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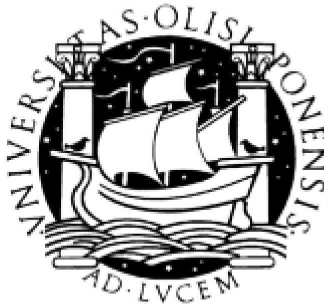
Departamento de Biologia Animal



"Viral modulation of interferon (IFN) responses to African swine fever virus (ASFV)"

Emanuel Nery de Oliveira Quartin Costa

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Dissertação Orientada Por:
Michael Parhouse (PhD)
Deodália Dias (PhD)

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List of Abbreviations

ADAR – Adenosine deaminase RNA I
AIM2 – Absent in melanoma 2
AP-1 – Activating protein-1
APOBEC – Apoplipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like
ASFV – African swine fever virus
ATF2 – Activating transcription factor 2
BVDV – Bovine diarrhea virus
CARD – Caspase activation and recruitment domain
CBP – CREB-binding protein
cig5 – CMV-inducible gene 5
CSFV – Classic swine fever virus
DAI – DNA-dependent activator of IFN-regulatory factors
DC – Dendritic cell
DExD/H – Aspartate-glutamate-any amino acid-aspartate/histidine
DHX – DexD/H-box helicase
DNA – Deoxyribonucleic acid
eLF – eukaryotic translational initiation factor
ER – endoplasmatic reticulum
FADD – Fas-associated DEATH domain
FMDV – foot-and-mouth-disease virus
GAF – IFN-γ activated factor
GAS – IFN-γ activated sequence
HHV – Human herpes virus
HIV – Human immunodeficiency virus
HPV – Human papillomavirus
HSV – Herpes simplex virus
IAP – Inhibitor of apoptosis protein
IFN – Interferon
IFI16 – IFN-inducible protein 15
IFNAR – IFN-α receptor
IFNGR – IFN-γ receptor
IKK – IκB kinase
IL-1 – Interleukin 1
IRAK – IL-1-associated kinase
IRF – IFN regulatory factor
ISG – IFN-stimulated genes
ISGF3 – ISG factor 3
ISRE – IFN-stimulated response element
JAK – Janus kinases
JEV – Japanese encephalitis
LRR – Leucine-rich repeat
LRRFIP1 – LRR flightless-interacting protein 1
MAPK – Mitogen-activated protein kinase
MAVS – Mitochondrial antiviral-signalling protein
MCMV – Murine cytomegalovirus
MDA5 – Melanoma differentiation associated gene 5
MGF – Multigene family

MHC – Major histocompatibility complex
NAC – nascent polypeptide-associated complex
NALP3 – NACHT, LRR and PYD domains-containing protein 3
NCLDV – Nucleo-cytoplasmatic large DNA virus
NF-κB – Nuclear factor kappa B
NK – Natural Killer
NLR – Nod-like receptor
NLS – nuclear-localization signal
OAS – Oligoadenylate synthase
ORF – Open reading frame
PAMP – Pathogen-associated molecular pattern
PKR – Protein kinase K
PML – Promyelocytic leukaemia
PRR – Pattern Recognition Receptor
PYHIN – Pyrin and HIN200 domain-containing protein
RD – Regulatory domain
RELA – Reticuloendotheliosis oncogene homolog A
RIG-I – Retinoic-acid-inducible gene I
RLH – RIG-I helicase
RNA – Ribonucleic acid
SeV – Sendai Virus
STAT – signal transduction and activators of transcription
TBK1 – TANK-binding kinase 1
TIR – Toll-IL-1 receptor
TLR – Toll-like receptor
TNF – Tumor necrosis factor
TRADD – TNF-receptor type 1-associated DEATH domain
TRAF – TNF-receptor associated factor
TRIF – TIR-domain-containing adaptor-inducing IFN-β
TRIM – Tripartite motif
TYK – Tyrosine kinases
UBC- Ubiquitin-conjugating enzyme
VCV – Vaccinia virus
VSV – Vesicular stomatitis virus
ZBP1 – Z-DNA binding protein 1

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Resumo

A imunidade inata constitui a primeira resposta dada por um hospedeiro quando atacado por agentes patogénicos. A resposta imune baseia-se em genes codificados na linha germinativa, chamados receptores de reconhecimento de padrões (PRRs). Estes conseguem distinguir o "Eu" do "não-Eu", reconhecendo padrões moleculares conservados ao longo da evolução dos vários agentes patogénicos, chamados padrões moleculares associados a agentes patogénicos (PAMPs). No caso dos vírus, um parasita intracelular obrigatório, os PAMPs mais importantes e mais estudados são o seu material genético, tal como o DNA genómico viral, RNA de cadeia dupla (ds) ou simples (ss) ou a estrutura RNA viral, 5'-trisfosfato-RNA. Existem vários PRRs, que podem ser agrupados em classes: os receptores transmembranares do tipo Toll (TLRs), os receptores citoplasmáticos do tipo RIG-I (RLRs), os receptores do tipo Nod (NLRs) e os receptores do tipo AIM2 (ALRs). Os PRRs iniciam uma sinalização em cascata que culmina com a activação de factores de transcrição, que entre outros, vão induzir a produção e excreção duma citoquina, o interferão (IFN).

Este grupo de citoquinas é composto por três classes, IFN tipo I (p.e IFN-α/β) , tipo II (p.e IFN-γ) ou do tipo III (p.e. IFN-λ). O IFN pode despoletar variados efeitos anti-virais. A cascata de sinalização estimulada pelo IFN inicia-se com a ligação do IFN ao seu respectivo receptor extra celular que ,através de fosforilações, permite a activação de receptores intra-celulares. Já no interior da célula, sinalizadores de transdução e ativadores da transcrição (STATs) são recrutados e fosforilados, o que permite a formação de homo ou heterodímeros que migram para o núcleo. No núcleo, as STATs ligam-se a zonas promotoras de genes estimulados pelo IFN (ISGs), para promover a transcrição de mais de 300 ISGs com propriedades anti-virais. No caso do estímulo causado por IFN do tipo I, os complexos formados pelas STATs vão ligar-se ao elemento de resposta estimulado pelo IFN (ISRE). No caso do IFNs do tipo II, os complexos ligam-se à sequência activada pelo IFN-λ (GAS). Os ISGs facultam ao hospedeiro diversas estratégias para combater a infeção viral.

Apesar de os mamíferos possuírem um sistema imune bastante evoluído, os vírus também têm evoluído estratégias para evitar e/ou manipular as defesas do hospedeiro, dedicando uma parte substancial do seu genoma a estas estratégias. Estas podem ir desde uma interferência global na expressão e/ou síntese de proteínas das células do hospedeiro, ou serem mais específicas, diminuindo o impacto dos IFNs. O estudo destas interações, pode não só ser útil para conhecer os mecanismos de infecção do vírus, mas também para perceber melhor os mecanismos de defesa do hospedeiro. Estes conhecimentos podem

permitir o desenvolvimento de terapias e tratamentos anti-virais ou mesmo anti-cancerígenos.

A peste suína africana (ASF) é uma doença que nos porcos domésticos (*Sus sacrofa*) é tipicamente hemorrágica e leva normalmente à morte do hospedeiro. Contudo, as infecções são assintomáticas nos hospedeiros naturais, o javali, o porco selvagem e a carraça, sendo esta última, um dos principais vectores de transmissão do vírus da peste suína africana (ASFV), tornando o seu controlo difícil sem uma vacina. Nos últimos anos, devido ao grande desenvolvimento urbano e consumo de carne de porco, têm havido surtos de ASF em África, causando perdas devastadoras. O ASFV é um virus de DNA de cadeia dupla, o único arbovírus de DNA e o único membro da familia *Asfaviridae*, infectando principalmente macrófagos e monócitos.

Tal como todos os vírus, o ASFV contém genes que manipulam a biologia da célula do hospedeiro, como por exemplo, genes que inibem a apoptose e respostas imunes controladas pelo factor nuclear kappa B (NFκB), entre outros. Contudo, ainda não foi demonstrado que algum gene do ASFV consiga inibir a resposta do IFN. Isto é surpreendente, pois o ASFV infecta macrófagos, um tipo de célula sensível ao IFN e porque a sua infecção persistente, é incompatível com uma resposta efectiva mediada por IFN.

O K205R é um gene do ASFV sem função definida, mas ensaios preliminares de luciferase mostraram que este gene consegue inibir a resposta do IFN. Contudo, os mecanismos utilizados pelo K205R nesta inibição são desconhecidos. O objectivo desta dissertação de mestrado é tentar perceber melhor estes mecanismos e determinar a sequência mínima necessária para que o K205R tenha o efeito observado.

O K205R foi isolado através de PCR, utilizando como molde o DNA genómico da estirpe do AFSV, BA71. Subsequentemente, foiclonado no plasmídeo pcDNA3, que contém um marcador molecular, a hemaglutinina (HA), a montante da zona de inserção do gene. Para determinar a extensão da ação do K205R, foram feitos ensaios de luciferase utilizando células transfectadas com repórteres de luciferase sobre o controlo dos promotores de IFN-β, ISRE e GAS. O K205R mostrou inibição para todos os reporteres. Para tentar definir a zona do K205R responsável pelo efeito observado, fez-se uma previsão da estrutura secundária da proteína do K205R, recorrendo à bioinformática, que permitiu identificar uma sequência “coiled-coil” putativa, uma estrutura secundária associada a interações entre proteínas. Também é sugerida uma sequência putativa para um sinal de exportação nuclear (NES). Com base nesta análise foram construídos quatro fragmentos do K205R e posteriormente clonados no pcDNA3.

Depois de se verificar a sequencia correcta de DNA de cada um dos clones e expressão das suas proteinas em células vero transfectadas , o passo seguinte foi verificar a localização celular destes fragmentos através de imunofluorescência nestas mesmas células. Esta experiência permitiu verificar que de facto, os fragmentos que não tinham a sequência putativa NES, em comparação com células transfectadas com o K205R inteiro, tinham uma maior acumulação nuclear.

Para estudar o mecanismo, e a que nivel o K205R actua para inibir a via de sinalização do ISRE, foi feito um “western blot” utilizando extractos proteicos de células VERO transfectadas com os diferentes fragmentos do K205R e posteriormente estimuladas com IFN-β durante 15 minutos e durante 45 minutos. Esta experiência permitiu verificar que a fosforilação da STAT1 diminui na presença do K205R, contudo, apenas um fragmento reproduziu este efeito. Este fragmento de 75 aminoácidos não contém a sequência, nem para a sequência “coiled-coil”, nem para NES.

Esta dissertação de mestrado apresenta resultados consistentes com a existência de um NES funcional na sequência do K205R, uma inibição da fosforilação da STAT1 mediada pelo K205R, mas também apresenta uma abordagem para determinar os mecanismos utilizados pelo K205R para inibir a indução e o impacto do IFN-β. Contudo, mais experiências têm de ser feitas para realmente se comprovar a existência de um NES, como por exemplo, ensaios de imunofluorescência de células transfectadas com K205R na presença de Leptomicina B, um inibidor da exportação nuclear. Também será necessário estudar as vias de sinalização inibidas pelo K205R que não foram abordadas neste trabalho, tal como a via de indução de IFN-β e a via do GAS.

Palavras-chave: Imunidade Inata, Interferão, Infeção, Virus, ASFV, K205R.

// Abstract

A key part of the innate response to virus infections is the interferon (IFN) response. This can limit virus replication and dissemination and is a critical factor in controlling virus infections, particularly persistent viruses. Many viruses encode proteins which interfere with induction of IFN and IFN-activated pathways and these can have important roles in virus pathogenesis and persistence. African Swine Fever (ASF) causes major economic losses in many African countries and is a threat to pig farming worldwide. There is no vaccine and therefore options for disease control are limited. In Europe, there is always the danger of accidental introduction of the virus, as indeed occurred in Portugal in 1957, causing severe financial losses. Thus, defining the mechanism of proteins involved in evasion of the host's defense response and in virus virulence is of extreme interest, so we can understand the virus and try to develop strategies to reduce ASF impact.

ASFV is a large cytoplasmic DNA virus which encodes between 160 to 175 open reading frames. Many of its genes are not essential for replication *in vitro*, but are host evasion strategies facilitating virus replication and transmission *in vivo*. These include proteins which inhibit host defence systems. Surprisingly, since ASFV can survive as a persistent virus, no ASFV proteins have been described which inhibit the IFN response. However, the early gene K205R, might have an impact on IFN response.

Luciferase assays, shown inhibitions of IFN induction (IFN-β) and IFN-signalling (ISRE, GAS) pathways. Using a bioinformatics tool (Jpred), we got a predication of K205R protein secondary structure. Based on this prediction, deletion mutant fragments of K205R were constructed and used in immunofluorescence and western blot assays. The immunofluorescence results suggest the presence of a functional nuclear export signal (NES) motif in the K205R protein sequence. Western blot experiments suggested that K205R is affecting the phosphorylation status of STAT1, in cells stimulated with IFN-β (ISRE pathway).

Although it was not possible to clearly determine the minimum sequence needed for all the functions of K205R, the results suggest that K205R inhibition of the impact of IFN type I, depends on a sequence within amino acids 130 and 205, which affects STAT1 phosphorylation. Further experiments should be done to investigate the mechanism of K205R inhibition in the pathways not covered on this thesis (IFN-β induction pathway and GAS pathway). The existence of functional NES also needs confirmation.

Key Words: Interferon, Virus, Evasion, ASFV, K205R, NES, ISRE, STAT

Introduction

The environment is full of pathogens that threaten the host with a large spectrum of infections. The first lines of defence against these threats are physical and chemical barriers: the skin, surface coating such as mucous secretions, tears, acid pH, etc. Most viral particles that land on the skin are inactivated by desiccation, acids or by other inhibitors formed by endogenous commensal microorganisms. Although these barriers represent a strong defence, they do not cover the entire host surface in contact with the environment, for example entry via the lungs and by the intestine, and they can fail (e.g. insect bites, bruised skin), or be evaded by a large spectrum pathological mechanisms. In order to stop the pathogenic invasion, a second line of defence has evolved in organism, called the Immune System (24)**.**

The primary function of the immune system is to detect structural features in the pathogens and mark them as distinct from host cells and thus to distinguish the self from non-self. This is essential for any immune response, as it permits the host to eliminate the pathogens without excessive damage to itself. Failure to recognize the self from non-self can result in autoimmune diseases (15).

The immune response to viral infection consists of an immediate innate and later an adaptive response. The innate response is the first line of defence and includes all defence mechanisms encoded by genes in the host's germline which functions continually in a normal host without any prior exposure to the invading pathogen (15). As these mechanisms are broadly expressed and rapidly activated in a large number of cells, most viral invasions are resolved by the innate immune system without the intervention of the adaptive immune system, a second line of defence. The adaptive response consists in gene elements that somatically rearrange to assemble antigen binding molecules (antibodies) with high specificity for individual pathogens and lymphocyte-mediated response, usually called the humoral and cell-mediated responses (24). The innate and adaptive responses are often described as contrasting and distinct phases of the immune response. However, they usually act together. Thus, the innate system can inform the adaptive system by producing cytokines and by cell-cell interaction between dendritic cells (DC) and lymphocytes in the lymph nodes, providing the adaptive immune system essential information about the nature of the invading pathogen, triggering the adaptive mechanisms more appropriate to control the infection. In fact, it is clear that the adaptive response cannot be established without instructions from the precedent (24).

Innate Immunity

The innate response is mediated by cytokines (soluble proteins such as interferons released from infected cells), local *sentinel cells* (dendritic cells and macrophages), a complex collection of serum proteins called *complement* and cytolytic lymphocytes called natural killer cells (NK cells). Other cells types, such as neutrophils and other granulocytic white blood cells, play an important role in innate defence in response to the initial burst of cytokines from dendritic cell, macrophages and infected cells (24). The innate defences relies on germline-encoded genes called the pattern recognition receptors (PRRs) (Figure 1), which recognizes a wide range of "non-self" targets, molecular patterns conserved through evolution in a wide range of pathogens, called pathogen-associated molecular patterns (PAMPs). These microbial molecules are evolutionarily conserved and hence shared between different microbial species (45, 91, 93)**.** In addition, most PAMPs are essential for microbial growth, therefore rarely modified by the microorganisms as means to avoid innate recognition. Along with identification of "non-self" molecules, another key principle of the innate recognition is the aberrant localization of specific classes of molecules, like the introduction of nucleotides (RNAs and DNAs) into endosomes and cytoplasm (24, 54).

All viruses propagate inside cells of the host they infect and depending on the virus, replication takes places in the cytoplasm or the nucleus, and is highly dependent of the involvement of cellular factors. The main viral PAMPs are glycoproteins of the virus particle and virus-derived nucleotide structures, being the latest particularly important for stimulation of innate antiviral defence (77)**.** Viral-derived Double stranded (ds) RNA, genomic viral DNA, single-stranded (ss) RNA and the viral RNA structure 5'-triphospho-RNA, which is normally not present in the cytoplasm due to the 5'-cap of cellular mRNA are the most important and studied viral PAMPs (51, 91).

The Toll-like receptors (TLRs) comprise the most studied family of PRRs. They are responsible for the recognition of a wide variety of microbial PAMPs, including virus, bacteria and fungi. They are constituted by transmembranar PRRs like TLR1, 2 and 4-6 and endosomal TLRs like TLR3 and 7-9 (55, 58, 118). The intracellular detection of viruses is also mediated by other cytoplasmatic sensors: the retinoic-acid-inducible gene I (RIG-I) helicase (RLH) family of proteins, which includes the RIG-I and the melanoma differentiation associated gene 5 (MDA5), which can sense RNA viruses (75, 115); Nod-like receptors (NLRs) family are also shown to engage with both DNA and RNA viruses, in particular the NLR NACHT, LRR and PYD domains-containing protein 3 (NALP3) (60); the absent in melanoma 2 (AIM2)-like receptor family, which senses DNA viruses (14); the DNA-dependent activator of IFN-regulatory factors (DAI), also known as Z-DNA binding protein I (ZBP1) (110); and KU70

(135). These receptors initiate a signalling cascade which culminates in the activation of transcription factors such as nuclear factor kappa B (NF-κB), interferon regulatory factors (IRFs) and activating protein-1 (AP-1) involved in the expression of inflammatory and IFN type 1 genes. (60, 97).

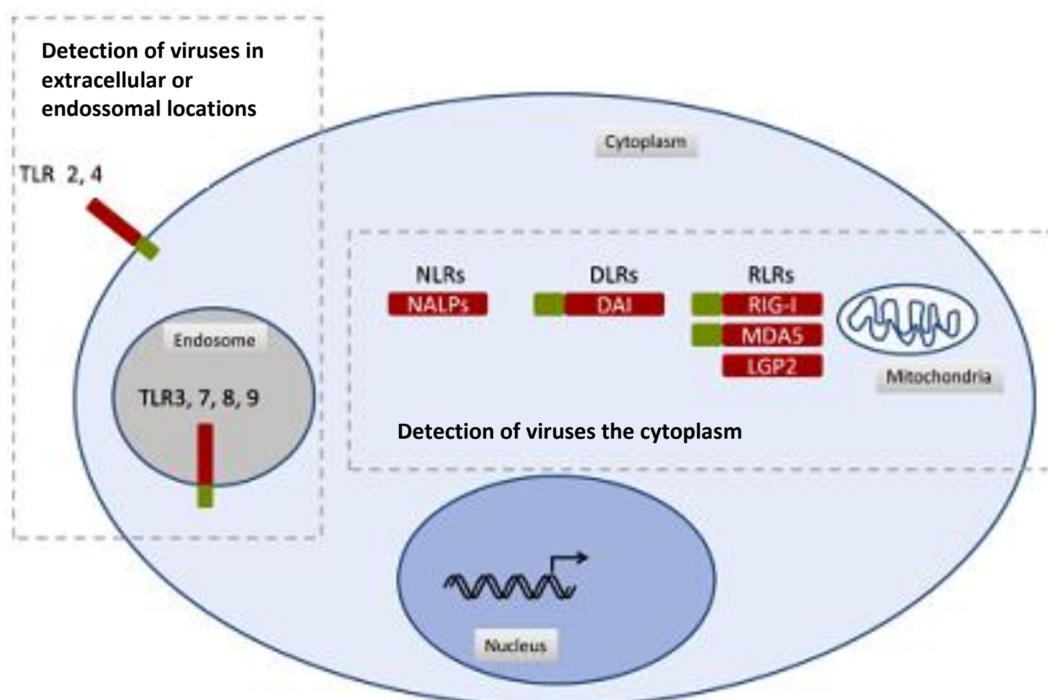

Figure 1: Pattern recognition receptors (PRRS) overview in viral infection. Adapted from (139)

The IFNs are a class II α-helical secreted cytokines that elicit distinct antiviral effects. They are grouped into three classes called type I, II and III, according to their amino acid sequence. Type I IFNs, discovered by Isaacs & Lindenmann in 1957 (53) **,** comprise a large group of molecules (IFN-α, -β, - ε, -τ, -δ,-κ) being IFN-α and IFN-β the most important in mammals concerning response to viral infection. There are multiple distinct IFN-α genes and one to three IFN-β genes. Type II IFN has a single member, the IFN-γ, also called "immune IFN", and is secreted by mitogenically activated T cells and NK cells rather than in direct response to viral infection. Type III IFNs were described more recently and comprise IFN-λ1, -λ2, -λ3, also referred to as IL-29, IL-28A and IL-28B, respectively (120). These cytokines are also induced in direct response to viral infection and appear to use the same pathway as the IFN-α/β genes to sense viral infection (85).

The signal transduction pathways initiated upon IFN binding to cognate receptors at the cell surface requires the activation, through tyrosine phosphorylation of intracellular receptors. This role is associated with the Janus kinases (JAKs), a family of tyrosine kinases

(TYK). Once phosphorylated, the receptors act as docking sites for the signal transduction and activators of transcription (STATs), which are phosphorylated upon recruitment to the receptor. Then, the STATs dissociate from the receptor, associated as homo- or heterodimers and migrate to the nucleus. In the nucleus, they bind to cis-acting elements found at the promoter regions of IFN-stimulated genes (ISGs) to promote the transcription of more than 300 ISGs (10).

Toll-like Receptors (TLRs)

TLRs are membrane-bound PRRs expressed by a variety of cell types, including epithelial cells, although antigen-presenting cells such as dendritic cells and macrophages are the cells most prominently expressing them (55**.** There are 10 known in humans (TLR1-10) and 12 in mice (TLR1-9 and 11-13). They are type I transmembrane proteins with ectodomains containing leucine-rich repeats (LRR) that mediate the recognition of PAMPs, transmembrane domain and an intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domain, that mediates downstream signal transduction. Depending on the TLR, the TIR domain is involved in recruiting various intracellular adaptors molecules, which also contain a TIR domain. Different combinations of the adaptor molecules give rise to specificity in TLR signalling (12). Viral PAMPs can be detected either intracellularly or at the cell surface. TLR2 and TLR4 are cell surface TLRs best known for their role in sensing bacterial and fungal PAMPs. However, TLR4 is also involved in the recognition of envelope proteins of some virus (3). The role of TLR2 in viral recognition and innate immunity was shown by demonstrating that mediates IFN I induction, in response to infection with vaccinia virus (VCV) and murine cytomegalovirus (MCMV) (7).

TLR3, TLR7-9 are localized in intracellular vesicles such as the endosome or lysosome and the endoplasmic reticulum (ER) and are traditionally more clearly related to anti-viral immunity than cell surfaces TLRs. TLR3 appears to represent a more general sensor of viral infections, through the detection of viral-dsRNA molecules (5), a by-product of viral replication and transcription for both RNA and DNA viruses. TLR7 and TLR8 recognizes ssRNA derived from RNA virus infections and TLR9 recognizes DNA viruses (20, 46, 59).

In the case of TLR7-9, endosome-mediated internalization of viruses or products of viral replication from lysed and/or apoptotic virus-infected cells (in case of TLR3) is a prerequisite for TLR-PAMP interaction. To expose the viral PAMP to the corresponding TLR, this process most likely involves degradation of a subset of virus particles in the endosome (33). TLR3 and TLR4 induction of type 1 IFN is mediated through the TIR-domain-containing adaptor-inducing IFN-β (TRIF). TRIF mediates the activation of IκB kinase ε (IKKε) and (TANK)-

binding kinase 1 (TBK1), which phosphorylates the IFN regulatory factor 3 (IRF3), resulting in its dimerization and translocation to the nucleus, where it promotes gene transcription. TRIF also mediates the activation of NF-κB and AP1 through the kinase complex IKK α/β/γ and the mitogen- activated protein kinase (MAPK) cascade, respectively (58). These three transcription factors (IRF3, NF-κB, and AP1) coordinate the transcriptional regulation of the IFN-β gene (125).

Induction of IFN-I through TLR2 and TLRs7-9 is mediated by the adaptor molecule myeloid differentiation primary response protein 88 (MyD88), which associates with the TIR domain of the TLRs, the interleukin-1 receptor–associated kinases (IRAK) 1, 2 and 4, and the tumor necrosis factor (TNF) receptor–associated factor (TRAF) 4 and 6. This results in downstream activation of IRF7, and of the IKK α/β/γ and the MAPK cascades, leading to NF-κB and AP-1 activation (58). IRF7 is functionally similar to IRF3 and mediates the induction of IFN-β but, unlike IRF3, it also initiates the general induction of the IFN-α genes (72). TLRs7-9 and IRF-7 appear to be constitutively expressed in only a subset of cells, the pDCs, which are characterized by high IFN production and can spearhead the early IFN response (57).

RIG-I-like Receptors (RLRs)

The two cytosolic PRRs, are RIG-I and MDA5 Highly relevant to viral-infection. They detect intracellular RNA species, and initiate downstream signalling and induction of cytokines (77). Both, RIG-I and MDA5, are homologous IFN-inducible proteins containing two amino-terminal caspase activation and recruitment domains (CARDs), a carboxy-terminal aspartate-glutamate-any amino acid-aspartate/histidine (DExD/H)-Box RNA helicase domain and a C-terminal regulatory domain (RD). The helicase domain and the RD interact with specific RNA species and the CARDs are responsible for downstream signalling and interaction with Mitochondrial antiviral-signalling protein (MAVS also known as IPS-1, VISA or Cardiff), which interacts downstream with TBK1-IKKi and IKK complexes, The adaptors TRAF3, TANK and TNF-receptor type 1-associated DEATH domain (TRADD) and the kinases TBK1 and IKKε are responsible for activation of NF-κB and the transcription factors IRF3 and IRF7 and subsequent synthesis of type I interferon (79, 130). Besides RIG-I and MDA5, the family of RLRs also includes a third member, LGP2, which lacks the CARD domains and may act as a negative regulator molecule, possibly by forming heterodimeric complexes with RIG-I and MDA5, although the precise mechanism by which it works is still poorly understood (84).

Cytosolic DNA Sensors

The identification of receptors and signalling components that mediate cytosolic interferon response has been the subject of intense study in the last years. The first molecule to be identified as a DNA sensor in the cytosol is DAI, a molecule that contains two binding domains for Z-DNA in the N-terminal and a centrally located region, presumably also having B-DNA-binding ability. The C-terminal region of DAI is essential for activation of downstream signalling pathways, mediating TBK1-IRF3 dependent type I IFN production (110). However, DAI-deficient mice and several cell types derived from them, including macrophages and mouse embryonic fibroblasts, have shown normal responses to synthetic DNA and DNA viruses, suggesting the existence of other cytosolic DNA sensors (123). In fact other cytosolic DNA sensors molecules have been described in the past few years, such as: Leucine-rich repeat (LRR) flightless-interacting protein 1 (LRRFIP1), which recognizes both cytosolic RNA and DNA, and subsequently recruits and activates β-catenin, which binds to IRF3 in the nucleus, contributing to the expression of IFN-β (129); RNA polymerase III, present in the cytoplasm, recognizes AT-rich DNA and transcribes it into RNA transcripts, recognized by RIG-I, activating its pathway (1, 17); DExD/H-box helicase 36 (DHX36) and DHX9, have been shown to sense CpG-A and CpG-B DNA, respectively, in the cytosol of human pDCs, recruiting MyD88 and leading to activation of IRF7 and NF-κB and subsequent IFN production (61); IFI16 and p204, IFN-inducible protein 16 (IFI16) and its closest murine homolog, p204, are members of the AIM2-like receptors family interferon, which belongs to inducible PYHIN (pyrin and HIN200 domain-containing proteins, also known as p200 or HIN200 proteins),. These proteins recognize DNA via its HIN domain and subsequently interacts with STING to activate TBK1-IRF3 complex, resulting in IFN-I production (119); Ku70, the more recently discovered, yet not completely characterized, cytosolic DNA sensor is part of heterodinamic Ku protein and induces the production of type III IFN, more precisely, the IFN-λ1. This induction is mediated by the activation of IRF1 and IRF7 (135).

IFN transcription control

As it was briefly mentioned above, the induction of IFN genes is dependent of signalling cascades, initiated by the activation of different types of PRRs. Despite the large diversity of PRRs found in cell membranes and cytosol and their signalling route, we can find common components downstream in the signalling cascades responsible for IFN production upon viral infection.

The best-studied model of IFN induction is the production of IFN-β. The induction of IFN I, is primarily regulated at the level of transcription and requires no new cellular protein

synthesis, where IRFs (mainly IRF3 and IRF7, but not only), NF-κB and c-jun/activating transcription factor (ATF)-2 heterodimer plays major roles. Prior to IFN-β induction, NF-κB and IRF3 are both cytoplasmatic and upon receipt of appropriate signalling, IRF3 is phosphorylated, causing conformational changes leading to its dimerization, which unveils its nuclear-localization signal (NLS). Translocated IRF3 remains in the nucleus until it is dephosphorylated. NF-κB is associated with its inhibitor, IκB, in the cytoplasm, signal generated during viral infection cause the phosphorylation of IκB and its subsequent ubiquitination and degradation by the proteasome, making the NLS of NF-κB accessible, thus allowing translocation of NF-κB to the nucleus. Optimal induction of IFN-β also required the binding of the c-jun/ATF-2 heterodimer to the promoter. The IRF3, NF-κB and c-jun/ATF-2 complexes assemble on the promoter in a cooperative manner to form the so called enchaseosome. This model predicts that each transcription factor binds to IFN-β with limited affinity and that cooperativity between these factors is required for optimal induction. However, the IFN-β can respond independently to each inducer, resulting in some degree of IFN-β production. The consensus view is that binding of either IRF3 or IRF7 is essential for induction (49, 50).

Induction of IFN-α is less well understood, but unlike IFN-β promoter, IFN-α genes promoters lack NF-κB sites, but contain several binding sites for the IRF family. The identification of the IRF family members which stimulates IFN-α genes is uncertain, but evidences show that IRF7 stimulates preferentially the IFN-α genes transcription, which is activated in a similar manner to IRF3. Upon viral infection, IRF7 is phosphorylated and translocated to the nucleus and forms a homodimer or a heterodimer with IRF3, and each of these dimers acts differentially on induction of IFN-I family members (49, 50).

Signalling responses to IFN

Type I IFNs binds to a common heterodimeric receptor of IFN-α (IFNAR) composed by IFNAR1 and IFNAR2. Prior to activation, the cytoplasmic tail of IFNAR1 and IFNAR2 is associated with TYK2 and JAK1, respectively. IFNAR2 is also associated with STAT2, which is weakly associated with STAT1 (95, 105, 113). Interferon binding to receptors induces their dimerization and subsequent phosphorylation of IFNAR1 by TYK2, creating a docking site for STAT2. Then, TYK2 phosphorylates STAT2 and STAT1 is phosphorylated by JAK1, enabling the formation of STAT1 and STAT2 heterodimer. In combination with IRF9, these proteins form a heterodimeric transcription factor known as ISGF3 (ISG factor 3). ISFG3 migrates to the nucleus, where it recognizes and binds to the IFN-stimulated response element (ISRE), present in the promoter region of ISGs (19, 121). ISGF3 formation and translocation to the

nucleus is dependent on the acetylation of all its components. In response to IFN stimulation, the acetyltransferase CBP (CREB-binding protein) acetylates IFNAR2, creating a docking site for IRF9, which associates to the receptor. Subsequently, IRF9, STAT1 and STAT2 are acetylated and the ISGF3 complex forms and migrates to the nucleus. IRF9 acetylation is also required for DNA binding, suggesting that acetylation may play an important role in the ISRE-mediated ISG induction (113). Another interesting recent finding demonstrated that exposure of cells to IFN-β is followed by IKKε activation, which phosphorylates STAT1, resulting in ISGF3 formation and subsequent migration to the nucleus (114).

Type II IFN also binds to receptors at the cell surface consisting of two subunits, the IFN-γ receptor (IFNGR)1 and IFNGR2, associated with JAK1 and JAK2, respectively. IFN-γ binding to receptors promotes the dimerazition of both subunits which activates JAK1 and JAK2. Then, each receptor chain is phosphorylated, creating docking sites for STAT1, which forms a homodimer and dissociates from the receptor chains after STAT1 phosphorylation, resulting in the transcription regulator IFN-γ activated factor (GAF).This complex is translocated to the nucleus where recognizes and binds to the regulatory sequence GAS (IFN- γ activated sequence) (19, 27). Of note, type I IFNs can activate, on a cell-type and context specific manner all seven members of the STAT family, leading to the formation of STAT homo/heterodimers, including STAT1 homodimer and subsequent induction of GAS promoted genes (106).

Type III IFNs signalling response is not fully understand, but is very similar to type I IFN response. The receptors chains are formed by the interleukin receptors, IL-10Rβ and IL28Rα and signal transducing requires JAK1, STAT1-2 and ISGF3 (137).

IFN-induced Antiviral state

The antiviral state is conferred by the transcriptional regulation of the ISGs, induced by IFN. These genes activate a set of antiviral processes to reduce or prevent viral replication in infected cells and their dissemination to neighbourhood healthy cells, limiting viral infection spreading and, when necessary, giving enough time to start the adaptive immune machinery to eliminate the virus and infected cells. Several hundred of genes are upregulated upon IFN induction, but no single gene is pivotal and for any given virus a subset of genes is required to protect the host from the virus. Many of them have been studied intensively, e.g. Protein Kinase R (PKR), 2’5’Oligoadenylate synthase (OAS), Mx family.

One of the first ISGs to be linked with an antiviral response was the dsRNA-dependent PKR. This enzyme is synthetized in an inactive form and, in response to the cofactor dsRNA, produced during viral replication, undergoes dimerizations and activation.

The best characterized substrate for PKR is the α subunit of the eukaryotic translational initiation factor 2 (eIF2α). This PKR phosphorylates eIF2α and prevents its recycling such that initiation of translations is halted. This interaction can also mark the cell for autophagy. Furthermore, the PKR is reported to be involved in other antiviral mechanism, including induction of apoptosis and cell-cycle arrest (111, 112, 134).

Another well studied ISG, the 2'5'OAS is also synthesized in an inactive form and uses dsRNA as co-factor. Its activation leads to RNase L activation, which degrades cellular and viral RNAs, preventing viral protein synthesis and in case of viral overload, the degradation of cellular dsRNA can lead to apoptosis or amplification of type I IFN by the RLRs (23, 70).

MX and the MX family of genes encode large GTPases related to dynamin. These proteins limit viral replication by interaction with nucleocapside-like structures and limiting their cell localization (43).

Many other ISGs have important antiviral responses and different strategies are applied to fight viral infection. They can improve the efficiency of the IFN response globally, like ISG15, which can protect against degradation of proteins important for innate immunity (e.g. JAK1, STAT1, PKR, MxA, RIG-I, etc) (69, 136); They can also interfere with viral replication. For example, the Promyelocytic leukaemia (PML) nuclear bodies, which interferes with chromatin structure and promoter accessibility, impairing the replication of both RNA and DNA viruses (26), or Viperin (also known as CMV-inducible gene 5, cig5), which disrupts the formation of lipid rafts, important in the assembling process of some virus (122). APOBECs (Apolipoprotein B mRNA editing enzyme-catalytic polypeptide-like) and TRIMs (tripartite motif) are constitutively expressed proteins but upregulated by IFN-α/β. These so called "restriction factors", can mutate viral genome and restrict replication of retroviruses, by cytidine deamination (APOBEC3F and 3G) (71, 103) or by interaction with viral capsides (TRIM5α) and subsequent formation of a complex which can be targeted for destruction by proteasomes (117). Another ISG involved in viral replication disruption is Adenosine deaminase RNA 1 (ADAR-1), which replaces adenosines with inosine in dsRNA, unwinding the dsRNA, disrupting viral replication (116).

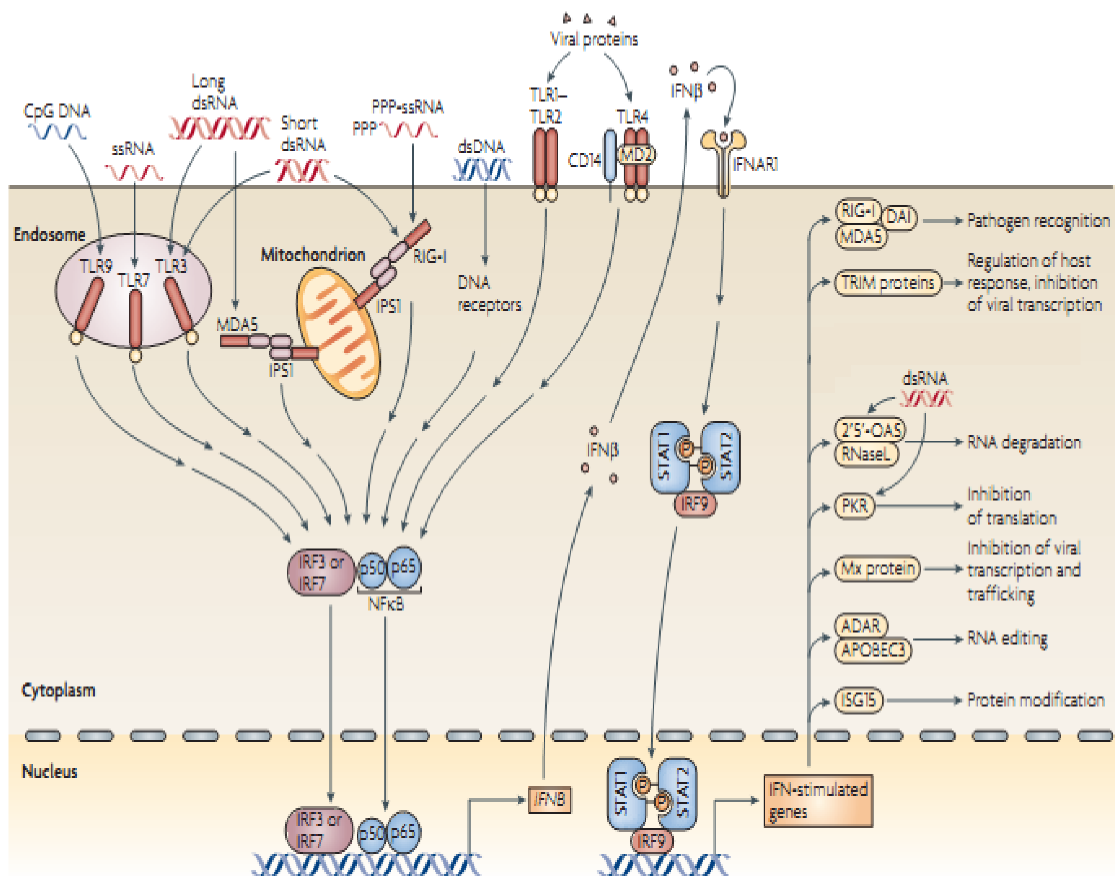

Figure 2: Schematic overview of type I interferon impact in response to viral infection. Recognittion of viral PAMPS through PRRs (TLRs, RLRs, cytoplasmatic DNA sensors) followed by production of IFN-β. Cellular and signalling response to IFN-β followed by production of differents ISGs. Adapted from (12)

Besides its important role in the innate immunity response (figure 2), the IFNs also play an important role in adaptive immune responses, providing the bridge between innate and adaptive immune response. Upregulation of class I major histocompatibility complex (MHC) molecules and components of the antigen-presenting machinery, are the most obvious examples. IFNs can also promote: maturation, activation and maintenance of NK-cells populations; maturation of DCs; proliferation of antigen-specific CD8^{+} T cells; effector mechanisms of cytotoxic T cells and cell division of memory cytotoxic T cells (48).

Viral evasion of IFN responses

In order to survive, viruses have evolved and dedicate a substantial part of their genome in strategies to circumvent the host defences. One of the main targets of these strategies is the IFN system, which constitutes a constant selective pressure in most viral infections, by its key role in detection, control and elimination of viruses. In the last years, many studies have been made regarding this topic and several molecular mechanisms (and respective involved proteins) have been described. We can summarise all these strategies into five main categories, by which viruses evade the IFN response:

1) Interfering globally with host-cell expression and/or protein synthesis;

2) Minimizing the induction IFN;

3) Inhibiting IFN signalling;

4) Blocking the action of ISGs;

5) Replication strategies largely insensitive to IFN action.

For each of these categories different molecular mechanisms have evolved in different viruses and most of the times a combination of more than one strategy has evolved in order to achieve efficient evasion of the IFN response. Consistent with this, most IFN antagonists are multifunctional proteins and their actions can vary at different stages of the virus infection and replication cycle (33, 96).

Interfering globally with host-cell expression and/or protein synthesis

The mechanism used by a virus to avoid the IFN system is a major factor influencing the molecular pathogenesis of a viral infection. This is especially obvious when it comes to viruses that have developed mechanisms to shut down cellular protein expression globally, including cellular gene transcription and mRNA processing or export. Viruses pursuing this strategy are unable to establish a persistent or latent infection and thus cause acute infections. This lifestyle limits the time for viral replication as a cell with inhibited protein synthesis will be die more rapidly or even be killed by other innate immune responses, such as the induction of apoptosis by tumor necrosis factor (TNF). Although many host-cell functions will be affected by inhibition of gene expression, a particularly important target for viruses is the IFN response. For example, mutation in proteins of Vesicular stomatitis virus (VSV) and foot-and-mouth-disease virus (FMDV), which are involved in inhibition cell-protein synthesis, generates attenuated strains with efficient IFN-induction (16, 28, 96).

Minimizing the induction of IFN

Given the pathway of IFN induction, virus can adopt two general strategies to keep IFN induction to a minimum without shutting down the entire cell, as discussed above. Viruses can either avoid detection by minimising their viral PAMPs or/and they can specifically block members of the IFN induction pathway. The most important viral PAMP is their genomic material, in particular dsRNA, which, until recently (127), was thought to be the only PAMP to clearly distinguish virus from host. Therefore, many viruses have adopted strategies focusing their genomic material, such as: tight control of viral replication and transcription in order to minimize production of dsRNA (e.g. transcribing gene in blocks in the same direction) or to “hide” from the host PRRs (e.g. positive-strand RNA viruses

replicating within intracellular membrane vesicles); encapsidating both genomic RNA and antigenomic RNA; protecting the 5' end of their mRNA, avoiding recognition by RIG-I; integrating their genome in host chromosomes; protecting dsRNA from host PAMPs by producing dsRNA-binding proteins that sequester dsRNA. This last strategy as additional advantage, as it also minimizes the action of dsRNA-dependent ISGs, like PKR and 2'5'OAS. There are many well studied examples of dsRNA-binding proteins produced by viruses, such as NS1 of influenza A virus, E3L of poxviruses, sigma3 of reoviruses, VP35 of Ebola virus and US11 of herpes simplex virus (HSV) (96, 126).

Some viruses have also evolved strategies that target specific components of the TLR and RLR signalling pathways. The NS3/4a protein of Hepatitis C virus blocks the TLR3 signalling by cleaving the TLR3 adaptor protein TRIF (65); VCV proteins A52 and A46 target multiple TIR proteins, including TRIF, to block TLR3 and TLR4 induction of IFN (44, 104). The V and C proteins of paramyxoviruses can inhibit the activity of MDA5 and RIG-I, respectively (6, 107). Further downstream, the NS3/4a protein of HCV can also inhibit the IRF3 signalling as it cleaves MAVS, disrupting its ability to signal to TBK1 and IKKε; the N^{pro} of both Bovine diarrhea Virus (BVDV) and classic swine fever virus (CSFV), target IRF3 for proteosome-mediated degradation. NF-κB is also targeted by some viruses, for example, the African swine fever virus (ASFV) encodes an IκB orthologue that inhibits the activity of NF-κB (41).

Inhibition of IFN signalling

As discussed before, the Interferon signalling is responsible for the induction of several antiviral cellular enzymes (such as PKR, 2'5'OAS, Mx, PML, etc) and also some adaptive immune functions, like the upregulation of MHC I molecules. Also, some of the components involved in this IFN signalling pathway are common to all IFN subtypes. Besides, virus-infected cells with blocked IFN signalling would become resistant to IFN production, so there are clear advantages to the viruses in inhibiting the IFN signalling pathways. Indeed, there are examples of virus proteins inhibiting all components of this signalling cascade, from receptor signalling to the formation and activity of IFN-induced transcriptions factors. For example, poxviruses sequester IFN by producing soluble IFN-α/β-receptor homologues that are secreted by infected cells (4). IFN-receptors can also be downregulated by viruses (e.g. the K3 and K5 proteins of Human Herpes Virus 8, HHV-8, targets IFNGR1 for ubiquitination, endocytosis and degradation) (66) or have their JAK kinases disrupted (e.g. NS5 protein of Japanese encephalitis virus, JEV, interferes with Tyk2, possibly by activating tyrosine phosphatases) (68). Interfering with STATs is also a strategy used by some viruses, for example, Sendai Virus (SeV) inhibits IFN signalling by sequestering STATs, increasing their

turn over and altering the pattern of STAT1 phosphorylation, by a set of proteins encoded by P/V/C gene, namely the C proteins (31, 32, 73). More downstream in the signalling cascades, the human papillomavirus (HPV) 16 E7 multifunctional protein, interacts directly with IRF9, preventing the formation of ISGF3 (8). However, the inhibition of IFN signalling by itself may not be enough, because the delay in virus replication induced by IFN should buy enough time to the host, so than it is able to mount an acquired immune response to help resolve the infection, and this may be why viruses that block IFN signalling also block IFN production (96).

Inhibition of IFN-induced antiviral enzymes (ISGs)

As already mentioned, some of the viral IFN antagonists are multifunctional proteins and we have already seen that in fact those dsRNA-binding proteins serve a second purpose, besides minimizing IFN induction, namely the inhibition of some ISGs. PKR, 2'5'OAS, Mx proteins, ISG15, PML and APOBECs are all IFN-induced antiviral enzymes, that can be target to efficiently circumvent IFN antiviral state. More than just dsRNA binding seems necessary, since, at least PKR can be activated in a dsRNA-independent way by the PACT (PKR-associated activator) and in many cases a direct interaction with PKR or 2'5'OAS/RNaseL system, has been demonstrated. For example the NS1 protein of influenza virus A that binds to directly to PKR (67) or the induction of RNase L inhibitor by human immunodeficiency virus (HIV) type 1 (78). ISG15, the ubiquitin-like protein can be targeted by the influenza B virus NS1 protein, which interacts with it and prevents the interaction of ISG15 with its substrates (131).

African Swine Fever Virus

African swine fever was first described by Montgomery in 1921, and is characterized by a typical haemorrhagic disease of domestic pigs (*Sus scrofa*). In contrast, the infection of the natural hosts, the bushpig (*Potamochoerus porcus*) and the warthog (*Phacochoerus aethiopicus*) is characterized by the absence of clinical symptoms, reflecting the long term host-pathogen co-evolution. There are different ASFV isolates, which share common biological features, and the pathogenesis of the disease may range from rapidly lethal to very attenuated and chronic disease (63). The ASFV also infects soft ticks of the species *Ornithodorus* (*O. moubata* and *O. erraticus*), where the virus can persist for long periods of time (9, 92). These ticks play an important role in the transmission of the disease by feeding on warthogs, hence acting as vectors in the sylvatic cycle. In the domestic pig, the virus is

usually transmitted directly between pigs, however ticks may represent an important reservoir of the virus (22).

The disease has been reported in several sub-Saharan countries and was introduced in Portugal in 1957 and 1960, where it remained endemic until the 1990s. On recent years, ASF was confined to African countries and Sardinia (22). There have been major outbreaks of ASF in Africa due to the increased urbanisation and pork consumption which, associated with the increasing commercial trade between countries, poses a constant threat to Europe. Recently, however, there was an ASF outbreak in Georgia , which had devastating consequences for pig industry and has spread to neighbouring regions. The genetic characterization of the ASFV isolate implicated in this outbreak suggested that it is closely related to isolates typically found in Mozambique and Madagascar (100).

Virus structure and genome organization

African swine fever virus (ASFV) is a large dsDNA virus, the only known DNA arbovirus and the only member of the family *Asfarviridae* (21, 22). The A*sfarviridae* it's a member of the nucleo-cytoplasmatic large DNA virus (NCLDV) superfamily, which also includes *Poxviridae*, *Iridoviridae*, *Phycodnaviridae*, *Mimiviridae* and *Marseilleviridae*. These families share similarities in its gene complement and replication strategy, which occurs at least partially in the cytoplasm. ASFV has more similarities with the *Poxiviridae*, in terms of replication strategy (56). The ASFV's virions have a complex multi-layered structure. The nucleoprotein core contains the viral genome, enzymes and other proteins necessary for the early stages of infection. This internal nucleoprotein is surrounded by a core shell and an internal envelope onto which the icosahedral capsid is assembled. It replicates in the cytoplasm and its genome varies in length between 170 and 190Kbp, containing terminal crosslinks and inverted terminal repeats. The variation in the genome length between different virus isolates is due to gain or loss of sequences in the left and right ends of the genome (21).

This virus contains a number of open reading frames (ORFs), ranging from 160 to 175 depending on the isolate. Of these, 110 are present as a single copy in the genomes of all isolates. The other ORFs belong to six different multigene families (MGF100, MGF110, MGF300, MGF360, MGF530 and P22 family) located near genome termini. The organization of these gene families suggests that they have evolved by a process of gene duplication and sequence divergence. Hence, the existence of multiple copies of several MGFs might give a selective advantage to the virus, representing a mechanism of virus immune evasion. In particular, the Vero adapted isolate BA71V and the low pathogenic isolates OURT88/3 and

NH/P68 have a deletion in the same region of the genome, which encodes 6 copies of MGF360 and 1 or 2 copies of MGF530 (22). Of the conserved ORFs, 39 encode proteins of known function, 42 contain motifs homologous to other proteins and 28 are of unknown function. Up to now, 17 ORFs have been identified as coding for structural proteins. As ASFV replicates in the cytoplasm, genes for enzymes and factors required for gene transcription and DNA replication are also included in the virus genome. There are many virus proteins that are non-essential for virus replication and are involved in interactions with the host, thus representing important factors for virus survival and transmission (22).

Pathogenesis and host immune response

Macrophages and the monocyte lineage are the cells primarily infected by ASFV, with some evidence that endothelial cells can also be infected later in the infection (109). The acute disease is characterized by massive apoptosis of lymphocytes and haemorrhagic pathology with extensive vascular damage, probably due to molecules released from the infected macrophages, although infected endothelial cells may contribute to the pathogenesis (22, 109). The extent of lymphocyte apoptosis correlates with the level of ASFV replication and the virulence of the virus isolate (87). In the bushpig, there are lower levels of apoptosis and absence of clinical signs together with a containment of virus replication (88). Therefore the level of lymphocyte apoptosis may be dependent on the amount of secreted cytokines, which in turn depends on the number of infected macrophages (87). In agreement with this hypothesis is the fact that increased levels of TNF-α, IL-1α, IL-1β and IL-6 were observed in sera from experimentally infected pigs, coinciding with the onset of clinical symptoms (101) and also an increased number of macrophages expressing these cytokines in areas of lymphocyte apoptosis (102). On the other hand, another study revealed that the transcriptional levels of TNFα and IL-6 were increased in macrophages infected with the low virulence NH/P68 isolate compared to the highly virulent L60 isolate, although has not been confirmed at the protein level (34). A more recent study suggests a new hypothesis for the differences observed between acute ASF disease in the domestic pig, and the tolerable ASFV infection in wild pig species. This study reports a polymorphic variation of RELA (p65; v-rel reticuloendotheliosis oncogene homolog A), of three amino acids, between warthog RELA and domestic pig RELA. This variation is reflected in reduced NF-κB activity *in vitro* for warthog RELA but not for domestic pig RELA. This activity variation of RELA may underlie the difference between tolerance and rapid death upon ASFV infection (90).

The immune response mounted after ASFV infection is highly complex and virus elimination probably requires both humoral and cellular immunity. Recovered animals are

usually resistant to challenge with homologous virus isolates, providing a model to study the mechanisms of protective immunity (63). Several experiments have shown that the passive transfer of antibodies from recovered, or convalescent pigs, delays the onset of clinical signs, reduces viraemia and increases survival rates after challenge with a related virulent isolate (124). In a later study, 85% of the animals receiving anti-ASFV antibodies survived infection with the E75 virulent isolate (83). These results suggest that antibody-mediated immunity is not by itself sufficient, but may play a role in protection. However, the generation of neutralizing antibodies during ASFV infections remains controversial. Three different ASFV neutralizing proteins have been proposed: antibodies against p72 and p54 inhibit virus attachment, while antibodies to p30 inhibit virus internalization (11, 42). However, in later studies it was shown that the immunization against p54 and p30 only conferred protection to 50% of tested animals (41), and the only detected effects were a delay in onset of clinical disease and reduction of viraemia (80).

Several studies were done to explore the role of cell mediated immune responses during ASFV infection. After experimental infection with the non-haemadsorbing, non-fatal NH/P68 isolate, a positive correlation was observed between the stimulation of NK activity and the absence of clinical symptoms, suggesting that NK cells play an important role in this model of protective immunity (63). In addition to NK cells as mediators of protection, the generation of ASFV specific cytotoxic lymphocytes was demonstrated in the NH/P68 model (74, 86). However, the immunization with a recombinant protein expected to stimulate ASFV-specific cytotoxic T lymphocytes activity, failed to protect against the infection with the highly virulent L60 isolate (64). On the other hand, established immunity of pigs was abrogated by blocking $CD8^+$ T cells *in vivo* with anti-CD8 monoclonal antibody, suggesting that $CD8^+$ T cell mediated immunity does play a role in protection (86).

Finally, both IFN-α and IFN-γ were shown to substantially reduce virus replication in swine monocytes and macrophages (25), and the cooperative action of both was able to cure lytically and persistently infected cells (89). Although these results were interpreted as evidence for a role of the IFN response in protection, the IFN treatment was done after 18h post-infection, a time at which the anti-viral state was already established. Importantly for the work described in this thesis, virus replication of ASFV in IFN-treated cells has been reported, an experiment which suggests that ASFV is able to subvert the Interferon response (89).

In conclusion, the immune response against ASFV is mediated by multiple mechanisms of both innate and acquired immune responses and another level of complexity is added with the ability of the virus to modulate these immune responses.

Modulation of host defense response

Large DNA viruses encode many proteins involved in the evasion of host immune responses. ASFV, contains approximately 90 proteins predicted to be involved in virus replication, therefore, the remaining 70 to 85 must include many proteins evolved for host evasion (22).

As ASFV replicates in macrophages the virus may interfere with both the initial innate and later acquired immune response to infection by modulation of macrophage immunoregulatory proteins and hence macrophage function. Indeed, one of the major strategies used by the virus is the manipulation of different signalling pathways that lead to the induction of transcription of cytokines (22).

One of the first evasion molecules described is the A238L protein with two dual functions: inhibition of NFκB (94) and NFAT activities (76). The A238L protein contains ankyrin repeats similar to those present in the IκB inhibitor of the host NFκB transcription factor in the centre of the protein (21). The mechanism suggested for the inhibition of NFκB mediated transcription of proinflammatory cytokines, chemokines, adhesion molecules and anti-apoptotic genes is through direct binding to NFκB and thus preventing its binding to DNA (94, 98, 108). The other function assigned to the A238L protein is the inhibition of calcineurin phosphatase activity and consequent inhibition of calcineurin activated pathways such as the activation of the NFAT transcription factor (76). In summary, A238L is predicted to act as a potent immunomodulatory protein with diverse inhibitory effects on the transcription of cellular genes regulated by NFκB and NFAT (22). In addition, the A238L protein also inhibits COX-2 expression (37), IL-8 induction and TNF-α expression (36, 94), expression of iNOS (39). Several of these functions are inhibited by targeting the p300 coactivator of transcription (36, 38).

A number of other proteins predicted to inhibit host signalling pathways are encoded in the ASFV genome. The ASFV j4R protein binds to the α-chain of nascent polypeptide-associated complex (α-NAC) (35). The α-NAC protein plays roles in both translation and transcription, more specifically as a co-activator of c-jun and is also a binding partner of Fas associated death domain (FADD). The interaction between J4R and α-NAC is therefore predicted to modulate the transcriptional activation of c-jun and TNF-α induced apoptosis (22). The ubiquitin-conjugating enzyme, UBCv, of ASFV has been shown to interact with a host nuclear protein SMCy and is involved in transcriptional regulation (13). The ASFV DP71L protein is similar to the neurovirulence-associated protein (ICP34.5) from herpes

simplex virus (HSV). Recently, comparisons between the known function of ICP34.5 and the unknown function of DP71L, have demonstrated that like ICP34.5, DP71L is required for the activation of PP1 phosphatase activity that is induced by ASFV infection (99) (Rivera et al., 2007). However, the latest studies indicates that DP71L is not the only factor required to control eIF2α phosphorylation, by PP1 (133). More recently described, the ORF I329l gene, encodes a highly glycosylated protein expressed in the cell membrane and on its surface. In dsRNA stimulated cells, I329L has been shown to inhibit NF-κB and IRF3 activation. The mechanism of I329L inhibition is yet to be fully determined. One study points TRIF as possible target of I329L protein, as overexpression of TRIF reverted NF-κB and IRF3 inhibition (138). While another study, based on structural and interaction analysis, suggest that I329L binds to TLR3, acting as an antagonist (47).

Inhibition of apoptosis is a common host evasion strategy used by viruses and ASFV has three proteins with this activity. The first protein, A224L, is similar to the inhibitor of apoptosis protein (IAP) family of apoptosis inhibitors, and has been shown to interact with caspase-3 and to promote cell survival (82). The second, the ASFV bcl-2 homologue A179L, has been recently demonstrated to bind to a specific Bcl-2 proapoptotic protein and in this way block the induction of apoptosis (30). Finally, the third protein, EP153R, is a C-type lectin homologue and the first to be described having anti-apoptotic properties, and might be involved in the control of the activity of cellular p53 (52).

Another mechanism used by ASFV to modulate host responses is to express transmembrane proteins with similarity to host cell adhesion proteins. The characteristic haemadsorption observed in ASFV infected cells is due to the interaction between a CD2 like protein encoded by the virus (CD2v or EP402R) and its ligand expressed on the surface of red blood cells (RBC). This virus protein is also incorporated into the virus particle and mediates attachment of the virus to RBC (29).

ASFV infection leads to the disruption of the trans-Golgi network with a consequently inhibition of MHC class I surface expression (81), thus providing a possible mechanism for evasion of cytotoxic T lymphocytes responses.

The modulation of the interferon response by ASFV has only been described in the comparison of transcriptional profiles of macrophage cells infected with wild type virus and a deletion mutant virus lacking six MGF360 and two MGF530 genes. A reduction in a 2 to 3 log on the virus titres was observed from the infection of macrophages with the mutant virus and early cell death was also observed. Microarray analysis revealed an up-regulation of several interferon stimulated genes (ISGs) mRNAs when the cells were infected with this mutant virus and in comparison with wild type, suggesting that MGF360 and/or MGF530

genes are involved in the inhibition of IFN response. Indeed, in contrast with the wild type virus infection, the mutant virus infected culture supernatant contained significant amounts of IFN-α (2). Notably, in porcine aortic endothelial infected cells, the IFN-α induced MHC class I expression is down-regulated (109).

However, no individual ASFV gene has been demonstrated to inhibit the IFN response. This is very surprising as the virus: 1) Resides in macrophages, a unique IFN sensitive cell and 2) because of its persistent infection, a lifestyle incompatible with an effective IFN response. Thus the focus of this work has been to characterize an unsigned early gene first described by Yáñez in 1995 (132) . K205R, shown to inhibit IFN response in luciferase assays (Correia, S., unpublished work). However, the mechanisms by which this ASFV gene modulates the IFN response is unknown. The aim of this thesis is to further understand how K205R can modulate the IFN response.

Materials and Methods

Production of ASFV and purification of viral genomic DNA

Cell culture

VERO cell line was grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) with 4.5g/L Glucose and 0.11g/L of Sodium Pyruvate, supplemented with 10% Fetal bovine serum (FBS, Gibco), penicillin(100u/mL)/streptomycin(100ug/mL) (Gibco) and 2mM of L-gluthamine (Gibco). All cells were maintained in a humidified atmosphere of 5% CO_2, 95% balanced air at 37 °C. Cell lines were passaged, using Trypsin-EDTA (Gibco) when enough confluence was observed, two/three times a week.

Production of ASFV

VERO cells were seeded in 150cm^2 Flasks (7x10^6 cells/flask) and the next day infected with 1x10^{-3}p.f.u/cell (plaque forming units) of BA71V strain of ASFV. After 9/10 days, cells were scraped and collected by centrifugation (1300rpm for 5minutes). The supernatant was centrifuged for 2 hours at 18 000rpm. The resulting pellet was resuspended in DMEM and stored in aliquots at -80ºC, or processed to extract viral genomic DNA.

Extraction of viral genomic DNA

After centrifugation, the virus was resuspended in 1mL of TE buffer (10mM TrisCl pH8, 1mM of EDTA). SDS (10% stock from Sigma-Aldrich) and proteinase K (10µg/mL stock from 50µg/mL) was added to a final concentration of 0,5% and 50µg/mL, respectively. Sample was mixed by vortex and incubated O/N at 37ºC. Subsequently, the DNA was extracted and purified using routine phenol-chlorophorm and ethanol precipitation protocols.

Plaque Assays

To determine the viral concentration, VERO cells (3.8x10^5/well in 6 well plate) were infected with serial dilutions of 1:10 (1mL DMEM) of the virus. On the next day, cells were covered with an overlay medium of DMEM containing 0.7% of agarose (4% stock from Gibco). After 5 days, overlay was carefully removed and cells fixed with 4% paraformaldehyde (PFA) for 10minutes, before being stained with Toluidine Blue (0.1% in 4% PFA), to facilitate the counting of the viral plaques.

Replication of viral DNA and preparation of the K205R fragments

The ASFV open reading frame K205R was amplified from BA71V DNA by PCR (Table1) using *Pfu* polymerase (Fermentas) and cloned into the pcDNA3 plasmid in frame with an amino-terminal influenza haemaglutinin (HA) tag using *EcoRI* and *XhoI* restriction sites, according to the Fermentas restriction enzymes protocol.

The K205R fragments were amplified using the same PCR conditions (Table1) and cloned using the same restriction site and protocol as for the complete K205R.

To confirm the correct size of the amplified PCR products, the samples were run on an agarose gel with a percentage (1%-1,5%) of the final PCR solution. After confirmation, the remaining PCR product was purified by DNA purifications columns (Qiagen) according to

Primer	Sequence
K205R 5’	5’- GC**GAATTC**ATGGTTGAGCCACGCGAAC-3’
K205R low 3’	5’-GC**CTCGAG**TTACTTCTTCTTCATCATCTC-3’
K205R129 3’	5’-CG**CTCGAG**TTAACTTGTTTTG-3’
K205R97 3’	5’-GC**CTCGAG**CTAAAGTGGAGTGCC-3’
K205R108 5’	5’-CG**GAATTC**CCGGCAAAAAAAA-3’
K205R75 5’	5’-GC**GAATTC**ACACCTAAAAAAAATCCC-3'

Temp. (ºC)	Time (sec)	Cycles
95	300	1
95	60	
42	60	30
72	200	
72	900	1

Table 1: PCR primers for K205R and K205R fragments and PCR standard conditions used for amplification using BA71V genomic DNA

manufacturer’s protocol. Purified DNA (insert) and pcDNA3 plasmid (vector) were digested and ligated according to manufacturer’s protocol of Rapid DNA Dephos & Ligation Kit (Roche). In order to prevent self re-ligation of the vector and consequent false positive colonies, this kit included a dephosphorylation step of the vector. Ligation mix was used to transform chemical competent E.coli DH5α strain. The ligation mix was added to competent cells and left on ice for 30 minutes followed by heat shock at 42ºC for 45 seconds and 2 minutes on ice, before plating in ampicillin agar plates, which were allowed to grow at 37ºC overnight. The resulting colonies were screened for successful ligation using restriction patterns of plasmid DNA, after *EcoRI/XhoI* digestion and agarose gel (0.7%-1.5% in 1x Tris-acetate-EDTA, TAE) electrophoresis. The DNA was stained with Redsafe™ and observed under UV light. In frame insertion of the genes was confirmed by DNA sequencing of the clones with correct restriction pattern.

The DNA quantifications were made using Nanodrop from Thermo Scientific

Luciferase Assay

Luciferase Reporters

The reporter plasmid for the IFN-β promoter [pIFΔ(-125/+72)lucter], the IFN-β responsive plasmid [p(9-27ISRE)4tkΔ(-39)lucter] and the IFN-γ responsive plasmid [p(IRF-

1*GAS)6tkΔ(-39)lucter] were kind gifts of Dr. S. Goodbourn. All these plasmids are fused with firefly luciferase gene. The pCMVβ plasmid contains a β-galactosidase gene under the control of human cytomegalovirus immediate early promoter.

Reporter gene assay.

VERO cells ($5x10^4$ cells/well, in a 24 well plate) were co-transfected with 100ng of reporter plasmid (IFN-β, ISRE, GAS,), 25 ng of β-galactosidase control plasmid and 300ng of pcDNA3HA with test gene or the empty pcDNA3HA plasmid, according to the Lipofectamine 2000 protocol (Invitrogen). Seventy two hours post-transfection, the cells were either stimulated for five hours or not stimulated with 25μg/mL Poly (I:C) (dsRNA analog), 1000U/ml human IFN-β or 100U/ml human IFN-γ, for cells transfect with IFN-β, ISRE and GAS luciferase reporters. The cells where then lysed and the luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer's protocol. The variations in the transfection efficiency were corrected by dividing luciferase values by β-galactosidase values.

Western blot and antibodies.

VERO cells ($2,5x10^5$cells/well in a 6-well plate) were transfected with 4μg of pcDNA3HA with test gene or 4μg of pcDNA3HA empty plasmid, using the manufacturer's protocol of Lipofectamine 2000 (Invitrogene). Seventy two hours post-transfection, the cells were stimulated or not stimulated with 1000U/ml human IFN-β during the indicated periods of time. Cells were then harvested and lysed in lysis buffer (20mM TrisHCl, 150mM NaCl, 1% Triton X-100, 2mM EDTA) containing a protease and phosphatase inhibitor cocktail (Roche and Calbiochem). Protein concentration of the lysates was quantified using Bradford reagent. Cell lysates were resolved on 8%-15% SDS-PAGE, transferred, using semidry method (constant voltage of 12, for one hour and an half) to a polyvinylidene difluoride (PVDF) membrane and analyzed by immunoblot assay using the primary antibodies: rabbit anti-STAT2 (C-20, Santa Cruz Biotechnology), rabbit anti-STAT1 (Upstate), rat monoclonal anti-HA HRP (horseradish peroxidase- conjugated, Roche), rabbit anti-phosphoTyr701-STAT1 (Cell Signaling), rabbit anti-phosphoTyr690-STAT2 (Abnova) and mouse anti-α-actin-HRP conjugated (Sigma). The bounded primary antibodies were then detected using HRP-conjugated secondary antibodies: mouse anti-rabbit (Invitrogen) and goat anti-mouse (Sigma). As a standard procedure, after transfer, all membranes were blocked with 5% milk TBS 0,05% Tween-20 (TBST) (Sigma Aldrich), for at least for 1 hour. Alternatively, for visualization of phosphorylated proteins, membranes were blocked with 5% Bovine Serum

Albumin (BSA) 0,05% TBST. Membranes were washed in TBST for 10 minutes(3x), after each antibody incubation. The membranes were developed with ECL solution (ThermoScientific).

Immunofluorescence and antibodies

VERO cells (2.5×10^5 cells/well in a 6 well plate) seeded on glass coverslips were transfected with 4µg of pcDNA3HA with test gene or empty pcDNA3HA, using Lipofectamine2000. Forty-eight hours post-transfection, cells were washed with phosphate buffer saline (PBS), fixed with 4% PFA for 20 minutes and permeabilized with PBS + 0.1% Triton X-100 (Sigma Aldrich) for 20 minutes. The cells were blocked with 5% goat serum PBS for thirty minute before antibody staining. Cells were incubated for 1 hour at room temperature for both primary and secondary antibodies. Antibodies used include: high affinity rat anti-HA primary antibody (Roche), diluted in 5% goat serum PBS; Texas Red dye-conjugated affinipure goat anti-mouse IgG. The DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI, 200ng/mL from Sigma) and examined using a fluorescence microscope Leica DMRA2.

RESULTS

Luciferase Assays

In order to determine if the K205R gene is able to manipulate the induction and impact of IFN, the viral gene was screened in luciferase reporter assays. In this system, cells are transfected with reporter plasmids containing the luciferase gene under the control of the IFN- β promoter, ISRE element or GAS element, together with pcDNA3 empty vector or pcDNA3HA-K205R, and the response is evaluated after stimulating the cells with the corresponding inducer: dsRNA analogue Poly I:C for the IFN-β luciferase, IFN-β for the ISRE luciferase and IFN-γ for the GAS luciferase. In this screening, the K205R protein inhibited all three luciferase assays (IFN-β, ISRE and GAS reporters).As can be seen (figure 3), the luciferase activities were clearly reduced in cells expressing K205R when compared with the control cells transfected with the empty plasmid, and stimulated with appropriate inducer.

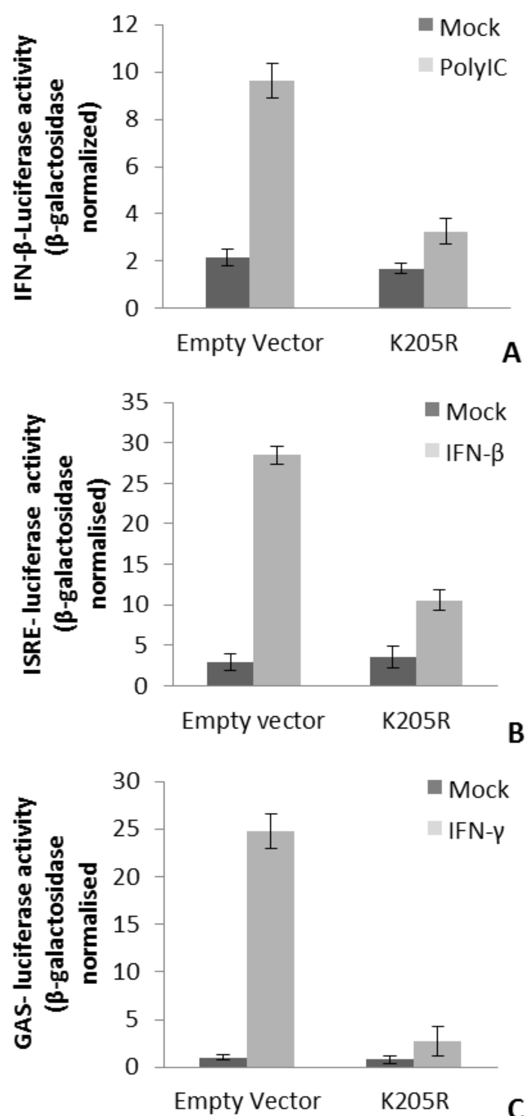

Figure 3: Luciferase assays results using K205R gene and three different luciferase reporters for IFN response. **(A)** IFN-β response stimulated with dsRNA analagog, PolyIC; **(B)** ISRE response, stimulated with IFN-β and **(C)** GAS response stimulated with IFN-γ

Bioinformatics

To define which region of the K205R protein might be impacting in each of the three signalling pathways that were shown to be inhibited in the luciferase assays, we used the Jpred program (18), which is a secondary structure prediction server (Annex 1 with prediction). This analysis uses algorithms that can predict three different protein structures (α-helix, β-strand and coiled-coil) with an accuracy of 81,5%. The analysis of the K205R predicted a coiled-coil motif with high probability. This particular coiled-coil motif is 34

amino acids long, starting at amino acid 40 and ending at amino acid 74. Coiled-coil motifs typically functions in protein-protein interactions. Considering that K205R might be interacting with proteins from the IFN signalling pathways and therefore inhibiting its response, the approach used here tried to possibly determine the minimum sequence needed for the K205R's observed effects. As K205R is a small protein, the strategy was to divide the protein in two parts of similar size without disrupting the coiled-coil motif. Two of the fragments (K205R129 and K205R108) contain an overlapping region and the other two (K205R97 and K205R75) are similar but exclude this overlapping region (figure 4). Fragments were named according to their protein size. In addition, K205R also contains a putative nuclear export signal (NES) between amino acid 80 and 89.

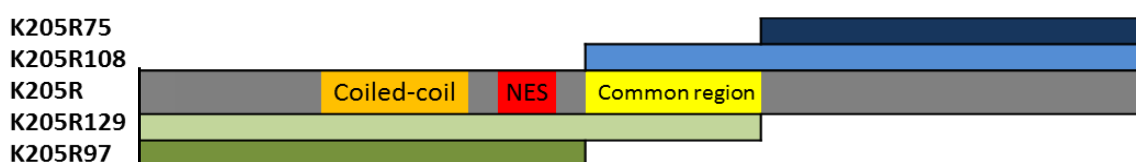

Figure 4: Schematic representation of K205R protein with putative motifs and K205R fragments

Replication of viral DNA and preparation of the K205R fragments

Vero cells were infected with BA71V and viral genomic DNA was extracted according to the protocol described above. The four different K205R fragments were amplified by PCR using the standard conditions already used for the complete K205R. The amplification of full length K205R was used as the positive control, while in the negative control, the DNA was replaced by MilliQ H_20. Samples were run in a 1% agarose gel with a constant 100 voltage.

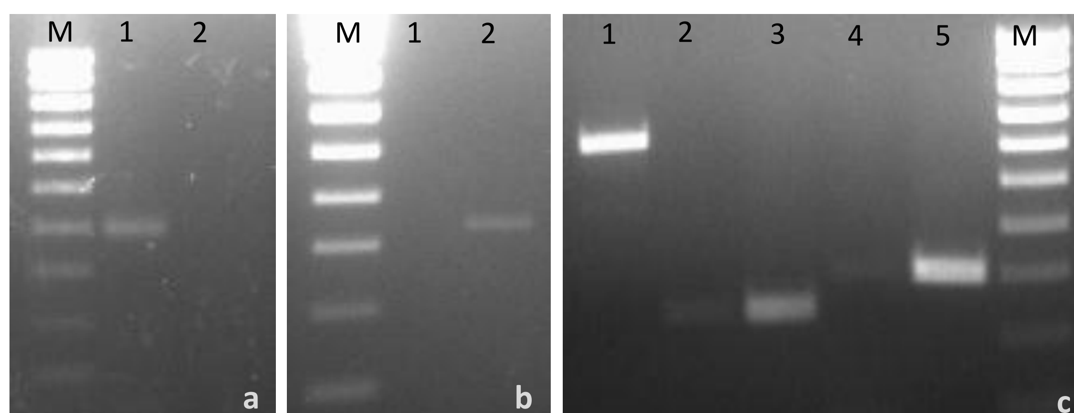

Figure 5: Amplification of K205R DNA fragments, visualized on 1,5% agarose gel electrophoresis, using DNA ladder Hyperladder IV **(M)**. K205R129 with 387pb **(a1),** K205R108 with 324bp **(b2)**, K205R75 with 225bp **(c3)** and K205R97 with 291bp **(c5)**. Each PCR fragment reactions contains a negative control in which DNA replaced with H_20 was also analyzed for each fragment, respectively **(a2)**, **(b1)**, **(c2)** and **(c3)**. Total K205R amplification was used as a control **(c1)**

The size of the resulting DNA bands corresponded to K205R total gene (618bp), to K205R129 (387bp), to K205R108 (324bp), to K205R97 (291bp) and K205R75 (225bp) (figure 5).The bands were excised from the agarose gel and the DNA extracted using QIAGEN columns. The different DNA fragments were digested with EcoRI and XhoI restriction enzymes, ligated into pcDNA3HA and transformed in E.coli DH5α strain. The twelve colonies were harvest and their plasmid DNA extracted and digested with same enzymes used for cloning in pcDNA3HA. In order to identify the colonies that contained the ligated inserts, these samples were run in a 1,5% agarose gel. From these twelve colonies, only five had a band with the desired size, near 600bp. To confirm correct insertion of k205R gene, three of plasmid DNA extracts were sent to sequencing and the selected one with the correctly cloned fragment was used for further experiments. (annex 2).

A similar protocol was followed for all K205R fragments. For fragments K205R129 and K205R97, it was used 5'primer of K205R with the respective 3'primer of each fragment (Fgure 5 **(a)** and **(c)**). In the case of the K205R108 and K205R75, 3'primer of K205R was used with respective 5' primer of each fragment (figure 5 **(b)** and **(c)**). Although the PCR empty controls of K205R75 and K205R97 had some amplification, the fragment's DNA was uncontaminated and with the expected size. Again, the resulting DNA was cloned into pcDNA3HA plasmid and transformed in E.coli DH5α strain. For each fragment, the DNA from up to three clones with the correct insert size (figure 6) was sequenced and only one correct clone was used for further experiments.

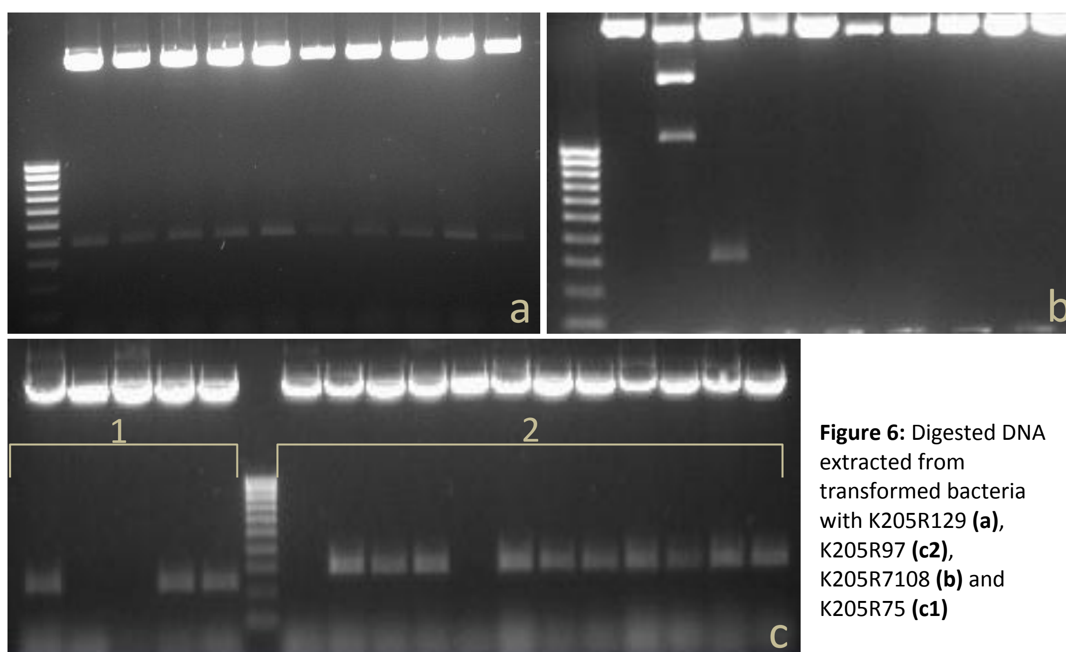

Figure 6: Digested DNA extracted from transformed bacteria with K205R129 **(a)**, K205R97 **(c2)**, K205R7108 **(b)** and K205R75 **(c1)**

The next subsequent step was to see if each K205R fragment clone, when transfected into cells resulted in protein expression with the expected size. Therefore, Vero cells were transfected with each K205R fragment, cells were lysed and run in a 15% SDS gel. The western blot anti-HA tag showed indeed, that each one of the fragments is beeing expressed and with te correct size (Figure 7).

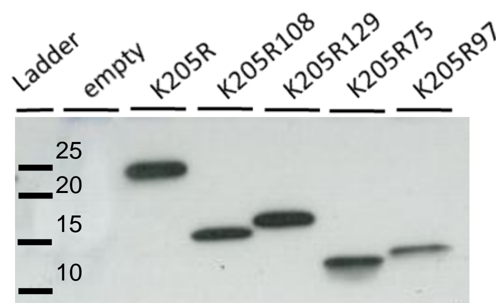

Figure 7: Western blot expression of K205R and K205R protein fragments in Vero cells, with expected molecular weight (ladder in kilodalton).

Immunofluorescence

The K205R protein localization at cytoplasm has been previously described (40). In order to understand which and if any part of K205R protein is important for its localization, Vero cells previously transfected with each K205R fragment were immunostained (figure 8). In fact, it was clear that the K205R fragments, that did not contain the putative NES, K205R108 and K205R75, localised in the nucleus. (figure 8). However, of the fragments which included the putative NES, only K205R129 was clearly expressed only in the cytoplasm. The K205R97 fragment localised equally in both, the nucleus and the cytoplasm.

STAT1 and STAT2 Western Blot

The K205R viral protein was shown to inhibit the three reporters in luciferase assay. Only the response to type I IFN (ISRE reporter) was further investigated in this study. This pathway can be disrupted by viruses targeting the ISGF3 complex, often impacting on STAT1 and STAT2. These two proteins can be targeted by the virus for degradation, altering phosphorylation levels and traficking of STAT to the nucleus (96). To further understand how and which K205R fragment could affect this signalling pathway, Vero cells were transfected with each fragment and, 48h post transfection, the cells were stimulated with IFN-β (to activate the ISRE pathway) for 15 minutes and 45 minutes before lysis and protein extraction. Western blot was made and the STAT1 and STAT2 protein levels and phosphorylation status were accessed by incubating the membranes with specific antibodies for phosphorylated STAT1 (Tyr701) and STAT2 (Tyr690), and for total STAT1 and STAT2 protein. Differences were only observed regarding STAT1 phosphorylation, which was parcially inhibited in the presence of full K205R protein and in the presence of K205R75 fragment, but no its total protein level. Regarding STAT2, no significant differences were observed, both in phosphorylation or protein level (figure 9).

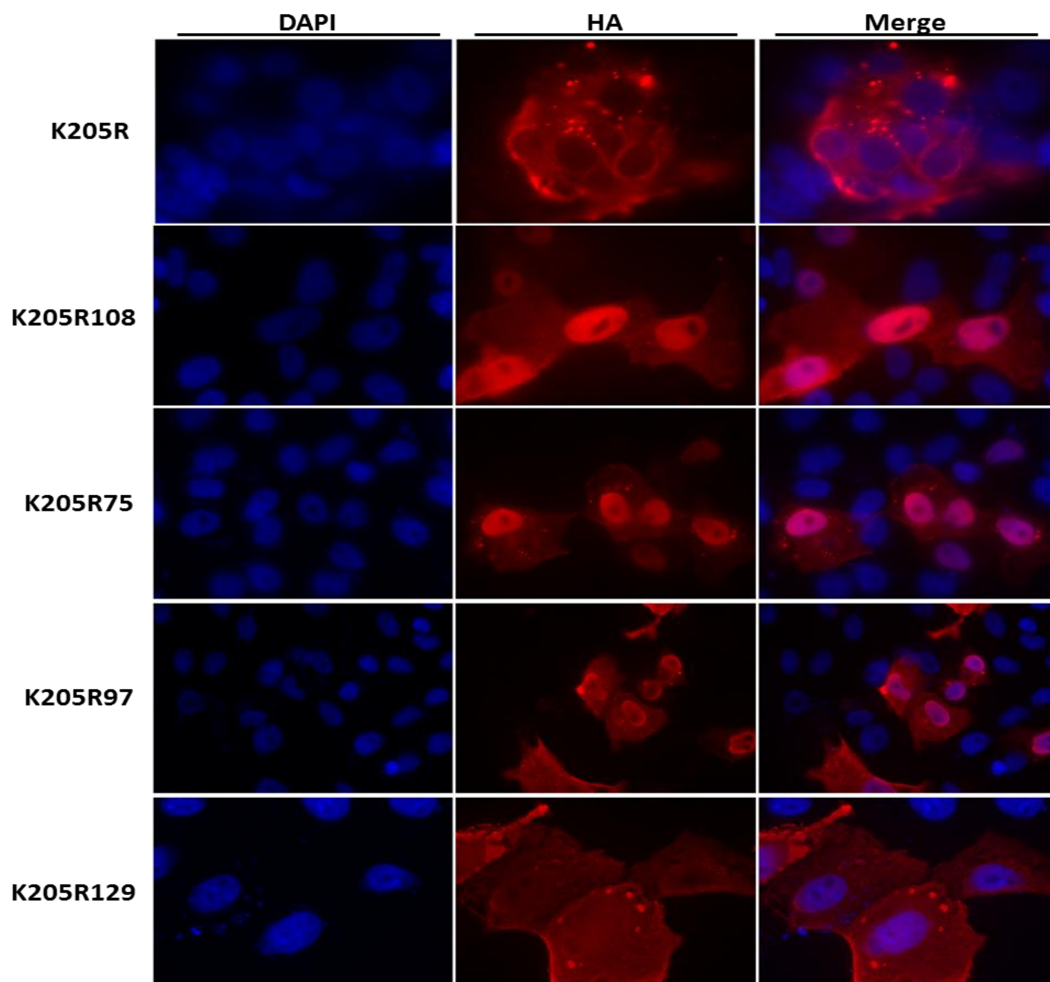

Figure 8: Imunofluorescence results. Vero cells were immunostained with DAPI for nucleus observation and test gene with Texas Red. Images taken using Leica's DMRA2.

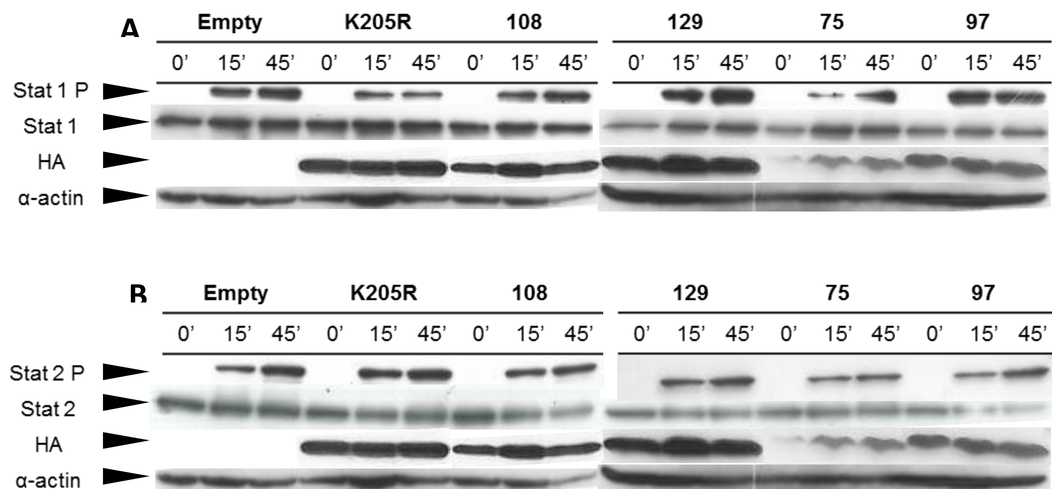

Figure 9: Western blot for STAT 1 and STAT 2, total protein and phosphorylation after IFN-β stimulus for the indicated times in cells previously transfected with full K205R and its fragments.

Discussion

The interferon (IFN) system is critical to anti-viral immunity. Not surprisingly, therefore, viruses have evolved a variety of different anti-IFN strategies, with the objective of surviving sufficient time to replicate The ASFV gene K205R is particularly interesting as it is able to not only inhibit the induction of expression of IFN-β, but also the impact of type I and type II IFN.

Luciferase assays showed that K205R not only seems to interfere with IFN signaling pathways, by inhibition of ISRE and GAS (Figure 3) in cells stimulated with IFN-β and IFN-γ, respectively, but also to inhibit the production of IFN-β in cells stimulated with dsRNA homolog, Poly I:C. Comparing the results of luciferase activity of cells transfected with the empty vector, with results from K205R transfected cells, we can see a significant (3-fold) inhibition of IFN-β and ISRE. In the case of GAS-luciferase activity, an 8-fold inhibition can be observed. These results encouraged us to further understand the mechanisms involved in K205R inhibitions of IFN responses.

Using a bioinformatics analysis of K205R protein, we obtained a prediction of pK205R putative secondary structures, which gave a clue about of possible K205R function. Interestingly, pK205R has a putative coiled-coil motif 34 residues long, between amino acids 41 and 75, with a prediction probability greater than 90%, according to Lupas prediction (18). The coiled-coil motif consists of two to five right-handed α-helices wrapped around each other into a left-handed helix to form a supercoil. This kind of motif is often associated with protein interaction. In fact, this prediction tool also suggests an α-helix structure for the first half (approximately) of pK205R, which corroborates with the coiled-coil prediction. This suggests K205R interacts with other proteins, namely with proteins involved in IFN production and IFN signaling pathways, according to the luciferase results. The analysis also suggested that K205R has a putative nuclear export signal (NES) between residues 80 and 89 (LGAIIAQLEI).

Based on this analysis, a series of deletion mutants of K205R was constructed using cloning techniques. As the K205R is a small protein, primers were design to divide pK205r by half. Two of the fragments (K205R129 and K205R108) contained a common region of 33 amino acids. The other two fragments (K205R97 and K205R75) correspond to the first ones but without the common region. After confirmation of correct amplification and cloning of each fragment in pcDNA3HA, in frame with the HA tag, each fragment was transfected into untreated VERO cells in order to confirm their expression and predicted molecular weight

(annex 2), using the western blot technique (described in methods). All fragments were expressed and with the predicted molecular weight.

The next was to see if the putative NES sequence influenced the localization of K205R. A immunofluorescence assay was done using Vero cells transfected with K205R or its fragments, all cloned with an HA peptide "immunotag" at the N-terminal. Cells were immunostained for the HA tag to visualize the fragments and with DAPI to visualize the cell nucleus. The entire K205R protein localizes mostly in the cytoplasm, but levels of K205R can be seen in the nucleus (figure 8). This suggests K205R is moving between the nucleus and the cytoplasm, presumably the result of a functional NES sequence. Consistent with this idea, cells transfected with fragments not containing the putative NES motif (K205R108 and K205R75), accumulate the K205R protein fragments in their nucleus. The K205R129 and K205R97 fragments, which include NES motif, on the other hand, are preferentially cytoplasmically located. The K205R97 fragment, which also contains the NES, is present in both nucleus and cytoplasm, but with more protein localized in the cytoplasm (figure 8).

These results suggest that the putative NES sequence is indeed a functional NES sequence. More experiments must be done to further define the minimal sequence necessary for the activity of the NES motif in K205R. As a first approach, point mutations in the putative NES motif and subsequent observation of cellular localization of mutated protein could be done. Another approach would be using cells transfected with full K205R protein in the presence of Leptomycin B, an inhibitor of nuclear export (62).

The putative coiled-coil motif of K205R suggests it may be interacting with other proteins, presumably with a role the IFN responses. At least two members of the STAT protein family play an important role in the signaling pathways activated in response to IFN. STAT 1 and STAT 2 are common targets of viral proteins that can sequester STATs, increase their turn over or alter the pattern of STAT phosphorylation, an important step for the formation of the two transcription factors, STAT1-STAT2 heterodimer and STAT1 homodimer, critical for the response to IFN-α/β and IFN-γ, respectively. Western blot results for IFN-β treated cells suggests that K205R is not affecting STAT1 or STAT2 turnover as total protein level of both STATs did not change with entire K205R or fragments. However, the level of phosphorylated STAT 1 seems to be lower in the presence of full K205R protein. Surprisingly the only fragment which maintains this effect on STAT 1 phosphorylation was K205R75, a fragment without the coiled-coil and NES putative motif. Even more suprising is the lack of effect in K205R108 transfected cells, as this fragment includes the K205R75 fragment. Despite this, western blot result suggest that K205R inhibition of ISRE pathway observed in the luciferase assays, might be due to an alteration of STAT1 phosphorylation

pattern, but this effect may occur without a direct interaction between STAT1 and K205R, as this effect is only preserved in the K205R75 fragment. It is also possible that K205R interacts with STAT1 but this interaction is not dependent of the putative coiled-coil motif. More experiments must be done to unveil the mechanism involved on K205R-dependent modification the phosphorylation status of STAT1. For example, a western blot at could can be done to investigate if K205R interacts or not with STAT1. By immunoprecipitating STAT1 and incubating it with α-HA antibody we can determine the existence the interaction. It is also possible to verify this by immunostaining STAT1 and K205R we can see their cellular localization and determine whether they colocalize, if so, confirm the possible STAT1-K205R interaction hypothesis. If no evidences of interaction are observed, K205R may be affecting a protein upstream in the signaling cascadeof STAT1 in the IFN-response signaling pathway, for example JAK1, which is responsible for STAT1 phosphorylation in response to IFN-α/β and also in response to IFN-γ, activator of the GAS pathway. In this case, JAK1 expression levels and turnover rates in the presence of K205R and K205R75 should be observed.

The same experiment done for ISRE pathway should be done for the GAS pathway, as K205R seems to affect this pathway in luciferase assays. I would expect to observe a similar result to the one observed for the ISRE pathway as they have STAT1 in common.

For technical reason it was not possible to do the luciferase assays done for K205R with its fragments, but it would be interesting to see which or if any of the fragments could inhibit IFN-β, ISRE and GAS signaling pathways. It would be expected to see an inhibition of ISRE pathway with at least K205R75 fragment.

The inhibition of IFN-β induction pathway by K205R observed in the luciferase assays was not further investigated in this thesis work. However it would be also very interesting to further investigate this signaling pathway. Besides repeating the luciferase assay with the fragments, experiments should be done to determine at what level of the IFN-induction pathway K205R was acting. Despite the PRRs sensing ASFV, IRF3 and/or IRF7 will always be involved on the IFN induction, so it would be of interest to determine if K205R affects them and later with its fragments, if changes were observed with K205R. This could be done using similar approaches used for the signaling responses to IFN, either by western blot and immunofluorescence to access protein levels and the phosphorylation status of IRF3/7.

After characterization of the K205R role in Vero cells, it should be confirmed if the same applies using an experimental model more closely related to ASFV host target, such as porcine macrophages, or the porcine macrophage IPAM cell line (128).

This work may be viewed as an approach to determine how the multifunctional ASFV gene K205R is able to inhibit both the induction and the impact of IFN. The K205r putative

secondary protein structure and functional motifs were explored by bioinformatics. Based on the bioinformatic data, deletion mutant fragments of K205R were constructed and used in immunofluorescence and western blot assays. The immunofluorescence results are consistent with the presence of a functional NES motif in the K205R protein sequence. Western blot experiments suggested that K205R is affecting the phosphorylation status of STAT1, in cells stimulated with IFN-β. Although it was not possible to clearly determine the minimum sequence needed for all the functions of K205R, the results suggest that K205R inhibition of the impact of IFN type I, depends on a sequence within amino acids 130 and 205.

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Annex 1: Results from Jpred analysis for K205R secondary structure

```
                : 1---------11--------21--------31--------41--------51--------61--------71--------81--------91--------101-------111-------121-------131-------141-------151-------161-------171-------181-------191-------201--
OrigSeq         : MVEPREQFFQDLLSAVDQQMDTVKNDIKDIMKEKTSFMVSFENFIERYDTMEKNIQDLQNKYEEMAANLMTVMTDTKIQLGAIIAQLEILMINGTPLPAKKTTIKEAMPLPSSNTNNDQTSPPASGKTSETPKKNPTNAMFFTRSEWASSKTFREKFLTPEIQAILDEQFANKTGIERLHAEGLYMWRTQFSDEQKKMVKEMMKK

Jnet            : ----HHHHHHHHHHHHHHHHHHHHHHHHHHHHH---HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----HHHHHHHHHEEEEEE----------HH-----------------------------------EEEEE------HHHHHH---HHHHHHHHHH--------HEE----EEEE-----HHHHHHHHHH--
jhmm            : ----HHHHHHHHHHHHHHHHHHHHHHHHHHHHH--HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH--HHHHHHHHHHHEEEEE---------HHHHH---------------------------------EEEEE------HHHHHH---HHHHHHHHHHH------HHH-----EEEE-----HHHHHHHHHH--
jpssm           : ---HHHHHHHHHHHHHHHHHHHHHHHHHHHHHH---EEEE----EE------------H-HHHHHHHHHHHH----EEEEE---EEEEEEEE----------------------------------------------EEEEE--------HHHHHH--HHHHHHHH-----------EE----EEEEE----HHHHHHHHHH--

Lupas 14        : -----------------------------------------------CCCCCCCCCCCCCCCCCCCCC---------------------------------------------------------------------------------------------------------------------------------------
Lupas 21        : ----------------------------------------cccccccCCCCCCCCCCCCCCCCCCCCCccccccc------------------------------------------------------------------------------------------------------------------------------
Lupas 28        : ----------------------------------------CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC------------------------------------------------------------------------------------------------------------------------------
```

Notes

Key:

```
Jnet            - Final secondary structure prediction for query
jalign          - Jnet alignment prediction
jhmm            - Jnet hmm profile prediction
jpssm           - Jnet PSIBLAST pssm profile prediction

Lupas           - Lupas Coil prediction (window size of 14, 21 and 28)

Note on coiled coil predictions  - = less than 50% probability
                                 c = between 50% and 90% probability
                                 C = greater than 90% probability
```

Annex 2: DNA and protein information of K205R and K205R fragments

K205R

Insert sequence(618bp):

ATGGTTGAGCCACGCGAACAGTTTTTTCAAGACCTGCTTTCAGCAGTGGATCAACAAATGGACACTGTAAAAAATGACATAAAAGACATCATGAA
AGAAAAAACATCTTTTATGGTGTCATTCGAAAACTTTATAGAACGTTACGATACCATGGAAAAAAATATTCAAGACCTTCAGAATAAGTACGAAG
AAATGGCGGCCAACCTTATGACCGTCATGACGGATACAAAAATTCAGCTTGGAGCCATTATCGCCCAACTTGAGATTCTGATGATAAATGGCACT
CCACTTCCGGCAAAAAAAACAACGATTAAGGAGGCTATGCCCCTACCTTCATCAAACACGAACAATGATCAAACGAGTCCTCCCGCCTCAGGCAA
AACAAGTGAAACACCTAAAAAAAATCCCACGAATGCAATGTTCTTCACGCGTAGCGAATGGGCATCCTCGAAAACTTTTCGAGAAAAGTTTTTAA
CACCAGAAATTCAGGCCATATTGGATGAGCAGTTTGCAAACAAGACCGGGATCGAAAGATTGCATGCCGAGGGTCTTTACATGTGGAGAACCCA
ATTCTCTGACGAACAGAAGAAAATGGTCAAAGAGATGATGAAGAAGTAA

Protein sequence:

MVEPREQFFQDLLSAVDQQMDTVKNDIKDIMKEKTSFMVSFENFIERYDTMEKNIQDLQNKYEEMAANLMTVMTDTKIQLGAIIAQLEILMINGTPL
PAKKTTIKEAMPLPSSNTNNDQTSPPASGKTSETPKKNPTNAMFFTRSEWASSKTFREKFLTPEIQAILDEQFANKTGIERLHAEGLYMWRTQFSDEQK
KMVKEMMKK

Protein size – 205 a.a.
Protein predicted molecular weight – 25 Kda

K205R129

Insert sequence(387bp):

ATGGTTGAGCCACGCGAACAGTTTTTTCAAGACCTGCTTTCAGCAGTGGATCAACAAATGGACACTGTAAAAAATGACATAAAAGACATCATGAA
AGAAAAAACATCTTTTATGGTGTCATTCGAAAACTTTATAGAACGTTACGATACCATGGAAAAAAATATTCAAGACCTTCAGAATAAGTACGAAG
AAATGGCGGCCAACCTTATGACCGTCATGACGGATACAAAAATTCAGCTTGGAGCCATTATCGCCCAACTTGAGATTCTGATGATAAATGGCACT
CCACTTCCGGCAAAAAAAACAACGATTAAGGAGGCTATGCCCCTACCTTCATCAAACACGAACAATGATCAAACGAGTCCTCCCGCCTCAGGCAA
AACAAGTTAA

Protein sequence:

MVEPREQFFQDLLSAVDQQMDTVKNDIKDIMKEKTSFMVSFENFIERYDTMEKNIQDLQNKYEEMAANLMTVMTDTKIQLGAIIAQLEILMINGTPL
PAKKTTIKEAMPLPSSNTNNDQTSPPASGKTS

Protein size – 129 a.a.
Protein predicted molecular weight – 16 Kda

K205R108

Insert sequence (324bp):

CCGGCAAAAAAAACAACGATTAAGGAGGCTATGCCCCTACCTTCATCAAACACGAACAATGATCAAACGAGTCCTCCCGCCTCAGGCAAAACAA
GTGAAACACCTAAAAAAAATCCCACGAATGCAATGTTCTTCACGCGTAGCGAATGGGCATCCTCGAAAACTTTTCGAGAAAAGTTTTTAACACCA
GAAATTCAGGCCATATTGGATGAGCAGTTTGCAAACAAGACCGGGATCGAAAGATTGCATGCCGAGGGTCTTTACATGTGGAGAACCCAATTCT
CTGACGAACAGAAGAAAATGGTCAAAGAGATGATGAAGAAGTAA

Protein sequence:

MAYPYDVPDYAEFPAKKTTIKEAMPLPSSNTNNDQTSPPASGKTSETPKKNPTNAMFFTRSEWASSKTFREKFLTPEIQAILDEQFANKTGIERLHAEGL
YMWRTQFSDEQKKMVKEMMKK

Protein size – 108 a.a.
Protein predicted molecular weight – 14 Kda

K205R97

Insert sequence(291bp):

ATGGTTGAGCCACGCGAACAGTTTTTTCAAGACCTGCTTTCAGCAGTGGATCAACAAATGGACACTGTAAAAAATGACATAAAAGACATCATGAA
AGAAAAAACATCTTTTATGGTGTCATTCGAAAACTTTATAGAACGTTACGATACCATGGAAAAAAATATTCAAGACCTTCAGAATAAGTACGAAG
AAATGGCGGCCAACCTTATGACCGTCATGACGGATACAAAAATTCAGCTTGGAGCCATTATCGCCCAACTTGAGATTCTGATGATAAATGGCACT
CCACTTTAG

Protein sequence:

MVEPREQFFQDLLSAVDQQMDTVKNDIKDIMKEKTSFMVSFENFIERYDTMEKNIQDLQNKYEEMAANLMTVMTDTKIQLGAIIAQLEILMINGTPL

Protein size – 97 a.a.
Protein predicted molecular weight – 13 Kda

K205R75

Insert sequence(225bp):

ATGGCTTACCCATACGATGTTCCAGATTACGCTGAATTCACACCTAAAAAAAATCCCACGAATGCAATGTTCTTCACGCGTAGCGAATGGGCATC
CTCGAAAACTTTTCGAGAAAAGTTTTTAACACCAGAAATTCAGGCCATATTGGATGAGCAGTTTGCAAACAAGACCGGGATCGAAAGATTGCAT
GCCGAGGGTCTTTACATGTGGAGAACCCAATTCTCTGACGAACAGAAGAAAATGGTCAAAGAGATGATGAAGAAGAAGTAA

Protein sequence:

MAYPYDVPDYAEFTPKKNPTNAMFFTRSEWASSKTFREKFLTPEIQAILDEQFANKTGIERLHAEGLYMWRTQFSDEQKKMVKEMMKK

Protein size – 75 a.a.
Protein predicted molecular weight – 11 Kda