reported to be involved in the evasion of innate immunity (JEV, TBEV, LGTV, WNV, see chapter 1, section 1.5.2.1.4). Discrepancies with Muñoz-Jordan`s data may be due to the experimental settings. In our work we have tried to maximise the biological significance of our system in several ways. First, aware of the high species specificity of the IFN response, we chose to employ human cell lines; also, K562 are cells of haematopoietic origin and therefore closer to DENV target *in vivo* (dendritic cells, monocytes, macrophages, see chapter 1, section 1.2.2). Next, when appropriate, we chose to

express some NS proteins in combination, in order to reproduce their function (NS2B+NS3) and their correct localisation/orientation (NS1+NS2A). Finally, we optimised our protein expression system in order to achieve expression levels comparable to the replicon and stable over time.

Inter-strain variability in DENV serotypes could also be responsible for the discrepancies observed in IFN antagonism in different reports. The possibility that there are strain-dependent differences in IFN sensitivity is supported by Umareddy and colleagues (Umareddy *et al.* 2008), who report that, differently from the NGC strain, the DENV-2 strain TSV01 is unable to subvert the IFN response. However, when we expressed TSV01 NS5 using our lentivirus delivery model, we observed the same inhibition of the IFN response that we observed for NGC NS5. Marked suppression of the IFN response by NS5 from a strain that is apparently sensitive to IFN is a puzzling observation: even hypothesising the existence of redundant IFN inhibitory mechanisms in the NGC strain that could be absent from the TSV01 strain, the strong inhibition observed in Figure 4.8 suggests that the presence of NS5 should be sufficient to block the IFN-induced antiviral activity. One critical element could be the level of expression of NS5 required for effective antagonism of the IFN-α mediated antiviral response. In Umareddy`s work (Umareddy *et al.* 2008) supernatant from A549 cells infected with TSV01 was shown to contain fewer viruses than supernatant from cells infected with NGC, suggesting lower levels of virus production. This implies lower levels of protein expression, and indeed Umareddy and colleagues show much lower levels of NS5 expression in cells infected with TSV01 than in cells

infected with NGC. Two opposite hypotheses could explain these observations: either lower viral replication is due to ineffective inhibition of the IFN response, or the ineffective inhibition of the IFN response is due to lower replication efficiency. Since from our work TSV01 NS5 inhibits IFN to the same extent as NGC NS5, it is tempting to hypothesise that the incapability of the TSV01 strain to inhibit the IFN response might be related to its less efficient replication and to lower NS5 expression levels, rather than to specific characteristics of NS5 itself. This hypothesis is supported by our own observations that lower NS5 levels correlate with a lower IFN inhibitory effect: on several occasions, we used constructs which gave low levels of protein expression, and in all these cases we also observed a reduced ability to inhibit the IFN response (data not shown). Conversely, when we tested by immunoblotting cellular levels of NS5 in cells transduced with TSV01 NS5 lentivirus, we showed expression levels comparable to NS5 expressed in the replicon. The lentiviral vector system that we established guarantees high levels of TSV01 NS5 expression, levelling out the expression differences observed in infection. As shown in chapter 3, NS5 expression in cells transduced with the lentivirus are comparable to NS5 levels in the replicon. Expression levels in BHK containing the replicon are in turn slightly lower than in BHK infected with DENV-2 NGC (A. Davidson, personal communications). Lentiviral transductions can therefore be employed as an invaluable tool to dissect the effects due to structural properties and expression levels.

Taken together these observations suggest that high expression levels of NS5 are required in order to inhibit the IFN response, but the hypothesis that a threshold level of NS5 could be required to inhibit the type I IFN has not been further tested in this work. To explore the correlation between DENV pathogenesis, the

inhibition of the IFN response, and NS5 expression levels would be of great interest, but it was beyond the objectives of this project.

4.5 CONCLUSIONS

In this part of the work we investigated the capacity of DENV-2 NS proteins expressed separately both to degrade STAT2 and to inhibit type I IFN. We showed that NS proteins expressed separately are not sufficient to induce STAT2 degradation. Reduced STAT2 levels are observed only in the presence of the replicon, where all DENV NS proteins are expressed together.

Despite its inability to reduce STAT2 expression, NS5 alone is a potent and specific type I IFN antagonist. NS5 prevents induction of type I IFN sensitive genes and inhibits the activation of an antiviral state by blocking IFN-α mediated signal transduction. We demonstrated the same inhibitory activity for NS5 from two different strains of the DENV-2, NGC and TSV01. The inhibition is specific for the type I IFN pathway, and the type II IFN response is not affected.

Chapter 5.

NS5 BINDS STAT2 AND PREVENTS ITS PHOSPHORYLATION

5.1 INTRODUCTION

Taken together, the data presented in chapter 4 demonstrate that DENV NS5 is a potent antagonist of the type I IFN that specifically blocks IFN-α/β mediated signal transduction. As described in chapter 1 (section 1.2.4.5), NS5 is a large multifunctional protein that includes both polymerase and methyltransferase domains. The observation that the same protein is responsible for such critical functions in virus replication and in the inhibition of IFN suggests a tight evolutionary relationship between viral replication and the evasion of innate immunity.

DENV NS5 has not previously been implicated as an IFN antagonist, but several papers have reported that NS5 proteins of other flaviviruses are involved in the inhibition of type I IFN (TBEV and LGTV (Best *et al.* 2005; Park *et al.* 2007; Werme *et al.* 2008); JEV (Lin *et al.* 2004; Lin *et al.* 2006); WNV (Laurent-Rolle *et al.* 2010)), however, the precise mechanisms of inhibition and the cellular components of the signalling cascade involved appear to be different for different flaviviruses. In cells infected by JEV, viral NS5 was reported to block Tyk2 phosphorylation in response to IFN (Lin *et al.* 2006). In contrast, direct interaction between LGTV NS5 and IFN receptors has been suggested (Best *et al.* 2005). The reported variations might suggest the existence of different mechanisms developed during parallel flavivirus evolution, but alternatively they may simply reflect differences in the experimental models. In keeping with this, inconsistent observations about the mechanisms of IFN inhibition have been reported for individual viruses: as discussed earlier in chapter 1 (section 1.5.2.2), for DENV

Muñoz-Jordan and colleagues suggest a block at STAT1 level (Munoz-Jordan *et al.* 2003), Ho *et al* propose inhibition of Tyk2 activation (Ho *et al.* 2005), while Jones *et al* show degradation of STAT2 (Jones *et al.* 2005).

Having identified DENV NS5 as a potent type I IFN antagonist, we next investigated the precise step/s of the IFN-α/β signalling pathway targeted by NS5, and explored the molecular mechanism of IFN inhibition. The major conclusion emerging from the first part of this chapter is that NS5 inhibits IFN-α signalling by binding the transcription factor STAT2, key component of the type I IFN transduction cascade, therefore preventing its phosphorylation. Inhibition of STAT2 phosphorylation following IFN treatment is also observed in cells expressing the DENV replicon; however, while in the presence of the replicon STAT2 is degraded via proteasome (Mazzon *et al.* 2009), we did not observe reduction of STAT2 levels in cells expressing NS5 alone.

Cells have developed sophisticated systems to dispose of proteins that are no longer required and degradation via proteasome of ubiquitinated targets is one of the most commonly employed. Ubiquitination is a reversible post-translational modification that results in the conjugation of ubiquitin -a small protein of 76 amino acids- to the target protein, usually on a lysine residue (reviewed in Leon and Haguenauer-Tsapis 2009). Different ubiquitination systems can be composed of different elements, depending on the different proteins and pathways involved, but the general mechanism requires three basic components: *E1*, the ubiquitin activating enzyme; *E2*, the ubiquitin conjugating enzyme; and *E3*, the ubiquitin ligase. The E3 ligase is normally a multifunctional complex itself, composed of one protein that specifically recognises the target and is in charge of the

specificity to the substrate, and one more complex/es of proteins recruiting E2. The ECS (Elongin C-Cullin-SOCS-box) E3 ubiquitin ligases, one of the most characterised E3 complexes, include the SOCS (suppressor of cytokine signalling) family of proteins, which are involved in feedback inhibition of JAK/STAT signalling pathways. In this E3 ubiquitin system, the protein responsible for the recognition of the target is usually responsible also for the assembly of the complex/es that binds E2, as it carries a "BC-box", i.e. a short consensus sequence responsible for the recruitment of the Elongin B/C complex (Figure 5.1). The Elongin B/C complex is in turn responsible for binding a further complex of proteins, which finally bridges to E2 through a RING domain. Figure 5.1 shows one of the best characterised of these complexes, composed of Cullin 2 or Cullin 5, and Rbx 1 or Rbx 2, the latter containing the RING domain (Yasukawa *et al.* 2008; Leon and Haguenauer-Tsapis 2009).

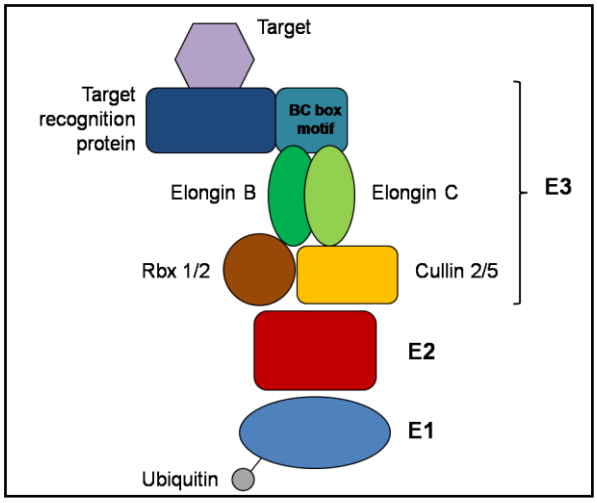


Figure 5.1|
Schematic representation of the ubiquitination complex. A BC-box motif in the protein that recognises the target recruits the E3 ligase complex which in turn recruits the ubiquitin activation enzyme (E1) and the ubiquitin conjugating enzyme (E2) (see text).

E3 ubiquitin ligases are also encoded by several viruses, in order to induce polyubiquitination and degradation of a variety of proteins, sometimes involved in apoptosis and cell cycle inhibition, or in the activation of the immune response (Ulane *et al.* 2005; Elliott *et al.* 2007; Isaacson and Ploegh 2009). Viruses are known that can induce ubiquitination and proteasome-mediated degradation of STAT proteins. Rubulavirus V protein assembles an E3 ubiquitin ligase by using cellular components, including the UV-damaged DNA binding protein 1 (DDBP1), Cullin 4A and STAT (Ulane *et al.* 2005). Respiratory Syncytial virus (RSV) NS1 and NS2 proteins also assemble ubiquitin ligase E3 complexes: in particular, NS1 has been shown to contain a BC-box and a Cullin 2 binding consensus sequence, and to interact with Elongin C and Cullin 2 *in vitro* (Elliott *et al.* 2007). In the second part of this chapter, we tested whether STAT2 degradation might be dependent on the assembly of a similar complex, with NS5 representing the component of the E3 ubiquitin ligase responsible for binding the target (STAT2), and some other DENV NS proteins responsible for the recruitment of other components of the E3 complex.

5.2 OBJECTIVES

- To identify which cellular component of the IFN-α signalling pathway is targeted by NS5
- to clarify the molecular mechanism by which NS5 antagonises the IFN response
- to test whether a combination of NS5 and other DENV NS proteins is responsible for STAT2 degradation.

5.3 RESULTS

5.3.1 NS5 inhibits type I IFN signalling downstream of STAT2 phosphorylation

The data presented in chapter 4 (Figure 4.6) show that expression of NS5 reduces STAT1 phosphorylation in response to IFN-α. It follows that NS5 inhibits the type I IFN signal transduction cascade at or before STAT1 phosphorylation. In order to investigate which step of the cascade is inhibited by NS5, we treated NS5 expressing cells with or without 100 IU/ml of IFN-α for 15 minutes. Cells transduced with empty lentiviral vector, replicon-expressing, and parental K562 cells were included as controls. Equal number of cells were lysed and proteins separated by SDS-PAGE. Immunoblots were performed using antibodies specific for different components of the type I IFN signalling cascade.

5.3.1.1 NS5 inhibits STAT2 phosphorylation in response to IFN-α

As expected, expression of either NS5 alone or DENV replicon reduced phosphorylation of STAT1 in response to IFN-α without affecting the levels of cellular STAT1 expression (Figure 5.2, A). In the classic type I IFN signal transduction pathway, the phosphorylation of STAT1 follows and is dependent on STAT2 phosphorylation. As described in Chapter 1, only STAT2 phosphorylation provides the docking site that orients STAT1 for phosphorylation and subsequent heterodimerisation with STAT2 (Li *et al.* 1997). Therefore, we next examined STAT2 phosphorylation in the presence and absence of NS5. As shown in Figure 5.2 B, STAT2 phosphorylation was markedly reduced in both NS5 and replicon

expressing cells. As expected, STAT2 levels were also greatly reduced in DENV replicon containing cells, in keeping with our previous data (Jones *et al.* 2005). In contrast, STAT2 levels were unaltered in cells expressing NS5 alone, indicating that expression of NS5 alone does not completely replicate the phenotype of replicon expressing cells (in which all the non-structural proteins are expressed together).

5.3.1.2 NS5 does not inhibit the IFN-α signalling cascade upstream of STAT2 phosphorylation

As described in chapter 1, early components of the IFN-α signalling pathway have been suggested to be antagonised by both DENV (Ho *et al.* 2005) and TBEV (Best *et al.* 2005; Werme *et al.* 2008). To test whether DENV NS5 targets IFN-α signal components upstream of STAT2 phosphorylation, we next tested the activation of the receptor associated kinase Tyk2. Activation of Tyk2 directly precedes phosphorylation of STAT2. Being a very early event in the signalling cascade, Tyk2 phosphorylation was assessed after stimulation with IFN-α for only 5 minutes. Lysate separation and immunoblotting were performed as above. In both replicon and NS5 containing cells, total and phosphorylated levels of Tyk2 were not reduced (Figure 5.2, C), suggesting that the signalling pathway upstream of STAT2 phosphorylation is intact. To further confirm that early components of the IFN-α signalling pathway are unaffected by DENV NS5, we also measured the expression of the type I IFN receptor IFNAR2c on the surface of cells containing NS5 by flow cytometry. As shown in Figure 5.3, expression levels of IFNAR2c in the presence of both NS5 and the replicon are comparable to parental K562 and cells transduced with empty lentiviral vector.

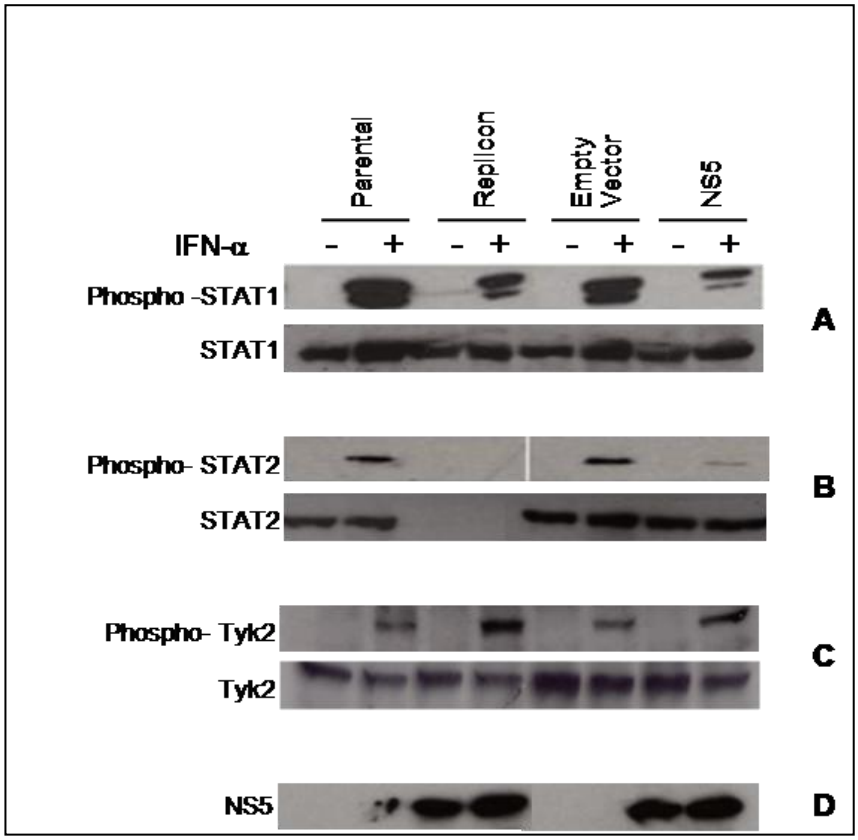


Figure 5.2|

NS5 blocks STAT2 phosphorylation. IFN-α signalling downstream of Tyk2 activation is inhibited in cells expressing NS5. K562 cells expressing NS5 and K562 cells containing DENV replicons were treated with and without 100 IU/ml of IFN-α for 15 minutes. Parental K562 and K562 cells transduced with an empty lentiviral vector were included as controls. Proteins in the cell lysates were separated by SDS-PAGE and total and phosphorylated levels of STAT1 **(A)**, STAT2 **(B)**, and Tyk2 **(C)** were analysed by immunoblotting. The expression of NS5 was tested with an antibody specific for NS5 **(D)**.

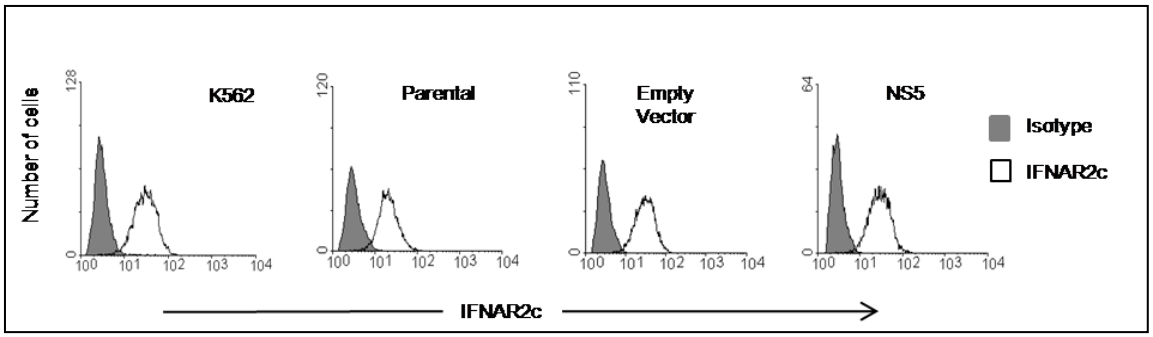


Figure 5.3|

NS5 does not alter IFN-α receptor levels on the cell surface. NS5 expressing cells and control cells were stained for the surface receptor IFNAR2c and analysed by flow cytometry. Cells stained with the secondary antibody alone (grey histograms) were included as negative staining controls.

5.3.2 NS5 binds STAT2

Taken together our data show that STAT2 phosphorylation in response to IFN-α is inhibited in both NS5 and replicon containing cells. While in replicon containing cells this could be explained simply by reduced levels of STAT2, this was not observed in NS5 containing cells. This was the only apparent difference between the phenotype of cells expressing NS5 and the phenotype of cells expressing all the non-structural proteins together (i.e. the replicon). We therefore hypothesised that NS5 expressed in isolation binds STAT2 and thus inhibits its phosphorylation and all downstream events in the signalling cascade, but additional factors, most likely other DENV NS proteins, are required to target STAT2 for degradation. In order to test whether NS5 binds STAT2, we investigated whether NS5 and STAT2 could be co-immunoprecipitated. Preliminary attempts at immunoprecipitation using commercially available STAT2 antibodies or a polyclonal anti-NS5 antibody (kindly provided by Dr Andrew Davidson) failed due to non-specific binding. We therefore decided to introduce a FLAG-tag at the C-terminus of NS5 (NS5-FLAG), enabling us to use an anti-FLAG antibody for immunoprecipitation.

5.3.2.1 Expression of NS5-FLAG

The NS5-FLAG gene was amplified by high-fidelity PCR from the plasmid pcDNA-3.1_NS5-FLAG (kindly provided by Dr Andrew Davidson) and the restrictions sites for BamHI and NotI, necessary for cloning into the lentiviral vector, were introduced in 5` and 3` ends respectively. The NS5-FLAG sequence was cloned into the lentiviral vector as described in chapter 2, and correct

sequences and insertions were confirmed by restriction analysis and DNA sequencing. K562 cells were transduced with NS5-FLAG lentiviruses, and GFP positive cells were isolated by FACS sorting (K562_NS5-FLAG). Expression of NS5-FLAG was confirmed by immunoblotting using both anti-NS5 and anti-FLAG antibodies.

5.3.2.2 NS5 specifically binds STAT2, but not STAT1 or Tyk2

The method used for immunoprecipitation is described in more detail in chapter 2 (section 2.4.3). Briefly, the lysate from 10 million cells was incubated with anti-FLAG affinity gel to allow NS5-FLAG binding to the anti-FLAG antibodies on the gel. Unbound proteins were washed away and NS5-FLAG was eluted by competition with 3X FLAG peptide: as we did not know the chemical characteristics of the putative interaction, we chose a gentler elution compared with other systems available (elution in lysis buffer or elution buffer). K562 cells expressing NS5 without FLAG were included as a negative control.

Immunoprecipitated samples were loaded onto an SDS-PAGE gel and immunoblotted with specific antibodies, as described in Figure 5.4. The whole lysates were also included as controls. Specificity of binding to the gel was confirmed by detection of NS5 and FLAG only in correspondence of the eluted sample NS5-FLAG (Figure 5.4, first and second immunoblots), suggesting no aspecific binding of NS5 to the affinity gel. We next tested whether NS5 bound to STAT2. As shown in Figure 5.4 (fourth immunoblot), we observed that STAT2, which was detected in the whole lysate of both NS5 and NS5-FLAG cells, after immunoprecipitation was detectable only in the presence of NS5-FLAG. This shows that STAT2 is co-immunoprecipitated in the presence of NS5-FLAG and

does not aspecifically bind to the affinity gel. In contrast, as shown in Figure 5.4 (third and fifth immunoblots), both STAT1 and Tyk2 were detected in the whole lysate of both cell lines, but neither STAT1 nor Tyk2 co-immunoprecipitated with NS5-FLAG.

This suggests that NS5 -directly or indirectly- specifically binds STAT2.

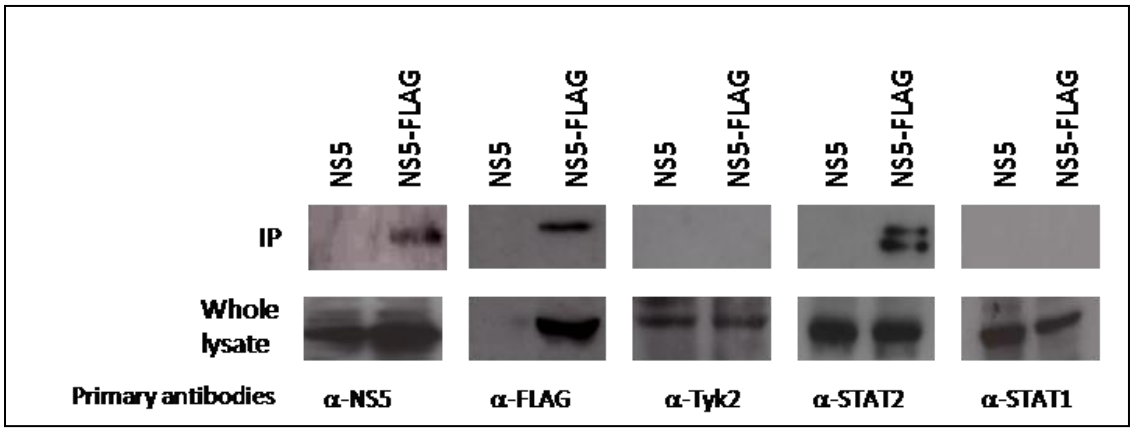


Figure 5.4|

NS5 specifically binds the transcription factor STAT2. Lysates from K562 cells expressing either NS5 or NS5 tagged at the C-terminus with the FLAG-peptide (NS5-FLAG) were immunoprecipitated with anti-FLAG antibodies. Proteins contained in the immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting using specific antibodies against NS5, FLAG, Tyk2, STAT2 and STAT1. Whole cell lysates were included for comparison. One representative experiment of four is shown. *IP: Immuno-precipitated samples*

5.3.3 Co-transduction of NS5 and other NS proteins is not sufficient to induce STAT2 degradation

The interaction between NS5 and STAT2, the same component of the IFN-α signalling cascade that is degraded in the presence of the DENV replicon, suggests that NS5, binding STAT2, might be one of the NS proteins required for targeting the transcription factor for degradation. However, since STAT2 is degraded only in the presence of the replicon, but not when NS5 is expressed alone, it is possible that some other DENV NS proteins are involved. In the next section, we investigate the possibility that, together with NS5, some other DENV NS protein/s are required to induce STAT2 degradation.

5.3.3.1 DENV NS4B contains a BC-box consensus sequence

As described in the introduction to this chapter (section 5.1), proteasome mediated degradation of target proteins requires the assembly of the ubiquitination complex. This is possible if the scaffold protein that specifically binds the target is also able to recruit other components of the proteolytic pathway. In the model described above, the link between the scaffold protein and the ubiquitination complex is provided by a BC-box motif on the scaffold protein, which recognises and binds the two cellular proteins Elongin B and Elongin C. The BC-box motif is a short consensus sequence composed of the following conserved residues: (TSP)LXXXCXXXΦ, where X can be any amino acid and Φ is a hydrophobic residue. The same motif has been recognised also in NS1 protein of RSV (Elliott *et al.* 2007), and it has been shown to recruit the complex of proteins required to induce proteasome-mediated degradation of STAT2. We therefore tried to identify

the same motif in some other DENV NS proteins by bioinformatic alignments. ScanProsite is a program that identifies specific motifs in amino acid sequences of interest. When we scanned DENV-2 polyprotein against the BC-box motif, amongst all DENV NS proteins we identified a BC-box sequence only in NS4B, between amino acids 88-99 (DENV-2). This suggests the presence of a site which can potentially bind Elongin and induce the assembly of the E3 ligase. We did not find the same sequence in DENV NS5, supporting the observation that NS5 alone is not sufficient to induce STAT2 degradation. As shown in Figure 5.5, the BC-box domain of NS4B is conserved in all four DENV strains.

Protein alignments described in the literature have also identified a different consensus sequence, downstream of the BC-box, which seems to be involved in the interaction with Cullin (Elliott *et al.* 2007). Such sequence, ΦXXΦXXXXXΦXXXΦ, is more generic and therefore is found several time along the polyprotein.

5.3.3.2 Co-expression of NS5 and NS4B does not reduce cellular levels of STAT2

To test whether NS4B is involved in the recruitment of the ubiquitination complex, we transduced K562 cells already expressing NS5 with the lentivirus encoding NS4B (K562_NS5+NS4B). A prerequisite condition for this experiment is that all cells, or at least the large majority, express both NS5 and NS4B. Since cells containing NS5 already express GFP, in order to assess the efficiency of transduction with the lentivirus NS4B, we transduced in parallel parental K562 cells and assumed that the percentage of K562_NS5 transduced by the lentivirus NS4B was the same as transduced (i.e. GFP positive) parental cells (Figure 5.6,

A). To test whether the combination of NS5 and NS4B induced STAT2 degradation, cells were lysed, proteins separated by SDS-PAGE and immunoblotted with an antibody specific for STAT2. Replicon-expressing and parental K562 cells, and cells transduced with empty vector, NS5 alone, and NS4B alone were included as controls. As expected (Figure 5.6, B) STAT2 levels in cells expressing either NS5 alone or NS4B alone were unchanged and comparable to parental cells and cells transduced with empty lentiviral vector, while reduced level of STAT2 were detected in the presence of the replicon. In contrast, we did not observe reduction of STAT2 levels in cells expressing NS5 and NS4B together, suggesting that co-expression of these two proteins is not sufficient to induce STAT2 degradation.

Figure 5.5|
NS4B "BC-box" motif is conserved between the four serotypes of DENV. CLUSTAL 2.0.11 multiple sequence alignment of the amino acid sequence of NS4B of DENV-1, DENV-2, DENV-3, and DENV-4. The conserved BC-box motif is shown in blue.

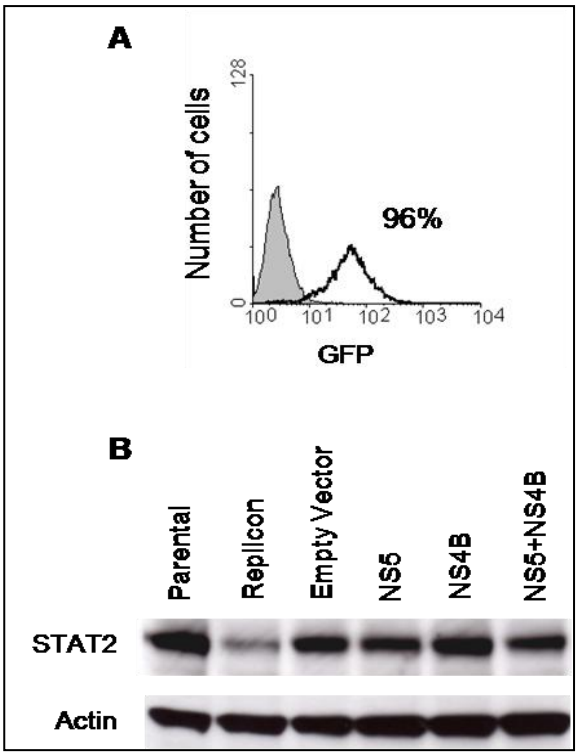


Figure 5.6|
STAT2 is not degraded in cells tranduced with DENV NS4B+NS5.
A. Transduction efficiency of the lentivirus NS4B. K562 cells were transduced at MOI 10. GFP expression (x axes) in transduced cells (black line) was measured by flow cytometry 72 hours after transduction. Untransduced cells (plain grey histogram) were employed as negative control. The percentage of GFP positive cells is calculated by comparison with the negative control. **B.** Comparison of STAT2 levels in K562 cells expressing DENV non-structural protein cassettes. Equal numbers of cells stably transduced with lentiviral vectors as indicated were lysed and proteins separated by SDS-PAGE. Cellular levels of STAT2 were analysed by immunoblotting. Parental K562 cells, K562 cells containing DENV replicons, and K562 transduced with an empty lentiviral vector, NS5 and NS4B were included as controls.

5.3.3.3 Combination of NS5 with other DENV NS proteins is not sufficient to induce STAT2 degradation

All DENV NS proteins have been suggested to take part in viral replication and to localise in close proximity to subcellular membranes (chapter 1, section 1.2.3.4). Therefore, each one of them could potentially interact or be in close proximity to NS5, to take part in STAT2 degradation. In order to test this possibility, we transduced NS5 expressing K562 cells with different lentiviral vectors, each one encoding a different NS protein cassette. Moreover, since the cytoplasmic form of NS5 has been shown to directly interact with NS3 (chapter 1, sections 1.2.3.4, 1.2.4.2, and 1.2.4.5), and in turn NS3 has been shown to interact with NS4B (chapter 1, section 1.2.4.4), we also super-transduced K562_NS5+NS4B cells (section 5.3.3.2) with the lentivirus encoding NS2B/NS3. All combinations tested are summarised in Table 5.1. Next, we tested cellular levels of STAT2 by immunoblotting in all cell lines. As above, equal numbers of cells were lysed, proteins separated by SDS-PAGE, and immunoblotted with an antibody specific for STAT2. Once again, no reduction of STAT2 levels could be observed for any of the combination tested (data not shown).

Two proteins	NS5+ NS1/NS2A	NS5+ NS2B/NS3	NS5+ NS4A	NS5+ NS4B
Three proteins	NS5+ NS4B+ NS2B/NS3			

Table 5.1|
Combinations of NS proteins tested in order to study STAT2 degradation. Efficiency of transduction was tested by transducing parental K562 in parallel and measuring the percentage of cells expressing GFP. In all cases, efficiency of transduction was higher than 80% (data not shown).

5.4 DISCUSSION

As covered in chapter 1, evasion of the innate immunity and in particular of the type I IFN is critical for the establishment of any viral infection. DENV replication is extremely sensitive to type I IFN, as IFN treatment before infection prevents viral replication (Diamond and Harris 2001). Under selective pressure from the innate immunity, DENV has evolved mechanisms to evade the IFN response. In chapter 4 we showed that DENV NS5 is a potent and specific IFN-α/β antagonist; in this chapter we elucidated the mechanism at the basis of such inhibition.

We first focussed on the step of the signalling cascade antagonised by NS5. After stimulation with IFN-α, we observed that, in the presence of NS5, both STAT1 and STAT2 phosphorylation are inhibited. In contrast, neither surface expression of the IFN-α/β receptor IFNAR2c nor Tyk2 phosphorylation are affected by NS5. This suggests that NS5 antagonises the IFN-α signalling pathway downstream of Tyk2 phosphorylation and upstream of STAT2 phosphorylation. The same phenotype is observed in replicon containing cells, but, differently from cells expressing NS5 alone, only in the presence of the replicon, i.e. all the NS proteins expressed together, we observed reduced levels of STAT2. We therefore hypothesised that NS5 blocked STAT2 phosphorylation by direct or indirect binding to the transcription factor, preventing its interaction with the kinase Tyk2. We also speculated that when other NS proteins are expressed, as in replicon containing cells, NS5 might immobilise STAT2 and other NS proteins concur to its degradation. In order to test whether NS5 binds STAT2, we generated a K562 cell line expressing a FLAG-tagged NS5, and

immunoprecipitated NS5-FLAG. By immunoblot we showed that STAT2 co-immunoprecipitates with NS5. The binding STAT2-NS5 is highly specific since neither STAT1 nor Tyk2 co-immunoprecipitate with NS5.

The possibility that STAT2 degradation might be induced by a combination of NS proteins was strengthened by the identification in DENV NS4B of a short consensus sequence (BC-box), which is known to be involved in the recruitment of the ubiquitination complex. The presence and function of a BC-box motif in DENV NS4B had never been described before and its capability of binding Elongin B/C has not been proved. However, when we tested whether the co-expression of NS4B and NS5 induced STAT2 degradation, we did not detect any reduction in cellular STAT2 levels. No degradation was observed even after super-transduction with the lentivector containing NS2B/NS3, which has been shown to interact with both NS5 and NS4B during viral RNA replication. Combinations of NS5 with the other NS proteins were also tested, but none had any effect on STAT2 levels. Even though it is possible that more than two NS proteins are required to induce STAT2 degradation, it is likely that simple co-expression of individual proteins is not sufficient to reproduce this phenomenon. However, the approach that we employed has several limitations and therefore, any observation is only speculative. Once again, as mentioned in chapter 4, we could not test the expression of each individual NS protein, and we do not have information about relative expression levels of each NS protein in cells already expressing NS5. Furthermore, it is possible that correct protein conformation, localisation, or interaction is obtained only following proteolytic processing of the polyprotein. While this work was in progress, a study by Ashour and colleagues (Ashour *et al.* 2009) also reported that DENV NS5 is a type I IFN antagonist.

Consistent with our observations, this work showed that the expression of NS5 alone is not sufficient to induce degradation of STAT2. Furthermore, STAT2 reduction is not observed even when NS5 is transfected into cells already expressing the polyprotein NS1-NS4B. In fact, according to Ashour and colleagues, NS5 is indeed sufficient to induce STAT2 degradation, but only if the N-terminal cleavage of NS5 is reproduced, mimicking the maturation process in the context of the DENV polyprotein. It is possible that the initial cleavage of NS5 is critical to correctly fold the N-terminal domain of NS, and this in turn might be critical for the recruitment of other cellular or viral proteins involved in STAT2 degradation.

Further experiments would be required to understand the role of the N-terminal domain of NS5 in STAT2 degradation, and the role of STAT2 degradation in DENV infection, but this was beyond the objectives of this thesis. We decided instead to further characterise the region of NS5 involved in STAT2 binding, and the role of NS5 nuclear localisation in the inhibition of the type I IFN. This will be the subject of the next chapter.

5.5 CONCLUSIONS

In this chapter we identified the mechanism by which NS5 inhibits cellular response to type I IFN. We showed that NS5 binds STAT2 and prevents its phosphorylation, and therefore any downstream event of the transduction signalling cascade. Differently from the replicon, which induces proteasome-mediated degradation of STAT2, NS5 does not affect cellular levels of any component of the signalling cascade. We hypothesised that in the presence of the

replicon other viral component, together with NS5, might be responsible for STAT2 degradation; however co-transduction with NS5 and other individual DENV NS proteins was not sufficient to reproduce this phenomenon. This suggests that NS protein expression in the context of the polyprotein might be critical to confer to the NS proteins the correct conformation, localisation or regulation.

Chapter 6.

IDENTIFICATION OF THE REGION WITHIN NS5 REQUIRED FOR TYPE I IFN ANTAGONISM

6.1 INTRODUCTION

In the previous chapters, we have demonstrated that DENV NS5 binds to STAT2 and inhibits its phosphorylation, thereby inhibiting downstream events in the type I IFN signalling cascade. As outlined in chapter 1, section 1.2.4.5, DENV-2 NS5 is a complex multifunctional protein of 900 amino acids (104 kDa), composed of a methyltransferase domain (MTase) and an RNA-dependent RNA polymerase (RdRp). At the N-terminal of the RdRp domain there is a region containing a bipartite nuclear localisation signal (NLS) and a nuclear export signal (NES). Both the MTase and the RdRp domains have been crystallised and the structures provide useful insights on their molecular functions.

The MTase domain (Figure 6.1) is composed of three subdomains: the core (amino acids 55-222, yellow in the figure) is the proper catalytic domain, while the N-terminal "appendage" (amino acids 7-54, red in the figure) provides a GTP-binding site, and the C-terminal "appendage" (amino acids 223-267, cyan in the figure), packed against the N-terminal one, works as stabiliser (Egloff *et al.* 2002).

The RdRp has the same structure of many other viral polymerases, with the typical pattern fingers, palm, and thumb (Figure 6.2). The finger subdomains (amino acids 273-315; 416-496; 543-600, blue in the figure) are very mobile regions; the palm (amino acids 497-542 and 601-705, green in the figure) contains the catalytic active site and is highly conserved amongst flaviviruses; and the thumb (amino acids 706-900, red in the figure) is the most structurally variable (Yap *et al.* 2007). The NLS region (amino acids 316-404) has been crystallised in complex with the RdRp, as it seems to be crucial in stabilising the RdRp. It localises between the fingers and the palm (Figure 6.2, in yellow), probably

modulating the open-close conformations of the enzyme (Yap *et al.* 2007).

Defining the region/s of NS5 that bind/s STAT2 and prevent/s its phosphorylation would add to our understanding of the role of NS5 in the evasion of the innate immunity, and might in future help the design of antiviral drugs able to prevent NS5 binding to its substrate, or of rationally designed live-attenuated vaccines unable to antagonise the IFN signalling.

6.2 OBJECTIVES

- To identify which domain -the MTase or the RdRp- of NS5 is responsible for IFN-α inhibition
- to investigate whether the region encompassing the NLS of NS5 has a role in IFN-α inhibition
- to test whether NS5 nuclear trafficking has a role in IFN-α inhibition.

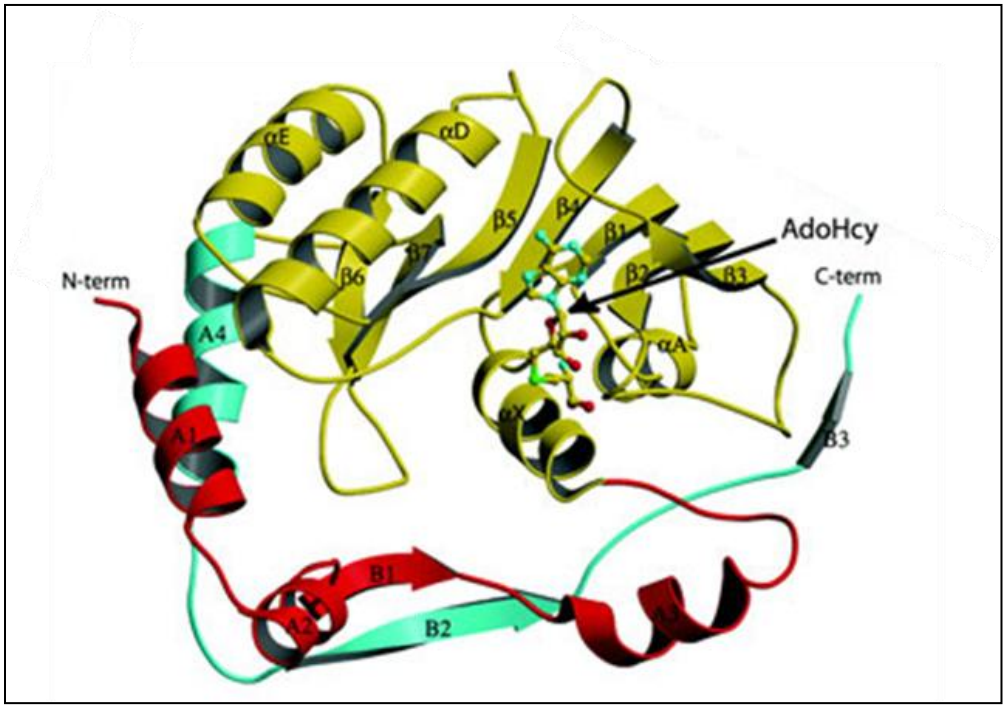


Figure 6.1|

Ribbon representation of DENV-2 NS5 MTase domain. The N-terminal domain (amino acids 7-54) is shown in red, the core domain (amino acids 55-222) in yellow, and the C-terminal subdomain (amino acids 223-267) in cyan. The structure is shown in complex with an S-Adenosylhomocystein (AdoHcy, sticks and balls representation), a co-product of the methyltransferase reaction (from Egloff *et al.* 2002).

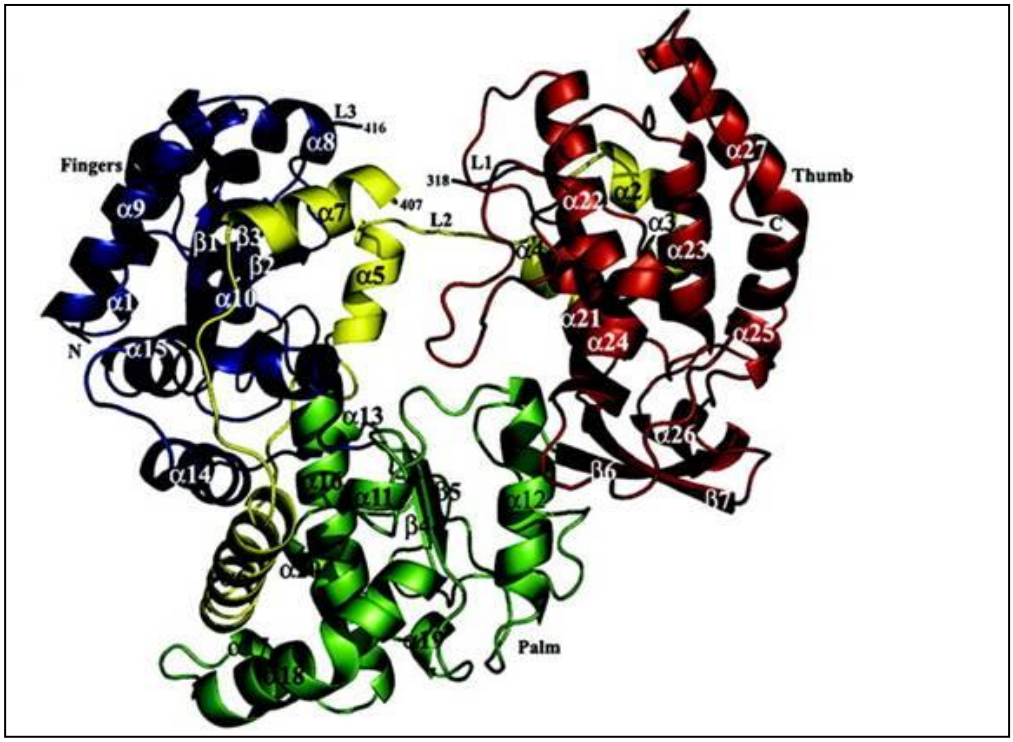


Figure 6.2|
Ribbon representation of DENV-3 NS5 RdRp (amino acids 273-900). The finger domains are shown in blue, the NLS in yellow, the palm in green, and the thumb in red (Yap *et al.* 2007)

6.3 RESULTS

6.3.1 Expression of the DENV NS5 RdRp domain alone is sufficient to inhibit the type I IFN response

6.3.1.1 Cloning strategy

In order to identify the domain of NS5 responsible for the inhibition of type I IFN, we separately cloned the MTase domain and the RdRp domain into the lentiviral vector described in chapter 3. Based on the published crystal structure (Malet *et al.* 2007), we aimed to expressed the MTase domain from amino acid 1 to 260 ($MTase_{1-260}$), and the RdRp domain from amino acid 270 to 900 ($RdRp_{270-900}$). A schematic representation of both constructs is shown in Figure 6.3 B. In brief, both domains were amplified by High-Fidelity PCR from the lentiviral vector containing the NS5 gene, and the restrictions sites for BamHI and NotI were introduced in 5` and 3` ends respectively. The sequences were first subcloned into the vector pGEM®-T Easy, then digested by BamHI and NotI restriction enzymes, and finally cloned into the lentiviral vector as described in chapter 2. Correct sequences and insertions were checked by double digestion (BamHI-NotI) and by DNA sequencing.

6.3.1.2 Development of K562 cell lines expressing the $MTase_{1-260}$ domain and the $RdRP_{223-900}$ domain

We used the constructs above to generate lentiviruses as described previously. This was successful for the construct $MTase_{1-260}$, but despite several attempts we were unable to recover lentiviruses encoding $RdRp_{270-900}$. The explanation for this was unclear, but was not investigated further. Instead, we attempted to generate

lentiviruses from a larger constructs ($RdRp_{223-900}$), which was chosen because it encompasses the entire polymerase domain (Figure 6.3 C). We successfully recovered lentiviruses encoding $RdRp_{223-900}$. Therefore, lentiviruses encoding $MTase_{1-260}$ and $RdRp_{223-900}$ were used to transduce K562 cells as previously described. We used GFP expression as a marker of successful transduction, but protein expression could be assessed directly only for the RdRp domain, since the anti-NS5 polyclonal antibody available to us only recognises the region between amino acids 397 and 778 (A. Davidson, personal communication). Detection of a band at the expected size (78 kDa) suggested that the $RdRp_{223-900}$ is correctly expressed (Figure 6.4, A).

6.3.1.3 The $RdRp_{223-900}$ is sufficient to inhibit cellular response to IFN-α

Cells expressing either $MTase_{1-260}$, or $RdRp_{223-900}$ were treated with 100 IU/ml of IFN-α for 24 hours and cell surface expression of the IFN-inducible molecules HLA-ABC was measured by flow cytometry, gating only on GFP positive cells (Figure 6.4, B). Cells expressing full-length NS5 and cells transduced with empty lentiviral vector were included as controls. As expected (Figure 6.4), in response to IFN-α, cells expressing full length NS5 but not cells transduced with empty vector, failed to up-regulate HLA-ABC molecules. In cells expressing $RdRp_{223-900}$ up-regulation of HLA-ABC was also reduced, although this effect appeared to be slightly less than in cells expressing full length NS5. In contrast, we did not observe reduced HLA-ABC expression in cells transduced with $MTase_{1-260}$. This implies that the RdRp domain of NS5 is sufficient to antagonise the type I IFN response, but maybe less efficiently than the full length NS5 protein.

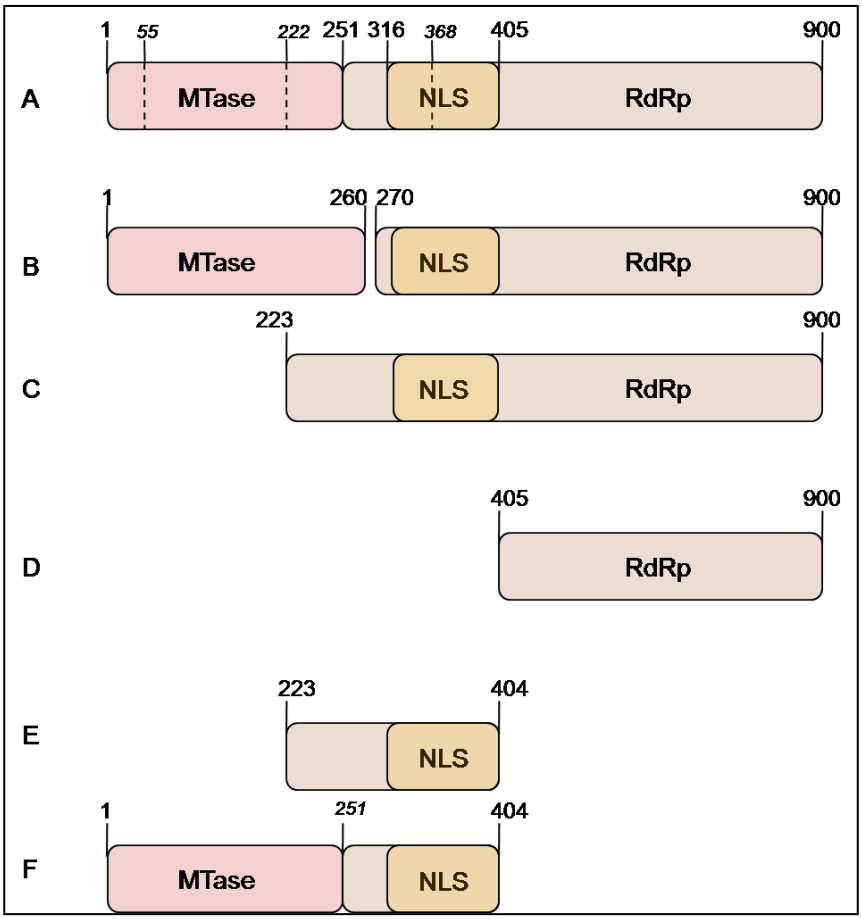


Figure 6.3|
Schematic representation of DENV NS5 sequence and of the truncated versions generated in this study. A. Full length NS5 proteins and its sub-domains: the MTase (amino acids 1-250), the RdRp (amino acids 251-900), and the NLS (amino acids 316-404). Dashed lines separate the N-terminal domain (amino acids 1-54), the core domain (amino acids 55-222), and the C-terminal domain (amino acids 223-250) of the MTase, and the β-NLS (amino acids 316-368) and the α/β-NLS (amino acids 369-404) of the NLS. **B and C.** Cloning strategy to individually express the MTase and the RdRp domains. The MTase was expressed from amino acids 1 to 260, while the RdRp was initially expressed from amino acids 270 to 900 **(B)**. Technical problems in lentivirus production led to the extension of the polymerase domain from amino acids 223 to 900 **(C)**. **D**. Deletion of the region 223-404, including the NLS, from the RdRp domain. **E and F**. Expression of the NLS region alone (amino acids 223-404, **E**) and at the C-terminal of the MTase domain (amino acids 1-404, **F**).

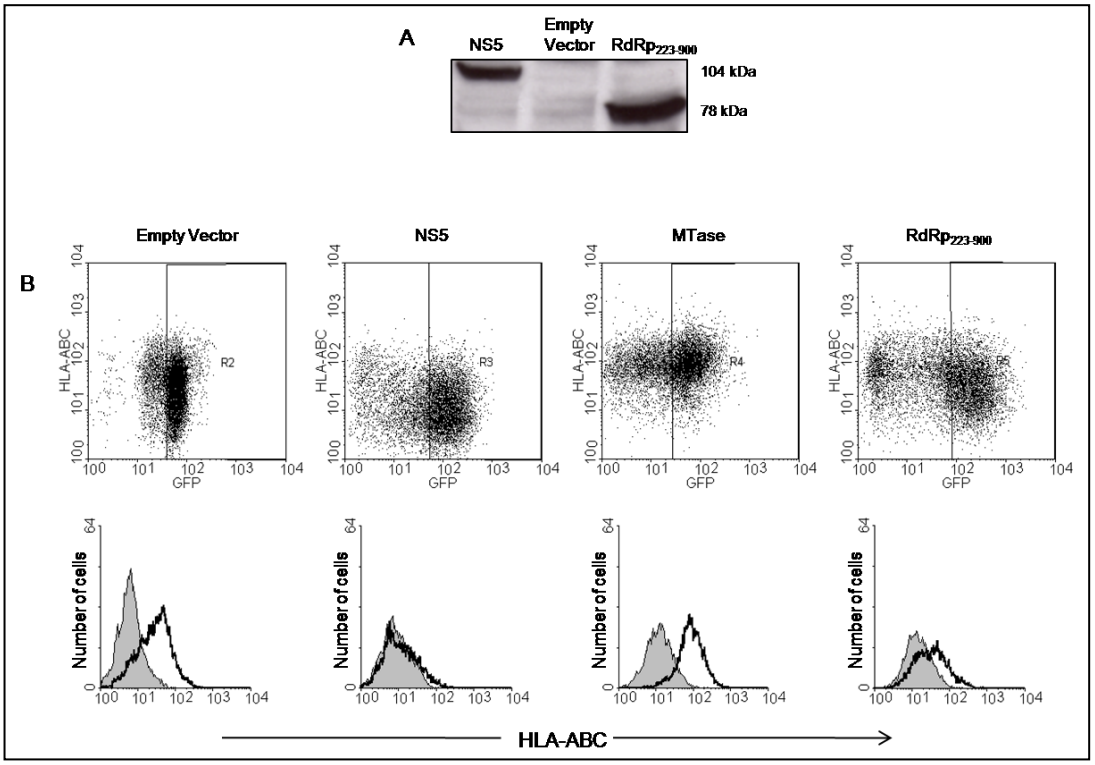


Figure 6.4|

IFN-α antagonism is mediated by the polymerase domain of NS5.

K562 cells were transduced with individual functional domains of NS5: the MTase (Methyltransferase 1-260) or the $RdRp_{223-900}$ (RNA dependent RNA polymerase 223-900). **A.** Expression of NS5 and of the RdRp domain was tested by immunoblotting. Cells were lysed and proteins separated by SDS-PAGE; immunoblotting was performed using an anti-NS5 antibody recognising the region 397-778. No antibodies specific for the MTase domain were available. Cells transduced with the empty vector were included as a control. **B.** Response to IFN-α was tested treating the cells for 24 hours with (open histogram and dot plots) or without (grey histogram) 100 IU/ml of IFN-α; cell surface expression of the IFN-α inducible molecules HLA-ABC was measured by flow cytometry, gating on GFP positive cells only. Dot plots show expression of HLA-ABC (y axis) after IFN-α treatment in the GFP positive fraction (right gate, x axis) and in the GFP negative fraction (left gate, x axis) for each transduced sample. Cells transduced with the empty vector and with full length NS5 were included as controls.

6.3.2 Deletion of the region containing the NLS abolishes the IFN antagonism

6.3.2.1 Cloning strategy and development of K562 cell lines expressing the $RdRp_{405-900}$ domain

In the previous experiment, we showed that expression of amino acids 223-900 of DENV NS5 is sufficient to inhibit type I IFN-mediated up-regulation of HLA-ABC. Residues 223-260 overlap the MTase construct previously tested, while residues 251-404 constitute the first finger of the polymerase domain and the NLS. The region from amino acid 223 to 404 therefore encompasses parts of both the MTase and of the RdRp domains, but in view of its location and composition the fragment 223-404 will subsequently be referred to as the "*inter-domain*". In order to test whether the "inter-domain" is absolutely required for IFN-α antagonism, we expressed the polymerase domain from amino acid 405 to 900 (Figure 6.3, D). As previously described, the required sequence was amplified by PCR from the NS5 gene, and the restriction sites for BamHI and NotI were introduced in 5` and 3` ends respectively. The sequence was subcloned into the vector pGEM®-T Easy, and finally cloned into the lentiviral vector, as described in chapter 2. Correct sequence and insertion were checked by double digestion (BamHI-NotI) and by DNA sequencing. The construct was successfully used to generate lentiviruses and transduce K562 cells, as described previously. Protein expression was assessed by immunoblotting: the presence of a band of the expected size (57 kDa) suggests that the protein is expressed (Figure 6.5, A).

6.3.2.2 The region 223-404 is critical to type I IFN inhibition

Cells expressing $RdRp_{405-900}$ were stimulated with 100 IU/ml of IFN-α for 24

hours, and expression of the IFN-inducible molecules HLA-ABC on cell surface was measured by flow cytometry, gating only on GFP positive (transduced) cells (Figure 6.5, B). Cells expressing full length NS5 and cells transduced with the empty lentiviral vector were included as controls. Once again, differently from cells transduced with empty vector, cells expressing full-length NS5 did not up-regulate HLA-ABC in response to IFN-α (Figure 6.5). In contrast, we did not observed reduced up-regulation of HLA-ABC in cells expressing $RdRp_{405-900.}$ This suggests that at least part of the "inter-domain" 223-404 must be expressed to retain the ability of the RdRp of antagonising the IFN response.

6.3.3 IFN inhibition occurs only when the "inter-domain" 223-404 is expressed in the context of the RdRp

6.3.3.1 Cloning strategy and development of K562 cell lines expressing the "inter-domain" 223-404 alone or at the C-terminal of the MTase domain

We next investigated whether the "inter-domain" 223-404 alone could inhibit the IFN response, or whether it could function as an IFN antagonist other than in association with the RdRp. We therefore cloned the "inter-domain" either alone (amino acids 223-404), or at the C-terminal of the MTase (amino acids 1-404) in the lentiviral vector. A schematic representation of the two constructs is shown in Figure 6.3 E and F. Cloning and transduction of K562 cells were carried out as described above. Although lentivirus transduction of cells could be assessed by GFP expression, expression of the relevant fragments of NS5 could not be tested directly because no antibodies that recognise these portions were available.

6.3.3.2 The "inter-domain" 223-404 alone or at the C-terminal of the MTase does not inhibit IFN-α response

Cells transduced with lentiviruses encoding either the "inter-domain" (amino acids 223-404) or the extended $MTase_{1-404}$ were stimulated with 100 IU/ml of IFN-α for 24 hours. Expression of HLA-ABC molecules on cell surface was then assessed by flow cytometry, gating only on GFP positive cells (Figure 6.6, A). Cells expressing full length NS5 and cells transduced with empty lentiviral vector were included as controls. As shown in Figure 6.6 B, only cells expressing full-length NS5 fail to up-regulate HLA-ABC in response to IFN-α; in contrast, in both cells transduced with the "inter-domain" and cells transduced with the extended $MTase_{1-404}$, up-regulation of HLA-ABC after IFN-α treatment is comparable to cells transduced with empty vector. Taken together these observations might suggest that the "inter-domain" (223-404) and the RdRp have to be expressed together in order to antagonise the IFN response. However, we cannot exclude the possibility that the "inter-domain" is simply not expressed, or does not adopt the correct conformation when expressed in a context different from the RdRp.

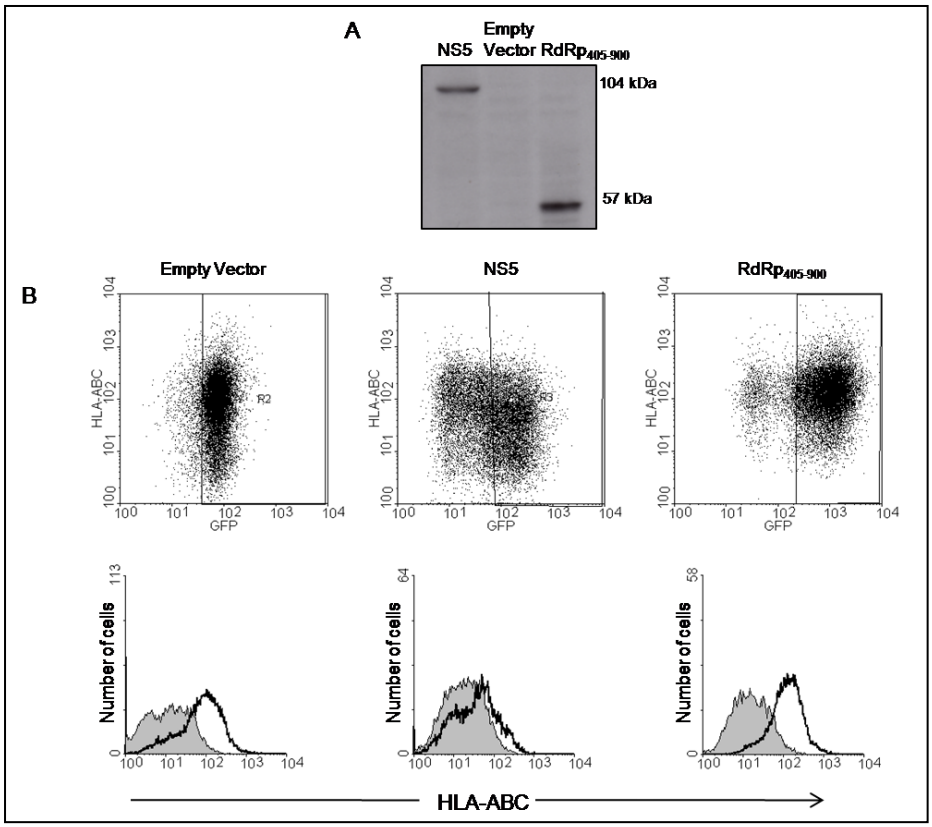


Figure 6.5|

Deletion of the region 223-404 of NS5 RdRp restores cellular response to IFN-α.

K562 cells were transduced with a truncated form of NS5 RdRp spanning from amino acid 405 to 900. **A.** Expression of NS5 and of the $RdRP_{405-900}$ domain was confirmed by immunoblotting. Cells were lysed and proteins separated by SDS-PAGE; immunoblotting was performed using an anti-NS5 antibody recognising the region 397-778. Cells transduced with the empty vector were included as a control. **B.** Cells were treated for 24 hours with (open histogram and dot plots) or without (grey histogram) 100 IU/ml of IFN-α and cell surface expression of the IFN-α inducible antigen HLA-ABC was measured by flow cytometry, gating on GFP positive cells only. Dot plots show up-regulation of HLA-ABC (y axis) after IFN-α treatment in the GFP positive fraction (right gate, x axis) and in the GFP negative fraction (left gate, x axis) for each transduced sample. Cells transduced with the empty vector and with full length NS5 were included as controls.

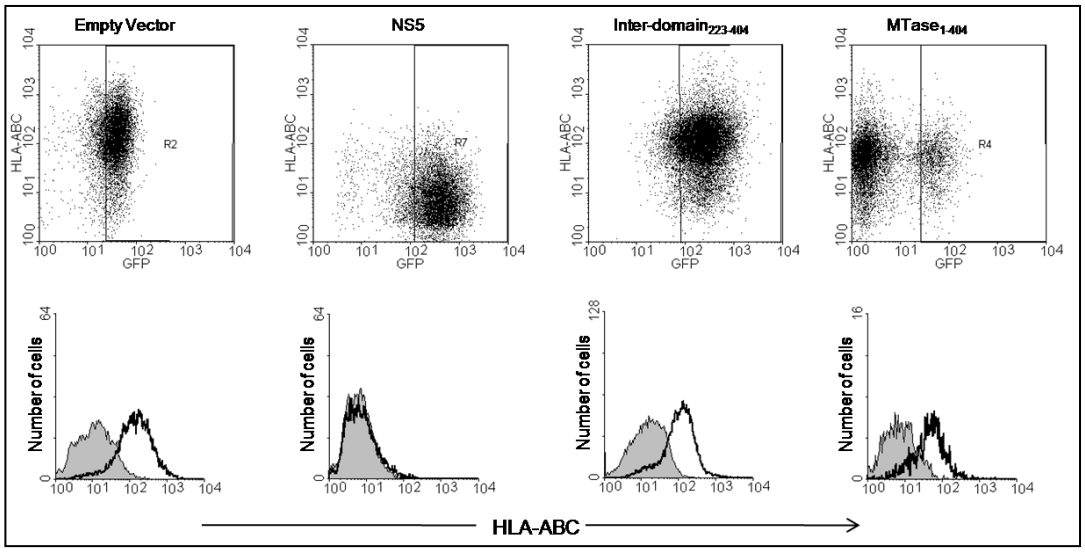


Figure 6.6|

Transduction of K562 cells with the region 223-404 alone or in association with NS5 MTase domain does not inhibit IFN-α response.

K562 cells were transduced with the "inter-domain" of NS5 alone ("inter-domain"$_{223-404}$) or at the C terminal of the MTase domain (MTase $_{1-404}$). Cells were treated for 24 hours with (open histogram and dot plots) or without (grey histogram) 100 IU/ml of IFN-α and cell surface expression of the IFN-α inducible molecules HLA-ABC was measured by flow cytometry, gating on GFP positive cells only. Dot plots show up-regulation of HLA-ABC (y axis) after IFN-α treatment in the GFP positive fraction (right gate, x axis) and in the GFP negative fraction (left gate, x axis) for each transduced sample. Cells transduced with the empty vector and with full length NS5 were included as controls.

6.3.4 Nuclear translocation of NS5 is not required for the inhibition of type I IFN signalling

6.3.4.1 Development of a K562 cell line expressing a mutant of NS5 that does not translocate into the nucleus

Since the "inter-domain" incorporates the NLS, we next considered whether the loss of IFN-α antagonism of NS5 RdRp after deletion of the "inter-domain" was due to the absence of nuclear translocation. In order to test this, we employed a mutant of NS5 (NS5_A1A2) in which five lysine residues (K371/K372 and K387/K388/K389) in the distal cluster of basic residues of the α-NLS, were replaced by alanine, preventing nuclear translocation (Pryor *et al.* 2007). Such a minor alteration selectively prevents nuclear translocation but does not disrupt the conformation of the NLS and does not alter any residues in the β-NLS, which has been shown to be critical for the interaction with the viral protease NS3 and for virus survival (Pryor *et al.* 2007).

The gene encoding NS5_A1A2 was amplified by high-fidelity PCR from the vector pcDNA-3.1_A1A2 (kindly provided by Dr Andrew Davidson) and cloned into the lentiviral vector as described previously. Correct sequence and insertion were checked by double digestion (BamHI-NotI) and by DNA sequencing.

The construct was then used to generate lentiviruses and transduce K562 cells as before. Published data show that NS5_A1A2 does not translocate into the nucleus (Pryor *et al.* 2007), but despite several attempts at optimisation, it was not possible to demonstrate this conclusively by immunofluorescence due to insufficient specificity of the available polyclonal anti-NS5 antibody in K562 and

to the particular cell morphology (K562 cells have a very large nucleus in relation to cytoplasm).

6.3.4.2 NS5 inhibition of cellular response to IFN-α is preserved in the absence of nuclear translocation

In order to examine whether nuclear translocation of NS5 is important for inhibition of type I IFN signalling, we treated K562 cells expressing NS5_A1A2 with and without 100 IU/ml of IFN-α for 24 hours and then measured expression of the IFN-inducible molecules HLA-ABC on cell surface by flow cytometry, gating only on GFP positive cells (Figure 6.7, A). Cells expressing wild type NS5 and cells transduced with empty lentiviral vector were included as controls. As in all previous experiments, we observed reduced up-regulation of HLA-ABC in cells expressing NS5, but not in cells transduced with empty vector (Figure 6.7, B). We also observed reduced up-regulation of HLA-ABC in cells expressing NS5_A1A2. This suggests that NS5 preserves its ability to inhibit the IFN response even in the absence of nuclear translocation, and that the importance of the region containing the NLS previously described (Figure 6.5) does not depend on its ability to direct NS5 into the nucleus.

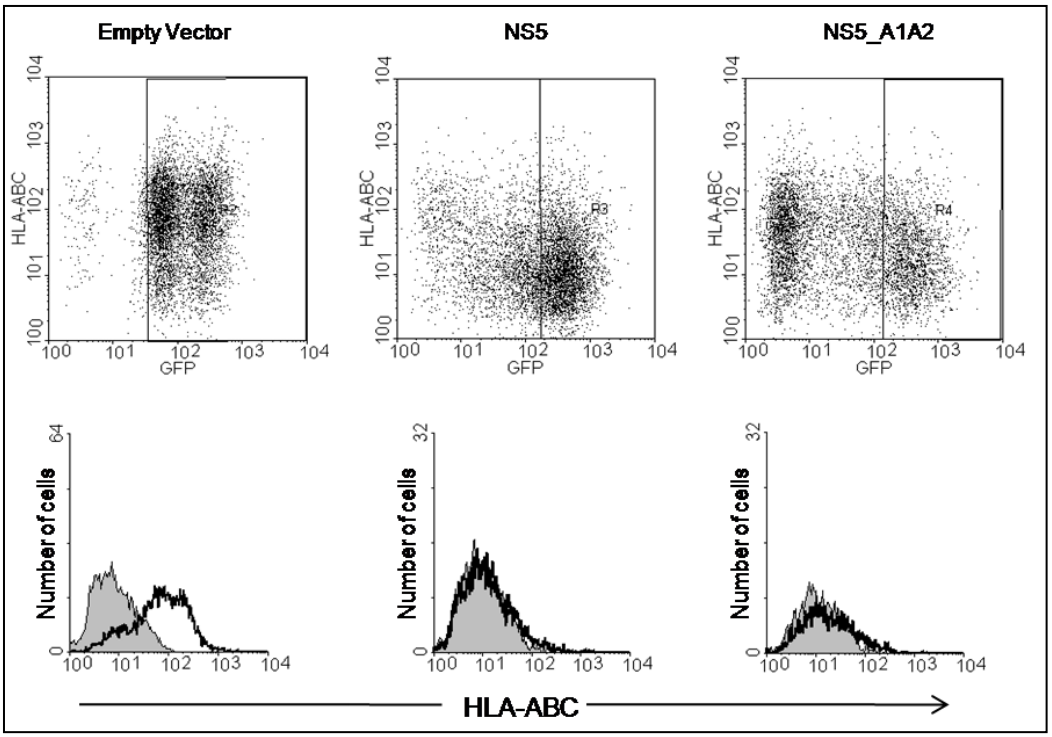


Figure 6.7|

IFN-α antagonism does not require NS5 nuclear translocation.

K562 cells were transduced with a mutant of NS5 unable to translocate into the nucleus (NS5_A1A2). Cells were treated for 24 hours with (open histogram and dot plots) or without (grey histogram) 100 IU/ml of IFN-α and cell surface expression of the IFN-α inducible molecules HLA-ABC was measured by flow cytometry, gating on GFP positive cells only. Dot plots show up-regulation of HLA-ABC (y axis) after IFN-α treatment in the GFP positive fraction (right gate, x axis) and in the GFP negative fraction (left gate, x axis) for each transduced sample. Cells transduced with the empty vector and with full length NS5 were included as controls.

6.4 DISCUSSION

Identifying the region of DENV NS5 that acts as an IFN antagonist is critical for complete understanding of the mechanism of inhibition. In this chapter we first aimed to identify which domain of NS5 -MTase or RdRp- is responsible for the inhibition of type I IFN signal transduction. For this purpose, we separately expressed the MTAse domain (amino acids 1-260) and the RdRp domain (amino acids 223-900) in K562 cells and tested cellular response to IFN-α. The exact N-terminal start of the RdRp domain is debated and still not clearly identified. Even though the sequence deposited in NCBI (accession number NP_739590.2) defines the RdRp of DENV-2 NS5 to start at amino acid 251, the crystal structure of DENV-3 NS5 could be obtained only by expressing residues 273-900 (Yap *et al.* 2007), and Malet and colleagues (Malet *et al.* 2007) suggest that in WNV the region 260-270 represents a linker domain between the MTase and the RdRp. According to these works, we initially decided to express the RpRp from residue 270, but for reasons that we did not further investigate, we could not rescue lentiviruses for this construct. We therefore decided to employ a different construct starting from residue 223. Although it overlaps the C-terminus of the MTase (amino acids 223-260), this construct was chosen because it includes the entire polymerase domain and because we were able to rescue sufficient titres of lentivirus.

After stimulation with IFN-α, we showed that only the RdRp when expressed alone retains the capability of inhibiting cellular response to type I IFN. We also observed that this inhibitory effect is slightly less pronounced than in cells

expressing full-length NS5: it is possible that different expression levels are responsible for this difference, or that the presence of the MTase domain is important to maintain the correct protein conformation required for optimal IFN-antagonism. Alternatively, as suggested for TBE virus (Werme *et al.* 2008), the MTase domain might allow the correct localisation of the polymerase on cell membrane in proximity of the IFN receptors, helping the interaction of NS5 with early components of the signalling cascade.

As discussed in chapter 4, a role for NS5 in IFN inhibition has been shown for other flaviviruses. Interestingly, the IFN inhibitory activity of NS5 of LGTV has also been mapped in the RdRp domain (Park *et al.* 2007). According to this work, the region involved in IFN antagonism in LGTV NS5 maps between residues 355 and 735. Site directed mutagenesis to alanine in the region between amino acids 342 and 735 suggests the involvement of two sequences in IFN inhibition: between amino acids 374-380 (375-381 in DENV-2) and 624-637 (622-636 in DENV-2) (Park *et al.* 2007). Even though alanine scanning mutagenesis does not distinguish whether a specific residue is critical because of its specific function or because of conformational issues, this work provides a first indication about the regions within the RdRp that are important in preserving the IFN inhibitory activity.

As described in chapter 1 (section 1.2.4.5), the RdRp includes a NLS and a NES. This region appeared particularly intriguing to us because NLS/NESs have been identified in some other viral proteins involved in IFN inhibition. Ebola virus VP24 protein interacts with karyopherin-α 1, 5, and 6, which are the same

NLS receptors for phosphorylated STAT1: such interaction prevents STAT1 translocation to the nucleus and therefore the activation of the IFN response (Reid *et al.* 2007). Even though a specific interaction between the NLS and STAT1 has not been shown, the P protein of Rabies virus, containing both a NLS and a NES, binds phosphorylated-STAT1, and retains it either in the nucleus or in the cytoplasm, preventing DNA binding (Vidy *et al.* 2007). Nsp2 of Semliki forest virus also inhibits IFN and a correlation with Nsp2 nuclear translocation has been shown (Breakwell *et al.* 2007). Finally, a direct correlation between the NES and STAT binding/retention has been identified for Nipah and Hendra viruses (paramyxoviruses): the V protein has been shown to bind both STAT1 and STAT2 through specific regions mapping inside the NES (Rodriguez *et al.* 2004). NLSs have also been identified in the suppressor of cytokine signalling SOCS1. SOCS 1 downregulates Jak-STAT signalling pathway in several ways: by proteasomal degradation of Jak, by competition with STAT for receptor domains, or by binding Jak2. SOCS 1 translocates to the nucleus through a bipartite NLS localising between the SH2 domain and the SOCS-box motif; full inhibition of IFN signalling is achieved in the presence of functional NLS and SOCS-box (Koelsche *et al.* 2009). All these examples suggest some correlation between NLS/NESs and proteins involved in the control of the IFN system, but whether NLS/NESs are only responsible for protein localisation or are directly involved in other inhibitory mechanisms is not clear.

Unfortunately, since the NLS does not localise at the beginning of the RdRp but only after the first finger, and since we were not able to express the construct 270-900, we could not test the specific role of the NLS alone (amino acids 316-404) in IFN inhibition, but we examined the entire region 223 to 404, which encompasses

also part of the MTase domain and the first finger of the RdRp domain (amino acids 273-315). According to Yap and colleagues (Yap *et al.* 2007), the first finger of the RdRp is composed of an α-helix, a β-sheet, and a segment of 35 amino acids which connects the finger with the first helix of the NLS, deeply buried in the thumb domain (see Figure 6.2). Residues 309-321 and 342-347 in the NLS form two flexible loops that link the fingers and the thumb and probably transmit conformational changes between the two domains and/or restrict their mobility. Interestingly, a similar structure has been identified in WNV RdRp, but WNV NS5, which has been shown to inhibit type I IFN response (Laurent-Rolle *et al.* 2010), has never been found in the nucleus (Malet *et al.* 2007); this might suggest an important role for the NLS region, beyond it nuclear translocation function.

In order to test whether this N-terminal region was involved in IFN inhibition, we expressed a truncated portion of the RdRp, starting from amino acid 405, and we showed that in the absence of the region 223-404 the RdRp domain loses its ability to inhibit IFN-α. This observation is difficult to interpret, since loss of IFN antagonism does not necessarily imply that the "inter-domain" is responsible for STAT2 binding; in fact, the "inter-domain" might simply have a role in preserving the correct conformation of the region involved in IFN inhibition. Even though we could detect protein expression at the expected size by immunoblotting, we cannot exclude the possibility that truncation of such a large region (181 amino acids) might destabilise the structure or introduce misfolding. Moreover, since the NLS constitutes only part of the deleted portion, we cannot conclude that IFN antagonism specifically depends on the NLS.

We finally tested response to IFN-α in K562 cells transduced with lentiviruses carrying either the region 223-404 alone, or associated with the MTase domain. After IFN treatment we could not detect any inhibition of the IFN signalling. Taken together, this and our previous observations suggest that neither the "inter-domain" nor the RdRp are dispensable for IFN inhibition. This is in agreement with the work of Park and colleagues on LGTV mentioned above (Park *et al.* 2007), which suggests that most of the polymerase is required to preserve the IFN antagonism (amino acids 355-735). However, our inability to prove the expression of both constructs calls for caution in the interpretation of these observations, since the proteins might be not correctly expressed. Tagging the constructs would only partially solve the problem, since correct folding would still remain dubious. Even though we could have tried to identify the minimal RdRp portion required to preserve IFN antagonism by progressive truncations at the N-terminal from amino acid 223, addressing functional issue by protein truncation always raises uncertainties, since any minimal structural alteration can potentially generate dramatic changes in protein folding and stability. For these reasons, we have decided to investigate the interaction between DENV RdRp and STAT2 directly by protein crystallography and ongoing work is currently focusing on this target.

In the previous chapters we have demonstrated that NS5 inhibits the type I IFN signalling by interacting with cytoplasmic targets (STAT2). Therefore we hypothesised that loss of IFN inhibition after deletion of the "inter-domain" is related to the conformation of the site involved in IFN antagonism rather than to NS5 nuclear translocation. To investigate this hypothesis we tested the response to

IFN-α in cells expressing a mutated form of NS5, in which five alanine substitutions in the NLS prevent its translocation into the nucleus (Pryor *et al.* 2007). We showed that even in the absence of nuclear trafficking, NS5 inhibitory effect on the IFN signalling is preserved, supporting the observation that the inhibitory activity of NS5 occurs in the cytoplasm, and that, if the region encompassing the NLS has a role in IFN inhibition, such function does not depend on nuclear entry.

6.5 CONCLUSIONS

Taken together these observations suggest that the site responsible for the inhibition of type I IFN response/STAT2 binding maps within the RdRp domain and that inhibition of type I IFN mediated signal transduction occurs in the cytoplasm, in keeping with the cytoplasmic location of the putative target STAT2.

The region spanning from the first finger of the polymerase domain to the end of the NLS seems to be critical to IFN antagonism, either preserving protein conformation, or being part of the inhibitory site itself. Possible alteration of protein stability and conformation cannot be excluded when using extended protein truncations. For this reason, in ongoing work, we are attempting to co-crystallise the complex RdRp-STAT2.

Chapter 7.

FINAL DISCUSSION

This project starts from the hypothesis that type I IFN inhibition and STAT2 degradation in DENV infected cells are due to some DENV non-structural components. This hypothesis is supported by the fact that both phenomena can be observed also in human cells stably expressing the DENV replicon, i.e. a self-replicating DENV RNA genome in which the genes coding for the structural proteins C, prM, and E have been removed. The initial aim of this thesis was to identify the NS protein (or combination of proteins) involved in the antagonism of the IFN system.

In the first part of this work, by using a lentivirus vector system we developed five K562 cell lines, each one stably expressing a different DENV NS protein (NS4A, NS4B, and NS5) or combination of proteins (NS1/NS2A, and NS2B/NS3) *(Chapter 3)*. Next, we tested the effect of each individual NS protein on both STAT2 degradation and IFN-α inhibition. Surprisingly, none of the DENV NS proteins individually expressed is able to induce degradation of STAT2, but we showed that the expression of NS5 alone is sufficient to inhibit type I IFN mediated signal transduction. Furthermore, we showed that the inhibition is specific for the type I IFN and that the IFN-γ signalling is unaffected. Inhibition of the type I IFN response in the presence of NS5 is not a peculiarity of the particular viral strain in use (DENV-2_NGC), since we could observe the same antagonistic effect in the presence of NS5 from a different strain (DENV-2_TSV01) *(Chapter 4)*. We demonstrated that inhibition of the IFN signalling pathway by DENV NS5 occurs at the level of STAT2 phosphorylation. Taken together with earlier data using DENV replicons, this suggests that STAT2 is the target for DENV inhibition of the type I IFN response. We hypothesised that NS5 could specifically bind STAT2, and demonstrated this by co-immunoprecipitation

(Chapter 5). To explain STAT2 degradation in the presence of the DENV replicon, i.e. of all the NS proteins together, we also hypothesised that while NS5 binds STAT2, some other NS protein might be required to direct STAT2 to proteasome-mediated degradation. By analogy with an unrelated virus, RSV (Elliott *et al.* 2007), whose non-structural components bind STAT2 and recruit the ubiquitination complex required for STAT2 degradation, we identified a short consensus sequence in DENV NS4B: such a sequence, called BC-box, is known to be involved in the recruitment of the ubiquitination complex. However, co-transduction of NS5 and NS4B, as well as different combinations of NS5 with other NS proteins, failed to induce degradation of STAT2, leaving the question unanswered *(Chapter 5)*. The final part of this work was dedicated to a preliminary work aimed at better defining the interaction between NS5 and STAT2. By truncating different domains of NS5, we showed that the RdRp domain is sufficient to inhibit the IFN-α signalling, and does not require the expression of the MTase domain. The inhibitory effect is less then when the full-length protein is expressed, suggesting that the MTase is probably important in preserving the correct conformation of NS5. Finally, we showed that the nuclear translocation of NS5 is not involved in the inhibition of the IFN response, but the N-terminal region of the RdRp encompassing the NLS is critical to preserve IFN antagonism *(Chapter 6)*.

These findings have been discussed at the end of each chapter, together with the limitations of our approaches and the questions that will lead to future studies, extending beyond the aims of this thesis. Therefore, the objective of this final discussion is to place our findings in the broader context of the current knowledge of DENV pathogenesis, and to suggest possible practical applications and future

work.

STAT2 binding and degradation

As outlined in chapter 1, when this project started little information was available about DENV inhibition of the IFN system, and neither the mechanism nor the NS proteins involved were entirely clear. Our finding that DENV NS5 is a potent type I IFN antagonist had never been reported before. Even though a role in IFN inhibition for the NS5 of other flaviviruses such as YFV (Lin *et al.* 2006) and TBEV (Best *et al.* 2005; Park *et al.* 2007; Werme *et al.* 2008) has been suggested, the mechanism of inhibition was unknown. Our observation that NS5 binds STAT2 and prevents its activation was entirely new. While this work was in progress, a parallel study by Ashour and colleagues (Ashour *et al.* 2009) reached a very similar conclusion. This is the first time that the same mechanism of IFN antagonism by DENV has been described by different investigators, using different experimental techniques, cell lines and DENV strains. This suggests that taken together our studies have identified a biologically relevant mechanism.

The lack of an adequate animal model has been a great hindrance to the study of DENV pathogenesis. Mice are not susceptible to DENV infection and this has been correlated to their effective IFN response (reviewed in Williams *et al.* 2009). Due to the high divergency between human and murine STAT2 (Park *et al.* 1999), we suggest that STAT2 might act as a critical restriction factor against DENV infection in mouse. Should this be the case, we hypothesise that a STAT2 KO mouse could provide an invaluable tool for the study of DENV pathogenesis, or for testing vaccines and antivirals. Further studies investigating the IFN inhibitory

properties of NS5 in different species would be required and are likely to provide interesting insights on cross-species transmission.

Type I IFN inhibition and DENV pathogenesis

This thesis constitutes an important step forward in defining the molecular pathogenesis of dengue. As outlined in chapter 1, high viral titres are associated with DHF, and depend on viral factors, host factors, and host pre-existing immunity. However, high viral replication would not be possible in the presence of a functional IFN response. We suggest that, by allowing rapid viral replication in the first stages of infection, inhibition of the IFN response is a crucial element determining DENV pathogenesis. As mentioned in chapter 1, a correlation between IFN antagonism and pathogenicity has been shown for WNV, where strong IFN antagonism is associated to more severe outcomes (Keller *et al.* 2006; Tobler *et al.* 2008). Moreover, recent work has shown that NS5 from the virulent NY99 strain of WNV is an efficient IFN antagonist, while NS5 of Kunjin virus, a naturally attenuated subtype of WNV, is a poor suppressor of IFN (Laurent-Rolle *et al.* 2010). Further studies would be required to test whether a correlation between IFN inhibition and DHF exists, and whether differences in pathogenicity and disease outcome observed for different DENV strains can be correlated to differences in IFN antagonism.

Having access to blood samples from DF and DHF patients, it would be possible to measure both NS5 expression and STAT2 levels in infected cells, and compare the activation of the type I IFN signalling pathway in cells expressing different levels of NS5. These experiments could be done by flow cytometry, and NS5 intracellular staining would also allow to distinguish between infected and

bystander cells. Viral titration on the same samples would also allow to correlate viral replication with NS5 expression and with IFN inhibition/STAT2 degradation, and finally to link these variables with disease severity.

Future work

The results of this study suggest new interesting lines of research. Of particular relevance would be the characterisation of the binding between NS5 and STAT2, and in ongoing work we are investigating such interaction by protein crystallography. In this thesis we narrowed down the region of NS5 involved in the IFN inhibition and suggest that future studies of the NS5/STAT2 interaction should focus on the RdRp of NS5. This information is particularly important since previous attempts to crystallise full-length NS5 have been unsuccessful, whereas the crystal structure of the RdRp domain alone has been solved (Yap *et al.* 2007). Furthermore, we have shown that the presence of the NLS is important to preserve IFN antagonism. No X-ray structure is available for full-length STAT2 and only the C-terminal portion has been crystallised (Wojciak *et al.* 2009). This suggests that expressing portions rather than full-length STAT2 might be of help in protein expression and crystallisation. Considering the specificity of the binding between NS5 and STAT2 we speculate that the C-terminal region is more likely to be involved in the interaction with NS5, since it does not have similarity with other domains on STAT1. However at present no further assumption can be made and we cannot exclude that the interaction between NS5 and STAT2 might be mediated by other cellular proteins.

Current work is also trying to identify the molecular mechanism behind STAT2

degradation, by testing the hypothesis that correct processing of the N-terminal of NS5 might be critical to direct STAT2 to the proteasome, as suggested by Ashour and colleagues (Ashour *et al.* 2009, see chapter 5). As discussed in chapter 4 and 5, since IFN inhibition occurs also in the absence of STAT2 degradation, we suggest that STAT2 degradation might have an important biological significance in viral replication, either allowing recycling of NS5, or maybe directly playing a role in viral replication.

Further work investigating the interplay between DENV and other components of the innate immune response would also be of great interest. Our findings show that DENV-2 NS5 selectively inhibits cellular response to type I IFN but not to type II IFN. In recent years a new class of IFN-related cytokines has been discovered and called type III IFN (Kotenko et al, 2003; Sheppard et al, 2003). These cytokines do not bind to the type I IFN receptor, but they still activate STAT1 and STAT2 signalling pathway, resulting in the formation of the ISGF3 complex and the activation of an antiviral response (Kotenko *et al.* 2003; Donnelly *et al.* 2004). Since NS5 binds STAT2, a downstream signalling components shared by both type I and type III IFN, it is tempting to hypothesise that NS5 probably also blocks the activation of type III IFN antiviral pathway. However, as discussed in Chapter 1, the association of STAT2 to the IFNAR receptor is critical for the activation of type I IFN response, and STAT2 localisation might be important in determining whether NS5 will be able to bind the transcription factor. To investigate whether NS5 is able to inhibit cellular response to type III IFN would therefore would add important information about DENV evasion of the innate immunity, but also provide interesting details about NS5 localisation and the mechanism of STAT2 binding. Further experiment could

be designed in order to test the antiviral response to type III IFN of both cells expressing NS5 alone and of cells containing the DENV replicon, and to verify whether a potential inhibitory effect is due to the same mechanism responsible for type I IFN inhibition. Conversely, should the virus be susceptible to type III IFN response, this would open new interesting lines of research aiming at further characterising not only the binding between NS5 and STAT2, but also the specificity of the two signalling pathways.

Potential applications

Defining the precise interaction between NS5 and STAT2 and identifying the residues of NS5 involved would provide a potential new target for antiviral therapeutics, designed to block the interaction between NS5 and STAT2. Furthermore, characterising the residues involved in STAT2 binding may enable reverse genetic engineering of DENVs that are disabled in their ability to inhibit IFN signalling; this would provide a potential new strategy towards the design of rationally attenuated vaccine candidates. Neither strategy would be sufficient to overcome the main problems associated with the use of antivirals or live-attenuated vaccines: in order to control viral replication an antiviral drug would still have to be administered at early stages of infection, and safety issues related to the use of a life vaccine, together with the risks associated to pre-existing immunity after secondary infections would still raise concern. However, this strategy of rational attenuation would have a particular advantage, since an engineered DENV unable to inhibit IFN could be easily grown at high titres in cells that do not produce IFN: this would overcome the typical low yield of attenuated vaccine strains, and, at the same time, it would still be much attenuated in humans, where the IFN response is functional.

In conclusion, this study opens several lines of research and represents an important step in defining the molecular pathogenesis of dengue. Since the absence of anti-DENV vaccines is mainly due to the complex pathogenesis of dengue disease and to the complex interplay between DENV and the host immune system, we believe that this work provides important clues to suggest potential new approaches to combat dengue disease.

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